Chapter-6

Discussion
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LSD’s are disorders which collectively constitute a significant burden in the community as collectively they constitute a prevalence of 1 in 5000. The present study here reports the use of biochemical markers for the diagnosis. The advent of Enzyme replacement therapy has changed the fulcrum of these potentially severe progressive non treatable disorders to potentially treatable phenotypes, if detected well within time. Certain countries have included LSD’s in the expanded newborn screening protocols. Of the treatable LSD’s, MPS1, Gaucher Disease, MPSII and Niemann Pick disease are commonly prevalent. Since there are pilot studies demonstrating significant prevalence of these disorders, a reliable biomarker would be a significant asset. To date, this is the only comprehensive investigation of biomarkers for Gaucher disease, Niemann Pick disease and MPS in patients of Indian ethnicity.

Evaluation of patients with Gaucher Disease

Enzymatic Analysis: Residual β-glucocerebrosidase (βGC) activity in GD patients. The routine diagnostic test for the confirmation of Gaucher disease is to perform an enzyme assay on mixed leukocytes and assess βGC activity, using an artificial substrate. While it is well known that this assay is not reliable for the identification of GD carrier status, due to overlap with the enzyme range found in normal individuals, it is, in the vast majority of cases, a reliable diagnostic tool for disease confirmation as levels in pathogenic ranges form the gold standard for diagnosis.
We also noticed significantly lower residual enzyme activity in Indian patients was noted. All GD patients (12/12) had a relatively low residual βGC activity (< 3.8nmol/h/mg protein) than the normal subjects (Table: 11). The median value of βGC activity in GD patients was 2.9 (1.9-3.8) compared to the median activity range in non affected individuals of 6.7 (4.5-10.0) nmol/h/mg protein.

**Biomarker Chitotriosidase, CCL18 activity in GD Patients:**

The greatest utility of a biomarker would be non invasive monitoring of a disease process and the evaluation of the efficacy of therapy. As mentioned previously there are certain characteristics of an ideal biomarker. We propose to discuss the efficacy of the biomarker proposed in our study i.e. CCL18/PARC in comparison with existing biomarker Chitotriosidase. Chitotriosidase and CCL18 are proteins that are massively produced and secreted by lipid-laden Gaucher cells. Increased plasma levels of these proteins give an indication of the total body load of storage cells. Determination of plasma chitotriosidase is already widely used and recommended for optimization of clinical management of Gaucher disease (Aerts et al 2003; Cox 2003; Baldellou et al 2004) and Niemann Pick disease (de Tandt et al 1996, Guo Y 1995). Plasma CCL18 may act as a valuable alternative surrogate marker for storage cells in the case of Gaucher patients who are deficient in chitotriosidase (Boot et al 2004; Deegan et al 2005) and in Niemann Pick patients (Brinkman et al 2005).
Chitotriosidase has long been used as a reliable biomarker for Gaucher disease. The first characteristic is that it should be easy to quantify reliably in clinically accessible samples. Since both assays are ELISA based, they are probably equally easy to perform. The second important criterion is it should be simple, rapid and reliable. Chitotriosidase estimation is fraught with methodological difficulty as chitotriosidase enzyme has both hydrolytic and trans glucosidase activities towards its substrate, chitotrioside. Thus it is not possible to assay the chitinolytic function of the enzyme under $V_{\text{max}}$ conditions and several dilutions are required. CCL18/PARC is liberated as a cytokine from the macrophages and not fraught with this difficulty but still requires serial dilutions. Genetic variation significantly affects chitotriosidase activity, as 6% of the general population as well as patients with GD demonstrate this variation. This occurs because of the presence of null alleles of the human chitotriosidase gene which have arisen as a result of a DNA insertional event (Boot et al 1998). Approximately 30% of the population, whether healthy or suffering from Gaucher disease are heterozygous for this null allele and their chitotriosidase activity is approximately 50% of levels found in individuals having 2 functional chitotriosidase genes. In homozygous state it is totally unreliable.

