A Review

Novel biomarkers for inborn errors of metabolism in the metabolomics era

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Inborn errors of metabolism arise due to deficiency of enzymes in metabolic pathways. Recent years have seen the onset of novel technologies like proteomics, metabolomics, lipidomics, and urinomics. In the past many novel biomarkers have been developed like succinylacetone for tyrosinemia, a number of organic acids for organic acidurias, acylcarnitines for the detection of fatty acid oxidation disorders and organic acidurias etc. With the advent of the -omics technologies, the number of novel biomarkers has increased dramatically. The use of techniques like mass spectrometry and NMR spectroscopy has led to a faster diagnosis of inborn errors of metabolism and smaller sample requirement. Hundreds of markers can be detected in a single urine sample, making diagnosis easier. There is an urgent need for implementing such techniques into routine practice in India.

Keywords: Acylcarnitine, Allo-isoleucine, Citrulline, Mass spectrometry, Magnetic resonance spectroscopy, Orotate, Succinylacetone.

Inborn errors of metabolism (IEM) result from the deficiency of enzymes in metabolic pathways. Most IEM are inherited as autosomal recessive disorders. More than 500 different IEMs are known.

Deficiency of an enzyme in a pathway can produce the effects such as a) accumulation of the substrate of that enzyme, b) the pathway may be directed to an alternate pathway, leading to accumulation of abnormal metabolites, and c) biochemically important products of the main pathway are reduced. These basic metabolic alterations are responsible for the diverse clinical manifestations1.

Metabolomics is the quantitative analysis of a large number of low molecular weight metabolites that are intermediate or final products of all the metabolic pathways in a living organism2. Any metabolic profiles detectable in a human biological fluid are caused by the interaction between gene expression and the environment. Genomics, transcriptomics, and proteomics are well-established technologies and are commonly used by many scientists. In comparison, metabolomics is an emerging field and has not reached such high-throughput, routine and coverage as other omics technologies (Fig. 1). Metabolome profiling is mainly hampered by its diversity, variation of metabolite concentration by several orders of magnitude and biological data interpretation. Thus, multiple approaches are required to cover most of the metabolites. Urinary metabolomics, for example, has been used in the recent years, for the discovery of a variety of biomarkers, including pediatric disorders3.

The two primary tools for metabolomics studies are mass spectrometry (MS) and NMR spectroscopy, which has distinct capacities and limitations, and being complementary, should ideally be used together. MS is more sensitive and has a wider coverage of the metabolome. However, analytical bias cannot be avoided in MS. Quantitation is also challenging by MS. NMR requires minimal sample
handling, allows easy quantitation and is a redundant method for metabolite identification. Metabolites normally have multiple characteristic NMR resonances and coupling patterns, and peak intensity is directly proportional to concentration. NMR metabolomics is used to identify the cause of disease process, and for rapid identification of biomarkers4.

The rise of the various “-omics” techniques give new scope and challenges in the screening, diagnosis, treatment, and monitoring of inborn errors of metabolism5. These techniques offer a “global view” on the various cellular biomolecules. The holistic strategies employed in such techniques are a clear contrast from the “reductionistic” approach practiced earlier.

Nuclear magnetic resonance spectroscopy (NMRS, MRS) and IEM

Metabolomics-based diagnosis: NMR spectrum of urine can identify more than 1000 metabolites simultaneously. This has been employed in the detection of markers in numerous inborn errors of metabolism5. NMR-based screening for inborn errors of metabolism is practiced as more metabolites can be detected. Fast, simple and cost-effective screening is possible. Neuropsychiatric disorders like schizophrenia, panic disorders, major depression, bipolar disorders and autism-spectrum disorders have been investigated systematically using NMR spectroscopy6.

