RESEARCH ARTICLE

Diagnosis of inherited metabolic disorders by selective metabolite testing: three years experience at a tertiary care center in Rawalpindi

Ayesha Hafeez[1], Aamir Ijaz[2], Nayyar Chaudhry[3], Oshaque Ali[4], Muhammad Tahir Khadim[5]

Abstract

Objective: To determine the pattern of various inherited metabolic disorders specifically through plasma amino acid and urine organic acid testing in high-risk paediatric population.

Method: The cross-sectional retrospective study was conducted at the Armed Forces Institute of Pathology, Rawalpindi, Pakistan, and comprised data from April 2015 to March 2018 of children referred to the Department of Chemical Pathology and Endocrinology for work-up of suspected inherited metabolic disorders. Complete clinical history, baseline biochemical investigations, plasma amino acid and urine organic acid profiles, where indicated, were collected. Quantitative plasma amino acid and analysis was carried out by Ion Exchange Chromatography on Biochrome 30+ amino acid analyser, and urine organic acid analysis by Gas Chromatography-Mass Spectrometry. Findings were linked to the identified disorders. SPSS 21 was used for data analysis.

Results: Of the 805 cases reviewed, 49(6%) had an inherited metabolic disorder. Male:Female ratio of the cases was 1.5:1, and the median age was 240 days (interquartile range: 1-15695 days). The most common presenting symptom was seizures 316(39.3%) followed by lethargy 283(35.2%). Of the diagnosed cases, aminoacidopathies were 28(57%) and in them, non-ketotic hyperglycaemia accounted for 7(25%.). There were 12(24.5%) cases of organic acidurias followed by 9(18.4%) that were other than the two diagnoses.

Conclusion: The cases of inherited metabolic disorder detected indicated significant prevalence. Non-ketotic hyperglycinemia was the commonest disorder diagnosed.

Keywords: Ion Exchange Chromatography, Aminoacidopathies, Inherited Metabolic Disorders, Organic Aciduria. (JPMA 70: 53; 2020). **https://doi.org/10.5455/JPMA.301908**

Introduction

Although individually rare, the aggregate incidence of Inherited Metabolic Disorders (IMDs) may approach 1 in 1,000 newborns.[1] In a country like Pakistan, where marriages between cousins have prevailed for centuries and the rate of consanguinity is high,[2] there is an increased risk of inheriting various genetic disorders. In healthcare setups facing resource-constraints and limited technical expertise, the diagnosis of IMDs poses a real challenge. Diagnosing these disorders is not always very straightforward and involves a multi-disciplinary approach. The most difficult task for a clinician is to know when to consider IMD and which tests to order for evaluation.[3] The signs and symptoms for various disorders are non-specific[4] and, moreover, other diagnoses like sepsis, anoxic ischaemic encephalopathy (in neonates), pulmonary haemorrhage etc., may accompany IMDs which makes the diagnosis even more challenging. In any case, the optimal outcome for these children depends upon early recognition of the signs and symptoms, expert evaluation, and prompt referral to a centre familiar with the evaluation and management of these disorders. Another stumbling block in diagnosis is the need for the exclusion of routine childhood illnesses in the first place, unfamiliarity with biochemical inter-relationships[5] and inappropriate sample collection or storage. In all cases, a battery of certain routine biochemical investigations need to be carried out before undertaking the more sophisticated investigations, which compounds the expenses involved. The specific metabolite testing includes Amino acid (AA) and urine organic acid (OA) analysis which helps in diagnosing mostly aminoacidopathies as well as organic acidemias.

[1,3,4,5]Armed Forces Institute of Pathology, Rawalpindi;
[2]Rehman Medical Institute, Peshawar.
Correspondence: Ayesha Hafeez. e-mail: ayeshahafeez30@yahoo.com

Plasma AA analysis by ion exchange chromatography (IEC) is a state-of-the-art technique for the quantitative analysis of AAs in various body fluids like plasma, urine and cerebrospinal fluid (CSF). Multiple AAs and their metabolites can be analysed in each run. Likewise, urine OA analysis on gas chromatography mass spectrometry (GCMS) is an excellent state-of-the-art technique for detecting various organic acidemias. Final confirmation relies on enzyme and genetic analyses.[6]

There is no programme available on the national level for the diagnosis of IMDs and only a few centres in Pakistan are currently undertaking testing for specific metabolites like AAs and OAs for IMD testing. The current study was planned to determine the frequency and pattern of IMDs detected in our setup.

