Amino Acid Profile and Lactate Pyruvate Ratio: Potential Adjunct Markers for Differentiating Inborn Errors of Metabolism

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Abstract Inborn errors of metabolism (IEM) lead to the physical and mental disability and death of infants, which can be prevented if treated early. Hence it is imperative in diagnosing these disorders at the earliest. The study is planned to differentiate suspected IEM by quantifying the lactate–pyruvate ratio (L/P ratio), Amino acid profiling by HPLC in addition to blood ammonia using the basic methodology in comparison to globally accepted mass spectroscopy technique and specific enzyme activity assays. The objective of this study is not to compete with the existing gold standard methodology rather makes the best of basic diagnostic modalities available. Five cases out of 100 referred cases of IEM suspicion showed the altered pattern of Aminogram correlating with high L/P ratio and Hyperammonemia, which were positive cases, as

confirmed by the clinician who referred. Hence study parameters could be used as preliminary protocol to investigate and screen the IEM.

Keywords Inborn errors of metabolism · Lactate–pyruvate ratio · Amino-acid profile · Hyperammonemia · Mitochondrial disorder

Introduction

Inborn errors of metabolism are the group of congenital disorders where mostly defect lies in a single gene coding for an enzyme. This defect leads to block in the normal metabolic pathway which in turn leads to the toxic accumulation of the intermediates or deficiency of essential intermediates. This toxic accumulation or deficiency of intermediates alters the physical and mental health of the infants, if not treated timely.

There are different types of IEM disorders, to name a few-amino acid disorders, urea cycle disorders, disorders of carbohydrate metabolism, Lysosomal storage, and mitochondrial disorders. Most of the symptoms of these disorders like poor feeding, vomiting, failure to thrive, developmental delay and diarrhea overlap with other IEM and also non-IEM disorders. This makes the first line of suspicion for IEM difficult.

The other challenge is availability and viability of worldwide accepted diagnostic modalities like tandem mass spectra techniques; whole genome sequencing and specific enzyme assay to screen and confirm IEM in India where the concept of mass newborn screening for IEM disorder is not a standard operational protocol [1]. There are very limited diagnostic laboratories in India which offer confirmatory tests for IEM disorders using mass

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spectrometry technique and specific enzyme activity assays in comparison to huge live births in India (19.3 births/1000 population, 2016 est.), the burden of which obviously delay the turnaround time for confirmation of the disease including the pre and post-analytical error as an added problem to it. Some of the treatable IEM disorders like Phenylketonuria, Galactosemia, and mitochondrial disorders require immediate intervention otherwise lead to permanent disability or fatal consequences. The focus of this study is to evaluate the IEM disorder at the earliest using basic techniques so that the intervention can be initiated much before the confirmatory diagnosis report to avoid irreversible damage. So this study planned to screen the suspected cases of IEM by quantifying the LP ratio, Blood Ammonia, and amino acid profile.

Lactate and pyruvate are normal intermediary metabolites in blood having an essential role in energy production and utilization. Thus their relative concentrations in blood reflect the nutritional and cytosol oxido-reduction status of an individual. Hyperammonemia is an important laboratory finding associated with IEM disorder. However, normal ammonia level doesn't exclude the absence of IEM disorder. Concentrations of various amino acids in plasma depend on the metabolic state of the individual and thus an altered plasma amino acid profile is a metabolic consequence of impairments in the electron transport chain in mitochondria and associated amino acid metabolism [2, 3].

Since the parameters mentioned has their role in overlapping symptoms of IEM from non IEM disorder, the objective of this study is to find the characteristic pattern of all three parameters: L/P ratio, Ammonia and amino acid profile in differentiating IEM disorders particularly mitochondrial and respiratory chain abnormalities from non IEM disorders.

Materials and Methods

This prospective experimental study was conducted for a period of 4 months in the year 2016 at Center for Excellence-IEM Laboratory, Department of Biochemistry, KMC Manipal, Karnataka, India. Institutional ethical clearance was obtained for the study. 100 blood samples which were sent for the analysis of suspected IEM disorders were taken after the accomplishment of requested analysis. Arterial blood was used for the estimation of lactate and Ammonia and levels were measured using an enzymatic method in Cobas 6000 autoanalyser. Estimation of pyruvate was done using enzymatic method [2]. Blood for lactate estimation was collected in fluoride tube, ammonia in EDTA tube and for pyruvate in tube containing ice cold perchloric acid (perchloric acid deproteinize the blood sample). Selected Amino acids were profiled by Reverse Phase HPLC

(Shimadzu) involving pre-column derivatization with o-phthalaldehyde (OPA) derivatization [4]. All the Chemicals used for the study were of Analytical grade and obtained from Sigma Aldrich and organic solvents were of HPLC grade and were from Rankem.