In vivo MRS data are available in a number of IEMs. A number of studies on rat and human subjects with phenylketonuria have been reported7,8. The brain lesions in glutaric aciduria type I have been investigated using MRI studies9. L-2-Hydroxyglutaric aciduria is a rare disorder characterized by increased excretion of 2-hydroxy glutaric acid and shows abnormal MRS proton signals10. MRI studies on patients with type II glutaric aciduria showed characteristic brain lesions, hypoplastic temporal lobes, and hypoplasia of corpus callosum11. 1H-MRS also showed a high choline-creatine ratio in the brain. Lactate, branched chain amino acids and oxo-acids were elevated in the brain in patients with maple syrup urine disease (MSUD)12. 1H-MRS of basal ganglia showed decreased NAA, inositol, and elevated glutamine/glutamate ratio. Decreased N-acetyl aspartate and increased myoinositol and choline were detected in 3-hydroxy 3-methyl glutaryl CoA (HMG CoA) lyase deficiency in a combined MRI/MRS study13. 1H NMR of body fluids was used to detect 3-Methylglutaconic aciduria type I. The analyte was detected in CSF, plasma, and urine14. MR spectroscopy has thus found wide applications in the diagnosis of IEM15.

Tandem mass spectrometry and IEM

Tandem mass spectrometry (TMS) is used widely in western countries for newborn screening of inborn errors of metabolism. Screening can be done on heel-prick blood samples with high sensitivity and specificity. A detailed description of the same is beyond the scope of this article. Interested readers are requested to refer several interesting reviews on the same16-18. Its use in India is still not very popular, however the trend in slowly changing

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Fig. 1—Schematic representation of workflow in metabolomics techniques (Reproduced with permission, Malet-Martino et al., J Pharm Biomed Anal. 2011)
with more and more centres interested in urine TMS for newborn screening.

**Biomarkers for inborn errors of metabolism**

*Sucinylacetone for tyrosinemia type I*

Tyrosinemia type I is an inborn error of metabolism due to defect in the enzyme, fumarylacetoacetate hydrolase. Many mutations in the FAH gene have been linked with tyrosinemia type I and currently at least 86 mutations have been linked with the disorder. There are severe liver and kidney failure associated with neurological manifestations and other symptoms, which start from infancy. The human gene is located on 15q25.1. The gene was isolated in 1991. Defects in the gene lead to spindle disturbances and segregational defects and sustained activation of extracellular signal-regulated protein kinase (ERK). Intracellular glutathione supplementation restores these defects by up to 80%.

If untreated, tyrosine and its metabolites build-up and accumulate and leads to toxicity. Other forms of tyrosinemia exist and are due to other enzyme defects. These include tyrosinemia type II and tyrosinemia type III. Worldwide incidence of tyrosinemia type I is 1 in 100000, though it is more common in Canada and Norway. The condition is inherited in an autosomal recessive pattern.

Table 1 Tyrosinemia type I is often asymptomatic at birth and can be detected by elevated tyrosine levels in the blood by either neonatal screening using tandem mass spectrometry or high-performance liquid chromatography (HPLC). However, tyrosine levels may be elevated in all types of tyrosinemias. Tyrosinemia type I can be treated successfully using nitisinone (2-(2 nitro-4-3 trifluoro-methylbenzoyl)-1,3-cyclohexanedione, NTBC). Molecular testing can be used for identification, but the exact identification of mutations is often more difficult and may be more time-consuming. Succinylacetone estimation is an easier way for the specific identification of tyrosinemia type I (Table 1).

IVS12 + 5G>A (1062 + 5G>A) splice site mutation responsible for more than 90% of HT-1 mutations in Quebec population. It is recommended that SUAC is a better NBS marker than tyrosine for the diagnosis of HT-1.