Materials and Methods

The cross-sectional retrospective study was conducted at the Armed Forces Institute of Pathology, Rawalpindi, Pakistan, and comprised data from April 2015 to March 2018 of patients referred from the paediatric outpatient department (OPD) and neonatal intensive care unit (NICU) to the Department of Chemical Pathology and Endocrinology for work-up of suspected IMDs.

Blood samples were collected for basic biochemical and specialised tests. Ammonia levels were collected in lithium heparin tube, and plasma lactate / glucose in sodium fluoride containers. Urine was collected in plain containers for non-glucose-reducing substances and qualitative metabolic screening ferric chloride 2,4-Dinitrophenylhydrazine (DNPH), cyanide nitroprusside and test for ketones. Blood complete picture (CP) was done on Sysmex Haematology analyser, while plasma ammonia, lactate and liver function tests (LFTs) were done on Clinical Chemistry Analyzer (Advia 1800-Siemens, Germany).

For plasma AA analysis, 2-3ml blood was collected in lithium heparin tubes and transported on ice to laboratory. The plasma was separated immediately by centrifugation at 3500 rounds per minute (rpm) for 5 minutes and frozen in aliquots at -80°C if there was a delay in analysis. AA analysis was carried on biochrome 30+ AA analyser (Biochrome® UK) based on ion exchange chromatography using lithium column (4.6mm diameter) for separation. Further, 5% sulfo-salicylic acid was added and the contents were allowed to stand for 30 minutes at 4^0C, followed by vortex centrifugation and filtration through a 0.2µm membrane. The filtrate was taken in sample vials to the analyser. Ninhydrin solution was utilised and after post-column derivatisation, the amount of colour produced was measured which was directly proportional to the quantity of AAs present in the sample. Coloured compounds were quantified at 440nm for hydroxypoline and proline, and at 570nm for the rest of the AAs. Individual AAs were detected based on their respective retention times, quantified by the area under the peak, and results were analysed by Open Lab control panel software version (Agilent Technology). Quality control was maintained in each run with 2 levels of controls (ClinChek® Recipe), with inter-assay and intra-assay coefficient of variation (CV) <1%, and L-norleucine (100mg; Sigma Life sciences) was utilised as the internal standard (ISD). All reagents were prepared using high-performance liquid chromatography (HPLC) grade water.

Urine OA screening was performed on GCMS (Agilent Technologies, USA, 7890A GC system, with 5975C inert Mass Selective Detector). For the purpose, 10ml of first-morning-voided urine was obtained in a plain container. Urine creatinine (mmol/L) was measured in all samples on Clinical Chemistry Analyser (Advia 1800-Siemens, Germany).

Liquid-liquid extraction was utilised to extract OA from the samples, followed by oximation, extraction and derivatisation. 3,3 dimethylglutaric acid (Sigma Aldrich) was utilised as the ISD. Results were accepted when the abundance of ISD in the chromatogram was >1.0 x 10^6.

For the diagnosis of phenylketonuria (PKU) we used a plasma phenylalanine (Phe) to tyrosine (Tyr) (Phe-to-Tyr) ratio of >3[7] and for diagnosis of hyperphenylalanemia (HPA), a Phe cut-off of <1200 µmol/L[8] was used in addition to the aforementioned ratio. Non-ketotic hyperglycinemia (NKH) diagnosis was based on raised CSF-to-plasma ratio of glycine (>0.02)[9] performed on parallel samples, in the absence of valproate therapy or a significant urine OA profile. Type 1 tyrosinemia was diagnosed based on clinical features, disturbed LFTs, markedly raised Tyr levels on plasma AA analysis and marked qualitative elevations of succinylacetone in urine by GCMS. Maple syrup urine disease (MSUD)[10] was diagnosed based on markedly elevated concentrations of branched chain AA leucine, isoleucine and valine and elevated concentration ratios of these AAs to others.

Data was analysed using Mass Hunter software utilising

Orgasid library[11] A questionnaire comprising complete clinical and biochemical data was filled out for all the samples. Results were analysed using SPSS 21. Data was expressed as mean and standard deviation (SD) for quantitative variables, and frequencies and percentage for qualitative variables.