Quality Assurance for the Biochemical Parameters

Split sample testing was done for blood pyruvate and amino acid profiling to assess variability, analytical errors, accuracy and reliability of the test results. For lactate and ammonia, the lab participated in External Quality Assurance Program and the Z score was satisfactory for these parameters.

Statistical Analysis

Data were compiled and appropriate statistical methods were used wherever necessary.

Result

The study participants were grouped based on biochemical parameters namely lactate pyruvate ratio (L/P Ratio) and plasma ammonia levels as in Table 1. The median values and the interquartile ranges for different parameters including amino acids is shown in Table 1. Individual amino acids of each group were sub grouped into two to report their relative frequency distribution under each category namely L = less than normal cut off range and H = more than the cut-off value in Fig. 1. Table 2 gives the biochemical investigation details obtained from the study for probable positive diagnosis of mitochondrial disorder and Pyruvate Carboxylase deficiency.

In Group 2 of Normal L/P ratio and high ammonia subjects, Methionine levels were reduced significantly ($p = 0.031$) compared to Group 1 with Normal L/P ratio and normal ammonia. Tryptophan with median = 129 $\mu\text{mol/L}$ (54.5, 620) in group 3 of high L/P ratio and high ammonia subjects showed significant elevation ($p = 0.047$) in comparison to group 4 of High L/P ratio and normal ammonia with elevation of tryptophan levels, median 80.9(31,111). Tryptophan in group 2($r = 0.591$, $p = 0.008$), group 3($r = 0.591$, $p = 0.008$) and group 4($r = 0.382$, $p = 0.001$) showed strong correlation with alanine levels. Though none of the amino acids showed statistically significant difference between groups: 1 and 3, 1 and 4, 2 and 3, 2 and 4, strong correlation was observed between the individual amino acids as well as between the amino acids and other parameters as shown in Table 3.

Table 1 Lactate–pyruvate ratio (L/P ratio) and plasma ammonia levels

Biochemical parameters	Group 1 Normal L/P ratio and normal ammonia (n = 15) Median (IQR)	Group 2 Normal L/P ratio and high ammonia (n = 12) Median (IQR)	Group 3 High L/P ratio and high ammonia (n = 19) Median (IQR)	Group 4 High L/P ratio and normal ammonia (n = 43) Median (IQR)	Normal range
Ammonia	48 (45, 54)	81 (80, 197.7)	96 (87, 191)	48 (30, 58)	18.7–80 µg/dl
Lactate	8.7 (7, 13.7)	13.35 (10.05, 13.5)	21.7 (12.8, 48.2)	18.2 (14.8, 26.9)	14–19.8 mg/dl
Pyruvate	0.38 (0.29, 0.58)	0.79 (0.58, 0.84)	0.3 (0.2, 0.44)	0.35 (0.25, 0.56)	0.3–2 mg/dl
LP ratio	21 (19, 22)	16.15 (12.7, 19.0)	80 (41, 169)	56 (41, 81)	16.6–22
Asp	16.7 (0–26)	26.95 (0, 46.2)	30.3 (0.09, 51.75)	7 (0, 23)	50–110 µmol/L
Ala	71 (28, 336)	37 (10, 922)	533.2 (21, 1378)	81 (21, 476)	30–100 µmol/L
Trp	95 (48, 371)	73 (12, 87)	129 (54.5, 620)	80.9 (31, 111)	30–80 µmol/L
Lys	81 (33, 112)	47 (16, 106)	89 (22, 1519)	86 (22, 330)	74–220 µmol/L
Met	18 (10, 100)	11 (8, 16)	13.4 (4, 30.5)	10 (7, 25.2)	22–60 µmol/L
Phe	89 (7.7, 182)	95.5 (90, 115.2)	74 (16, 137.1)	56 (9, 156)	72–100 µmol/L
Tyr	228 (27, 583)	228 (1.025, 615.7)	228 (45, 839)	246 (2.25, 545)	44–142 µmol/L
Ala/Lys	0.81 (0.6, 2.4)	0.6 (0.37, 17.2)	0.7 (0.5, 5.4)	0.6 (0.2, 6.2)	< 3
Ala/Phe+Tyr	0.09 (0.04, 1.82)	0.04 (0.03, 7.78)	1.73 (0.03, 5.2)	0.07 (0.04, 2.89)	< 4

Mann–Whitney U test, Median values with IQR1 and IQR 3 * total 100 sample was recruited for analysis but only 89 are considered for statistical analysis. The reason to exclude them from the study is an insufficiency of the sample to perform all the parameters

Fig. 1 Bar diagram showing low and high cut off limits for aminoacids

Discussion

The L/P ratio reflects cytoplasmic NADH/NAD ratio and thus the diagnostic marker for cytosolic oxido reduction state [5]. When there is a block in pyruvate oxidation either because of respiratory chain dysfunction or deficiency in pyruvate dehydrogenase complex enzyme activity, pyruvate metabolism takes an alternate pathway of lactic acid and alanine production or gluconeogenesis leading to lactic

acidosis and hyperalaninemia in case of former alternate route and hyperglycemia in the later. Elevated lactate concentrations in blood may occur in spite of fully functional PDH complex in case of respiratory chain dysfunction. The increased rate of anaerobic Glycolysis taking place in cytosol and subsequent compromise of energy metabolism will increase the NADH/NAD-ratio and thus elevated lactate levels in cytosol which mirror the elevated L/P ratio in blood [6].