The extent of elevation in plasma CCL18 level in symptomatic patients is far less compared to the elevation in chitotriosidase concentration. The application of plasma CCL18 level is therefore of particular interest for those Gaucher and Niemann Pick patients in whom chitotriosidase is lacking due to a homozygosity for the common gene defect.
At present it cannot be excluded that plasma CCL18 levels can also be markedly increased due to other pathologies. It should be clear that measurement of the plasma CCL18 level for primary diagnosis of Gaucher and Niemann Pick disease should therefore not be advocated. Only in those cases in which the disease has been confirmed by demonstration of glucocerebrosidase deficiency or gene defects; the monitoring of plasma CCL18 level useful to obtain an impression of Gaucher and Niemann Pick cell burden. Determination of CCL18 in plasma samples kept frozen at -20 C gives reproducible results. The relative ease with which plasma samples can be collected and stored in a home setting is an attractive aspect. In sharp contrast to the observations made for chitotriosidase, CCL18 levels in plasma samples of Gaucher and Niemann Pick patients correlated well with these cases (Deegan et al 2005, Brinkman et al 2005). However neither Chitotriosidase nor CCLL8 demonstrated any increase over time during which the disease burden is likely to have progressed. The predictive value of CCL18/PARC is difficult to define as none of our patients could afford enzyme replacement therapy. Stability is another important criterion. This is of paramount importance as most laboratories estimate enzymes using either leucocytes or fibroblasts while some require serum. So limiting factors using enzyme assays include stringent time lines and need for leukocyte preparation. This has been overcome now with estimation in dried blood spots but the technology is available only in a select handful of centers in the country. Both chitotriosidase and CCL18 are robust and stable markers.
Specificity is the next most important criterion of a biomarker. Chitotriosidase activity is elevated not only in patients with GD but also in Niemann Pick disease Type A and B and iron overload situations like thalaseemia. CCL18/PARC is elevated in acute coronary syndromes, leukemias, Niemann Pick disease, beta Thalassemia and rheumatoid arthritis presumably because of its release from macrophages in conjunction with other interleukins (Yildiz et al 2013). Spectacular elevation of a biomarker, specifically in relation to a given disease, has enormous advantages for clinical diagnosis as well as for monitoring disease activity. Thus, both CLL18 and Chitotriosidase share the same limitation of relative non specificity but perform very well in patients with GD due to their spectacular elevation. Indian experience for Chito also suggests being a robust marker with significant elevations being seen in patients with GD, NPA, and NPB. (Sheth et al 2010)

The ideal biomarker should reflect the total burden of the disease at all sites. Thrombocytopenia is often recalcitrant in GD and may be related to functional hypersplenism, as well as to infiltration of the bone marrow and infarction crises in the bone as independent clinical manifestations (Boot et al 2004). The ideal biomarker should be similarly elevated as a result of storage-related inflammatory changes and infiltration occurring, for example, in the liver, spleen, bone marrow and lungs, so that its elevation gives an overall view of the generalized effect of the disease rather than its manifestation at a single site or in a single organ (Boot et al 2004, Colburn et al 2000).
However neither Chitotriosidase nor CCLL8 demonstrated any increase over time during which the disease burden is likely to have progressed. The predictive value of CCL18/PARC is difficult to define as none of our patients could afford enzyme replacement therapy. However, with experience of enzyme replacement therapy for Gaucher disease in our country, availability of ideal biomarkers could really be rewarding (Nagral et al 2011). We therefore conclude that CLL18/PARC is as reliable as Chitotriodosidase for monitoring patients with GD but of significant advantage in population deficient in the genetic polymorphism causing a homozygous state where chitotriosidase would be completely unreliable for diagnosis and monitoring of the disease.