Results

Of the 805 cases reviewed, 49(6%) had an inherited metabolic disorder. Male:Female ratio of the cases was 1.5:1, and the median age was 240 days (interquartile range [IQR]: 1-15695 days). Rate of consanguinity was 563(70%). The most common presenting symptom was seizures 316(39.3%) followed by lethargy 283(35.2%) (Table 1). Age was divided into three categories 0-1 year 29(59%) cases, 1-5 year 14(28%) cases and 5-11 years 6(13%) cases. Of the diagnosed cases, 38(78%) were received from intensive care setups, 4(9%) from non-intensive, and 6(13%) from the OPD. Seizure was the commonest presenting feature in children <1 year of age 11(39%), while delay in developmental milestones was the commonest feature among those aged >1 year

Table-1: Frequency of Individual presenting features in suspected Inherited Metabolic Disorder (IMD) cases (n=805).

Presenting Complaint	n(%)
Seizures	316(39.3)
Lethargy	283(35.2)
Affected sibling	250(31.1)
Developmental delay	239(29.7)
Vomiting	208(25.8)
Poor suckling	173(21.5)
Loose motions	156(19.4)
Jaundice	141(17.5)
Mental retardation	111(13.8)
Hypotonia	107(13.3)
Smelly Urine	30(3.7)
Hepatosplenomegaly	25(3.1)
Dysmorphism	23(2.9)
Skin lesions	15(1.9)

Table-2: Frequency of biochemical derangements in suspected Inherited Metabolic Disorder (IMD) cases (n=805).

Biochemical feature	n(%)
Raised ammonia level	389(48.3)
Disturbed LFTs	176(21.9)
Raised lactate levels	138(17.1)
Altered ABGs	126(15.7)
Positive Non -glucose reducing substances	22(2.7)
Positive ketone bodies in urine	2(1.5)

LFT: Liver function test, ABG: Arterial blood gas.

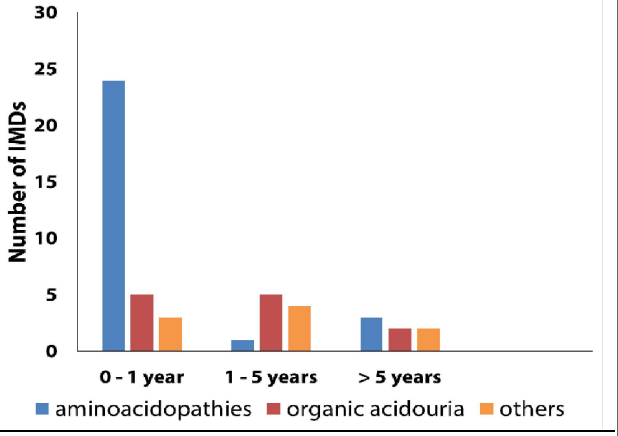

Figure: Number of diagnosed aminoacidopathies, organic acidouria and other Inherited Metabolic Disorder (IMD) cases in different age groups.

10(20%). Raised ammonia levels 232.2±65.8 and disturbed LFTs, mainly raised bilirubin, were the most striking biochemical derangements (Table 2).

Out of 49 diagnosed IMD cases, 28(57%) had aminoacidopathies and 12(24%) had organic acidurias.

Among the aminoacidopathies, there were 4(14.3%)HPAs, 3(10.7%)PKUs, 7(25%) NKHs, 2(7%) hyperprolinemia cases, 5(18%) cases of tyrosinaemia Type 1, 4(14.3%) urea cycle defects, and 3(10.7%) cases of homocystinuria.

Organic acidurias included 3(25%) cases of glutaric aciduria Type 1, 4(33.3%) methylmalonic aciduria, 2(16.6%) propionic academia and 3(25%) cases of maple syrup urine disease (MSUD).

Cases diagnosed with disorders other than aminoacidopathies and organic aciduria were 6(12%); 1(16.6%) each of glycogen storage disease (GSD), Fanconi syndrome, and congenital lactic acidosis, and 3(50%) alkaptonuria. Finally, 2(4%) cases were suspected of having mitochondrial disorder, and 1(2%) of having pyruvate carboxylase deficiency. The prevalence of IMDs in children aged <1 year was the highest (Figure).

Of the total specimen, 45(5.5%) were either recalled or repeated for various reasons, such as non-availability of clinical data 22(2.8%), gross haemolysis 11(1.2%), or icterus 12(1.5%). Hyperbilirubinaemia was the most common analytical challenge, whereby we found analytical interference in the form of multiple peaks around retention times of some of the AAs.