Table 2 Positive case 1 to 4: mitochondrial disorders and 5 pyruvate carboxylase deficiencies

Positive case	L/P ratio	Ammonia (µg/dl)	Lactate (µg/dl)	Asp (µmol/L)	Ala (µmol/L)	Trp (µmol/L)	Lys (µmol/L)	Met (µmol/L)	Phe (µmol/L)	Tyr (µmol/L)	Ala/Lys ratio	Ala/Phe + Tyr ratio
1	79.7	20	34.3	2	189	20	6	2	9	0	31.5	21
2	90	53	31	0	465	30	7	8	6	23	66.4	16.03
3	169	82	69	30	1130	28	42	13	0	188	26.9	6.01
4	500	31	148	239	2646	9	477	22	9.5	4	5.5	196
5	155	87	82.3	0.12	3353	329	4878	18149	37.5	4	0.7	80.8

Total 100 samples with suspicion of IEM disorder which was referred for blood gas analysis or other biochemical parameter were taken for the study after their analysis. But only 89 samples were sufficient to process for all the parameters mentioned in the study and same number is used for the final data analysis. Although, the separations of 20 amino acids were accomplished in the present method, we have focused only those amino acids which fall into the scope of this study. Increased concentration of Glutamine and Glutamate were not taken into the consideration as they are invariably elevated in hyperammonemia condition and also on delayed analysis.

Of the total samples, 62 samples showed elevated lactate pyruvate ratios (group 3 and 4) in plasma, 31 cases showed elevated lactate levels in plasma which is a nonspecific marker of mitochondrial disease as reported by Haas et al. [7, 8] and only 7 out of these 31 elevated lactate population are found to fall into hyperalaninemia category following characteristic pattern in amino acid profile that is increased alanine: lysine ratio more than 5 (normal < 3:1) and alanine: phenylalanine + tyrosine more than 6 (normal ratio < 4:1) supporting the hypothesis of abnormal increase in plasma alanine levels in case of mitochondrial disease. Alanine elevation alone can never be a marker for mitochondrial disease as it can also be elevated post metabolic crisis due to physiologic stress and regression. Thus alanine quantification along with lactate pyruvate ratio, blood lactate concentration and clinical symptoms could be an adjunct markers for identification of mitochondrial disease [7]. However elevated alanine with decreased Lysine, Tryptophan, and Methionine further confirmed four of the total IEM disorders in the study as mitochondrial disorder. So we could confirm 4 mitochondrial disorders from the findings of our study which was later on counter verified with the treating clinician.

Another interesting pattern which was observed in the study was significantly low aspartate levels along with increased lysine and alanine levels in a patient with hyperammonemia and high LP ratio suggesting the pyruvate carboxylase deficiency. This can be further confirmed by estimating citrulline levels [9].

Significant persistent elevation in tryptophan in correlation to alanine in all the three groups (2, 3 and 4) supports the basis for clinical findings of neurological and behavioral symptoms like central fatigueness, hyper aggressiveness, hyper sensitivity, and facial dysmorphism which are commonly observed in IEM disorders.

Apart from the above discussed conditions, detailed analysis of amino acid profile in a particular case could give additional information which will help in understanding the underlying cause for the specific symptoms. This can be illustrated by the observation in a condition called chronic fatigue syndrome which is non IEM disorder. Elevated free tryptophan/branched chain amino acids ratio was observed in this condition as reported by Castell LM, et al. In the present study the confirmed four mitochondrial cases showed significantly high levels of tryptophan along with alanine. However we have not analyzed the statistical relationship between tryptophan and branched chain amino acids. Tryptophan/branched chain amino acid ratio appears to have a potential, as an additional marker to confirm and monitor mitochondrial disorder (impaired energy metabolism) [10].

Thirty one cases of elevated lactate–pyruvate ratio but without elevation in lactate were symptomatic and some were already under treatment. This necessitates the repeat test with post glucose load which may aid the confirmation of the diagnosis. With respect to amino acid profile, these thirty one cases did not show the expected alteration which could be due to the effect of therapy/the dietary status. Results of the present study suggest that dietary and therapeutic factors should be taken into consideration while sampling for the study of above parameters in the suspected IEM cases.