**CCL18 in Niemann Pick Disease**

Given the similarity of the storage cells in NPD and Gaucher disease, we studied Gaucher plasma markers (chitotriodosidase and CCL18) in two NP patients. Analysis of plasma specimens revealed markedly increased levels of chitotriodosidase and CCL18 in both patients. In both children, plasma chitotriodosidase and CCL18 were clearly elevated above normal values. The both chitotriodosidase and CCL18 may also serve as markers for the formation of pathological lipid-laden macrophages in NPD, in analogy to Gaucher disease. The availability of sensitive plasma surrogate markers may be of great value for diagnosis and monitoring the efficacy of enzyme supplementation therapy that is currently being developed. (Brinkman et al 2005)
Evaluation of patients with MPS

Residual enzyme activity of MPS type I and type II

All MPS type I patients (6/6) had a relatively low residual α-L-iduronidase activity (< 2.0 nmol/h/mg protein) than the normal subjects (Table: 19). The average α-L-iduronidase activity in MPS type I patients was 1.06 (±0.75) nmol/h/mg compared to the average activity range in non affected individuals of 27.9 (±12.86) nmol/h/mg. All MPS type II patients (11/11) had low enzyme Iduronate 2 sulfatase activity (< 2.5 nmol/h/mg protein) than the normal subjects (Table 20). The average Iduronate 2 sulfatase activity in MPS type II patients was 0.75 (±0.64)nmol/h/mg compared to the average activity range in non affected individuals of 23.5 (±5.54) nmol/h/mg.

Glycosaminoglycans, Biomarker Heparin Cofactor II - Thrombin and Mucopolysaccharidoses:

Similar to our design for Gaucher Disease we evaluated the utility of the new biomarker in comparison to the existing biomarker. Currently the diagnosis of MPS is confirmed using both urinary GAG levels and blood levels of deficient enzyme. However these methods are labor intensive and time consuming. Blood enzyme levels are not suitable after enzyme replacement therapy for monitoring disease progression as levels can fluctuate due to the presence of recombinant enzyme. Urinary GAGs are the primary biomarker as they represent the primarily storage material (Derrick et al 2008).
The primary blocks in MPS are the catabolism of GAGs hence they behave as primary biomarker. The reasons for limited utility of GAG’s as biomarkers needs clear understanding of their structure. The deficiency in all subtypes of MPS primarily involves the enzyme hydrolases which metabolize GAGs only from the non reducing end of the molecule. Thus in a particular subtype of MPS the GAG which accumulates would have the same non reducing end. MPS II would presumably lead to accumulate of GAGs in tissues with an 2-sulfatated iduronate residue (Clarke et al 2012).

Dermaton Sulfate, Keratan Sulfate, Heparan Sulfate and Chondroitan Sulfate are the broad category of GAG’s. GAG’s vary in chain length (10-150 disaccharides units), N sulfation, O-S sulfation, N- acetylation and epimerization of saccharides units within the chain. The widely used DMB dye binding assays simply measure the depolarized GAG’s. GAG’s are negatively charged and the dye is a positively charged reporter molecule with close affinity due to difference in polarity. The detection of the quantity of GAG,is based on the amount of reporter bound to the sample compared to dye bound with a known quantity of purified GAG’s. This assay is therefore unlikely to differentiate between different types of GAG’s (de Jong et al 1991).

GAG levels are fraught with variability due to the age and the hydration status of the patients. Also the estimation of total urinary GAG relates to non specificity of its measurement given the fact that it is a reflection of the renal tubular involvement/dysfunction rather than total body burden.
Langford smith have suggested that dermatan sulfate and chondroitin sulfate ratio (DS/CS) in urine would be more informative but these ratio is likely to be less reflective of the total disease burden and dependant on renal function (Kia Langford et al 2010). Another limitation is the variation with age which for urinary GAG’s in particular is maximal in early childhood. This is precise point of time where it could be of maximal utility making reliable cut offs a difficult proposition. Evaluating urinary GAGs in 35 South American patients with MPS type II reported higher urinary GAG in 18 patients with neurological involvement than those without it (p= 0.042) (Schwartz et al 2007). This states their limitation to correlate with the total body burden. Considering these limitations there emerged the need to evaluate another biomarker.