Medical therapy for HT-1 with nitisinone was discovered incidentally. Nitisinone blocks the catabolic pathway of tyrosine and leads to a reduction in the accumulation of toxic tyrosine metabolites. It has to be supplemented with a low-protein diet, which is deficient in tyrosine and phenylalanine. Nitisinone therapy has to be started early in life to avoid sequelae; ideally in the newborn period. Hepatocellular carcinoma, liver and kidney dysfunction, rickets, and neurological crises can be prevented if nitisinone therapy is started in the newborn period. Reversible eye symptoms may occur as a side-effect of nitisinone, but other side effects are rare. Some patients have an impairment of neurocognitive development. Spot tests are available for detecting nitisinone.

Studies from Europe show that selective screening for HT-1 is not as effective as NBS for the detection and prevention of HT-1. SUAC inhibits the enzyme that metabolizes δ-aminolevulinic acid and hence δ-aminolevulinic acid accumulates, which is responsible for the neurological effects of HT-1 (porphyria-like effects) when treated with tyrosine and phenylalanine-restricted diet alone. These disappear when treated with NTBC.

Urinary concentrations of SUAC are much more than blood and hence urine is commonly used for its estimation. In a study from India on 3 patients with HT-1, SUAC was found to be elevated. Early treatment would require a diet low in phenylalanine and tyrosine, administration of 2NTBC, and liver transplantation. Liver transplantation is an effective treatment for TT1 with good quality of life. The major indications are non-response to NTBC, the risk of malignancy and poor quality of life-related to dietary restriction and frequency of blood sampling.

SUAC concentration is affected by hematocrit values, and to a lesser extent, by blood volume. A study from the UK found that SUAC concentration remains elevated even after liver transplantation. The

<table>
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<tr>
<th>IEM</th>
<th>Biomarker</th>
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<tbody>
<tr>
<td>Maple Syrup Urine Disease</td>
<td>Alloisoleucine</td>
<td>LC-MS/MS</td>
<td>Very high (Up to 100%)</td>
<td>Very high (Up to 100%)</td>
</tr>
<tr>
<td>Tyrosinemia Type I</td>
<td>Succinylacetone</td>
<td>LC-MS/MS</td>
<td>Very high (Up to 100%)</td>
<td>Very high (Up to 100%)</td>
</tr>
<tr>
<td>Fatty acid oxidation disorders</td>
<td>Acyl carnitine</td>
<td>LC-MS/MS</td>
<td>Very high (Up to 100%)</td>
<td>Very high (95%)</td>
</tr>
<tr>
<td>Urea cycle disorders</td>
<td>Urine orotate, Plasma citrulline</td>
<td>LC-MS/MS</td>
<td>High</td>
<td>High</td>
</tr>
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success of treatment and follow up has also been done by assessing NTBC levels\textsuperscript{33}.

Prenatal diagnosis of HT-1 is possible by detection of SUAC in amniotic fluid\textsuperscript{34}. Phenylalanine and homogentisate increase the concentration of succinylacetoacetate and succinyl acetone both in serum and urine in patients with hereditary tyrosinemia and therefore increase the excretion of 5-aminolevulinate. Both phenylalanine and homogentisate cause a tubular proteinuria. Their metabolites maleylacetoacetate and fumarylacetoacetate are the toxic compounds in hereditary tyrosinemia. The patient with the highest excretion of succinyl acetoacetate and succinylacetone has the slightest tubular proteinuria, whereas the one with the lowest excretion of these compounds has the more pronounced tubular proteinuria\textsuperscript{35}.

**MSUD, Allo-isoleucine**

Maple Syrup Urine Disease (MSUD) is due to the deficiency of the enzyme, branched chain keto acid dehydrogenase (BCKAD) complex. It catalyses the second step in the metabolism of branched-chain amino acids (BCAA), valine, isoleucine, and leucine. BCKAD has 4 sub-units, E1a, E1b, E2 and E3. Defects in any of the sub-units of the enzyme can lead to accumulation of BCAA and branched-chain keto acids (BCKA)\textsuperscript{36,37}. MSUD can be of classic and intermediate types. Corresponding to the enzyme sub-unit deficiencies associated, it may also be classified into type 1a, 1b, and 2. BCKDHA gene encodes sub-unit 1a, BCKDHB encodes 2a, and DBT encodes sub-unit 2.