Conclusion

The results of the present study suggest that IEM disorders could be evaluated at the earliest with basic facilities so that dietary modifications can be initiated and the setbacks could be intervened early with appropriate fluids till the

Table 3 Correlation between the parameters within group

	Group 1		Group 2		Group 3		Group 4	
	r	p	r	p	r	p	r	p
L/P ratio with Phe	–	–	– 0.662	0.019	–	–	–	–
L/P ratio with Ala	–	–	–	–	0.591	0.008	–	–
L/P ratio with Ala/(Phe + Tyr)	–	–	–	–	0.550	0.015	–	–
Ammonia with Try	0.563	0.029	0.645	0.024	–	–	–	–
Ammonia with Ala	–	–	0.821	0.001	–	–	–	–
Ammonia with Ala/(Phe + Tyr)	–	–	0.765	0.004	–	–	–	–
Ammonia with Ala/Lys ratio	–	–	0.955	0.00	–	–	–	–
Lactate with Phe	– 0.516	0.049	– 0.882	0.000	–	–	–	–
Lactate with Tyr	–	–	–	–	– 0.519	0.023	–	–
Lactate with Ala/(Phe + Tyr)	0.551	0.033	–	–	–	–	–	–
Pyruvate with Asp	–	–	– 0.770	0.003	–	–	–	–
Pyruvate with Ala	–	–	– 0.586	0.045	–	–	–	–
Pyruvate with Lys	–	–	– 0.647	0.023	–	–	–	–
Pyruvate with Met	–	–	– 0.765	0.004	–	–	–	–
Pyruvate with Trp	–	–	– 0.586	0.045	–	–	–	–
Pyruvate with Ala/(Phe + Tyr)	–	–	– 0.647	0.023	–	–	–	–
Pyruvate with Phe	–	–	–	–	– 0.696	0.001	–	–
Asp with Ala	–	–	0.706	0.010	–	–	–	–
Asp with Met	–	–	0.708	0.010	–	–	–	–
Asp with Phe	– 0.567	0.028	0.708	0.010	–	–	–	–
Asp with Try	0.671	0.006	–	–	–	–	0.325	0.033
Asp with Ala/(Phe + Tyr)	–	–	0.585	0.046	–	–	–	–
Ala with Tyr	– 0.528	0.043	– 0.821	0.001	–	–	–	–
Ala with Lys	0.613	0.015	–	–	0.689	0.001	–	–
Ala with Ala/Lys ratio	0.784	0.001	0.706	0.010	–	–	0.652	0
Ala with Ala/(Phe + Tyr)	0.887	0.000	0.938	0.000	0.814	0.000	0.709	0.000
Ala with Try	–	–	0.821	0.001	0.591	0.008	0.382	0.011
Ala with Phe	– 0.627	0.012	–	–	–	–	–	–
Trp with Lys	–	–	0.703	0.011	0.645	0.003	0.427	0.004
Trp with Phe	– 0.618	0.014	–	–	–	–	–	–
Trp with Ala/Lys	–	–	–	–	–	–	–	–
Trp with Ala/(Phe + Tyr)	–	–	0.938	0.00	–	–	–	–
Lys with Ala/Lys ratio	–	–	–	–	–	–	– 0.385	0.011
Lys with Ala/(Phe + Tyr)	0.629	0.012	–	–	0.491	0.033	–	–
Phe with Tyr	–	–	–	–	0.667	0.002	–	–
Phe with Ala/Lys ratio	– 0.661	0.007	–	–	–	–	– 0.365	0.016
Phe with Ala/(Phe + Tyr)	– 0.518	0.048	–	–	–	–	– 0.315	0.04
Tyr with Try	–	–	– 0.645	0.024	–	–	–	–
Tyr with Ala/(Phe + Tyr)	– 0.786	0.001	– 0.765	0.004	–	–	– 0.406	0.007
Tyr with Ala/Lys ratio	–	–	– 0.955	0.000	–	–	–	–

Spearman correlation co-efficient—correlation between parameters within group, r = correlation coefficient, p = significance

confirmation of diagnosis is achieved. Thus further deterioration in the babies' health status can be slowed down.

Statistically significant correlation and the consistent pattern of variation observed between the amino acids in

relation to LP ratio and hyperammonemia helped us to narrow down the 100 high risk cases to 2 categories. Four of which were of mitochondrial disorders and one suggestive of pyruvate carboxylase deficiency. In addition,

detailed analysis of amino acid profile in a particular case could give additional information which will help in understanding the underlying cause for the specific symptoms of IEM disorders. However this detailed analysis warrants focused study of specific conditions.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Statement Ethical clearance approval obtained before the start of the study (IEC 817/2016).

Informed Consent Informed consents are not applicable. Residual samples after completion of the requested laboratory analysis were anonymized and used for the study. Study results were reported to sample anonymizer who conveyed the reports to the treating clinician.

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