This utility of HC II T was initially demonstrated in murine models by Derrick et al. 2008 and also demonstrated in the MPS patients. The first criterion for evaluating a biomarker resides in its reliability to reflect the disease. We found a significant difference in the levels of HCII T found in healthy controls with median being 39 (12-60 ng/ml) compared to the disease group being , 455 ng/ml (330-550ng/ml) in MPS type I and 340 ng/ml (265-530ng/ml) in MPS type II (p < 0.001). There was no overlap between healthy controls and patients with disease with HCII T levels being 5-10 folds higher. Similar results have been reported by (Langford smith and Randall et al 2008.)
Regarding the efficacy to adequately predict course of disease we had the following observations. We found an increasing level with medians being 455 ng/ml, 440.2 ng/ml, and 510 mg/ml in MPS Type I, and 340 ng/ml, 356.7 ng/ml and 352.5 ng/ml at the end of the recruitment, close of 1st year and close of 2nd year. This gives an increasing trend but we could not demonstrate a statistically significant figure. The plausible reason for this could have been the short duration of follow up or inclusion of attenuated phenotype which may demonstrate slow progression and wide variability in the course of disease.

Regarding specificity, HC II T belongs to a protease inhibitor (serpin) family (D.M. Tollefsen et al 2007). Each serpin regulates the activity of its protease though a “suicide mechanism”, where the protease initiates proteolysis of the serpin but cannot complete the reaction, thereby forming a covalent linkage between the two proteins. The interaction of dermatan and heparan sulfate proceeds when a negatively charged GAGs displaces the regulating arm of the HCIIT that subsequently binds to exosite 1 of thrombin and exposes HCIIT reactive central loop to thrombin active site (Huntington et al 2003). Through this interaction GAGs are able to increase HCIIT reactivity with thrombin by 3 orders of magnitude. The specificity for dermatan sulphate needs further elaboration. Dermatan sulfate is selectively accelerated and this may be related to the need to prevent thrombin activation in intravascular spaces; where 40% of HCIIT is localized [Huntington et al 2003, He et al 2002]. Thus we selected patients with MPS I and II where the primarily GAGs stored is dermatan sulfate with some amount of keratan sulfate.
Specifity of HCII T to a particular subgroup: We evaluated the utility of MPS as a valuable biomarker to distinguish between MPS I and II. We could not demonstrate any significant difference in HCII T levels amongst patient with MPS I and II. Through the understanding of the pathophysiology of the disease and the support obtained by NMR data suggest distinct sulfation pattern in DS stored in patients with MPS I as compared to patients with MPS II we could (Harmatz et al 2006) have expected otherwise. The probable explanation could be small sample size, this being a rare disease or the wide variation innate to the disease itself demonstrated by wide interquartile range in these 2 sets of MPS which may have objectively limited the statistical interpretation of the data.

Another important criterion in evaluating a biomarker is the ease of monitoring and its reliability for monitoring response during therapy. Though we had the limitation that we could recruit only one patient who demonstrated a statistically decline from 330ng/ml to 135ng/ml levels probably this reflects one the most potential use of this marker. Another group studied plasma derived GAG’s which were found to decrease with ERT regardless of age and severity of disease (Langford et al 2011). This may limit their utility as a reliable marker for evaluating response to therapy. Clarke et al 2012 demonstrated significant fluctuation in HCIIT levels which remained above normal range despite normalized in urinary GAGs. The authors found that in patients with antibodies to recombinant idurosulfase HCII T levels was reasonably high, at times, even reaching pretreatment values. These elevations were minimally reflectd in urinary GAG levels. This indicated that HCIIT assay may reflect rapidly the changes in clinical status of the patients.
To conclude HCIIT is a reliable screening biomarker for this complex multisystemic group of disorders. This is a reliable secondary biomarker that is a reflector of altered cellular tissue homeostasis, hence is likely to be more informative. However, considering the limitation to differentiate well between subtypes and to predict the course in attenuated phenotypes, we need to move from organ or pathway specific biomarkers. We need to comprehend that rather than focusing on ‘one stop-shop biomarker’ we need to utilize a panel of biomarkers to correlate with prediction, typification, disease progression and response to therapy.