Elevated plasma levels of BCAA and allo-isoleucine are seen within 12-24 h in classic MSUD. Clinical features are seen in the first week of life and include ketonuria and ketonemia, irritability and poor feeding, encephalopathy with lethargy, apnea, and stereotyped movements. Within ten days coma and central respiratory failure ensues. Intermediate MSUD is due to partial deficiency of BCKAD enzyme and has a better outcome with response to treatment with thiamine. These patients exhibit intermittent symptoms of deficiency of the BCKAD enzyme, and these include severe metabolic encephalopathy precipitated by catabolic stress\textsuperscript{38}.

Management includes dietary restriction of leucine, formula feed deficient in BCAA, judicious supplementation of isoleucine and valine, and frequent clinical and biochemical monitoring. Treatment of metabolic decompensation is with sufficient calories, insulin, isoleucine, and valine. Orthotopic liver transplantation is effective for the treatment of classic MSUD. Pregnant women with MSUD should also have strict control of BCAA and frequent biochemical monitoring\textsuperscript{39}.

MSUD can be diagnosed by elevated levels of BCAA or the non-protein amino acid, L-allo-isoleucine (>5 μmol/L), which is seen in all forms of MSUD\textsuperscript{40}. On some chromatographic runs, allo-isoleucine coelutes with isoleucine, making interpretation difficult. NBS programs by TMS are available for the detection of BCAA and allo-isoleucine. Urine testing by dinitrophenylhydrazine (DNPH) was used prior to the emergence of TMS technique; even though less sensitive and specific, it can be used as a screening test in centres where advanced testing facilities are not available. Residual enzyme activity in fibroblasts can be measured and will be less than 3% in classic type, and 3-40% in intermittent-type MSUD\textsuperscript{41}.

Defects in BCKDHA account for 45% of cases, BCKDHB 35%, and DBT gene for 20% of cases of MSUD. Many mutations and allele-variants of these genes have been recognized. Differential diagnosis of MSUD includes non-ketotic hyperglycinemia, urea cycle disorders, and rarely methylmalonic acidemia and propionic acidemia. Typical neuroimaging features may be seen on magnetic resonance imaging (MRI), but the patients during the acute phase are usually too unstable to be subject to the MRI procedure. Hence biochemical diagnosis remains the keystone to the diagnosis\textsuperscript{42}. Inheritance of MSUD is an autosomal recessive manner. Heterozygotes (carriers) are clinically asymptomatic.

Allo-isoleucine/phenylalanine ratio is considered as a useful biomarker for the diagnosis of MSUD\textsuperscript{43}. Studies suggest that second-tier NBS screening with allo-isoleucine help to improve detection of variant forms of MSUD\textsuperscript{44}. Hepatocyte transplantation tends to restore allo-isoleucine levels towards the normal\textsuperscript{45}.

Allo-isoleucine is derived from transamination of isoleucine \textit{in vivo}. It may be present in normal individuals, but even then, only in trace amounts and not normally detected easily. It is elevated in all forms of MSUD. It is especially important in the detection of variant forms of MSUD. It is not elevated in diabetes mellitus. Transient elevation of branched chain amino acids occurs in ketotic hypoglycaemia and starvation, but these conditions are not associated with elevation of allo-isoleucine. The levels tend to get elevated even before the clinical onset of symptoms, and before acute metabolic crisis sets in and hence it would be a useful NBS marker\textsuperscript{40}.
BCAA are subject to diurnal variations including dietary variations, however, allo-isoleucine is unaffected by such variations.46.

**Acylcarnitines as a biomarker for IEM**

Plasma acylcarnitine levels are used for the diagnosis of fatty acid oxidation disorders and organic acidurias and for their follow-up and evaluation of treatment. In newborn screening, C5, C6, C8 and C10 acylcarnitine levels are commonly employed in different algorithms.47,48.

Fatty acid oxidation plays a major role in energy production during periods of fasting. The acyl groups in fatty acids can be conjugated with carnitine, to form acylcarnitine, which are measured by tandem mass spectrometry. Acylcarnitine analysis can be used to diagnose more than 20 inborn errors of metabolism, including fatty acid oxidation disorders and organic acidurias.48.

Fatty acid oxidation disorders include carnitine palmitoyltransferase I (CPTI) deficiency, medium-chain 3-ketoacyl-CoA thiolase (MCKAT) deficiency, dienoyl-CoA reductase deficiency, short-chain acyl-CoA dehydrogenase (SCAD) deficiency, medium/short-chain 3-hydroxyacyl-CoA dehydrogenase (M/SCHAD) deficiency, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency & trifunctional protein deficiency, very long-chain acyl-CoA dehydrogenase deficiency [MADD]; glutaric acidemia type II)47,48.

Organic acidurias that can be detected by acylcarnitine analysis are glutaryl-CoA dehydrogenase deficiency (glutaric acidemia type I), propionic acidemia, methylmalonic acidemia, isovaleric acidemia, 3-hydroxy-3-methylglutaryl-CoA carboxylase deficiency, 3-methylcrotonyl carboxylase deficiency, biotinidase deficiency, multiple carboxylase deficiency, isobutyryl-CoA dehydrogenase deficiency, 2-methylbutyryl-CoA dehydrogenase deficiency, β-ketothiolase deficiency, malonic aciduria ethylmalonic encephalopathy and glutamate formiminotransferase deficiency (formiminoglutamic aciduria).47,48.

Confirmatory diagnosis usually requires further biochemical studies like enzyme assays or molecular diagnosis. Rarely false-negative results may also occur. For MCAD deficiency and certain other disorders, calculation of acylcarnitine species’ ratios is often employed. These are usually included in the algorithm itself. Dietary factors, anabolic/catabolic state and treatment status can affect acylcarnitine values and can confound the results. Fasting is however, not required for acylcarnitine analysis as it can lead to catabolic stress and becomes a confounding factor. Many modifications of the acylcarnitine analysis technique have been suggested.49-53.

**Differential diagnosis of urea cycle disorders**

Urea cycle disorders (UCD) are characterized by high plasma ammonia levels above 150 μmol/L, normal anion gap, blood glucose and in many cases, metabolic alkalosis. There are mainly five disorders, carbamoyl phosphate synthetase (CPS) deficiency, ornithine transcarbamoylase (OTC) deficiency, argininosuccinate lyase (ASL) deficiency, citrullinemia, and argininemia. If the defect in one of the proximal enzymes of the urea cycle, they are known as proximal UCD and if the latter enzymes are deficient, they are known as distal UCD. Differential diagnosis of UCD is possible by the use of plasma citrulline and urine orotate. Citrullinemia, as the name suggests is characterized by high plasma citrulline. Elevated citrulline levels are also seen in ASL deficiency. Low citrulline levels are seen in CPS and OTC deficiencies. Out of which, urine orotate is elevated in OTC deficiency and low or normal in OTC deficiency. Using a combination of the markers, urine orotate, and plasma citrulline, along with blood ammonia, and blood gases, differential diagnosis of UCD can be made, even without employing tandem mass spectrometry.54.

**Conclusion**

Many biomarkers have been discovered which have made the diagnosis of inborn errors of metabolism accurate and easy. Techniques like tandem mass spectrometry and MR spectroscopy have unleashed a diagnostic armamentarium, which was unknown prior to the advent of these techniques. However, not much work has been done in the Indian sub-continent in this important category of disorders. There is an urgent need for more focus and more research. With a different genetic background, there is still a high chance that more biomarkers might be discovered in this exciting area of clinical chemistry. It needs to be remembered also that the discovery of biomarkers is often a serendipitous process. This is however not a substitute for hard work and application.
References