Discussion

The data presented by the current study is unique in the

sense that it belongs to one of the few centres in Pakistan equipped with facilities for testing specific metabolites like AA and OA for diagnosing IMDs. In most of the local data presented, these tests have been outsourced abroad.[12,13] Our results have shown a significant proportion of patients (14.2%) who were diagnosed as NKH which is in accordance with literature. Only one patient was on valproate therapy, but at the time of sampling he had been off-treatment for at least 4 weeks. Most of the cases diagnosed were aged <6 months. We also diagnosed a rare presentation of IMD in a child who had seizures in the 8th year of life and later investigations revealed encephalopathy due to atypical NKH.[14] Measurement of glucosylceramide synthase (GCS) enzyme activity in liver biopsy specimen[15] is an invasive procedure and molecular testing facility was not available in our centre so we used the raised CSF-to-plasma glycine levels in all patients .

Out of the five cases of type 1 tyrosinemia diagnosed, one of the cases had a full-blown renal involvement with phosphaturia, amino aciduria and glycosuria. Another was a case of 9-year-old child presenting with rickets and cirrhosis and the plasma AA analysis showed elevated Tyr and Phe levels along with moderate elevation of methionine levels. This AA profile was similar to one case reported in a study.[16] Two of the five children we diagnosed had markedly elevated alpha fetoprotein levels. This biochemical finding was similar to that reported earlier.[17]

For diagnosis of PKU, in our laboratory, we used a plasma Phe-to-Tyr ratio of >3.For diagnosis of HPA, a Phe cut-off of <1200 μmol/L was used in addition to the ratio described earlier. MSUD was diagnosed based on markedly elevated concentrations of branched chain amino acids leucine, isoleucine and valine and elevated concentration ratios of these AAs to others. CSF leucine and isoleucine levels were also markedly elevated in all cases much in accordance with literature.[18] Mitochondrial disorders were suspected in 2 patients on the basis of clinical history, examination, biochemical findings and findings of brain MRI.

In the current study, 49(6%) cases were diagnosed as IMDs. Data from Pakistani population is still limited. The prevalence of IMDs in our study closely matched that reported by an earlier study by Sherazi NA et al.[11] Seizures and coma were the most common presentations in our study, and that also matches literature.[19] We did not find a very high frequency of storage disorders in patients referred to us. This contrasts with a study which found a much greater frequency of GSDs and Gaucher disease compared to other IMDs.[20] This difference in prevalence can be explained keeping in mind the fact that the data of earlier study was from Gastroenterology, Hepatology and Nutrition departments of a paediatric hospital. A study showed a high rate of consanguinity[21] which is a finding similar to that seen in our study (70%). Similarly positive history of a previously affected sibling was 35.5%, much in accordance with our study. It was seen that aminoacidopathies like NKH, type 1 tyrosinaemia and various organic acidurias encompassed most of the high-risk cases which, again, is in accordance with literature.[22]

We faced several analytical challenges during the course of workup of these cases referred for suspected IMDs. A total of 45 samples were either recalled or repeated for various reasons such as non-availability of clinical data, presence of gross haemolysis or icterus. Haemolysis yielded rise in multiple amino acids like glycine, serine, lysine, glutamic acid and taurine which made the interpretation difficult. Arriving at a provisional diagnosis for any IMDs needs a constellation of biochemical results rather than a single investigation. A report of plasma AAs can be meaningfully interpreted in the presence of relevant clinical and biochemical data. This aspect was taken care of by close collaboration with the treating/referring paediatrician. The samples with gross haemolysis were not analysed and recalled. This is due to the fact that haemolysis may cause increases in certain AAs like serine, glycine, taurine, aspartic acid, glutamic acid, ornithine and decreased arginine. Moreover, haemolysis, along with certain other factors is found to influence sample stability.[22,23] Bilirubin remained the most common analytical challenge.[23,24] Analysis was recommended to be carried out/repeated after bilirubin levels had settled.

Some of the cases were regularly followed-up. Four out of the seven cases diagnosed as NKH expired despite the initiation of treatment. One of the cases diagnosed as atypical NKH was managed during admission with anti-epileptics to which seizures responded but abnormal movements settled partially. Oral folic acid and vitamin B12 were also added to his medications. His condition had improved on discharge and he was advised regular follow-up, and genetic counselling sessions were carried

out with the parents.[25] Another 5-year-old diagnosed as homocystinuria was started on pyridoxine, folic acid and a low-methionine diet. However, he showed no biochemical response to pyridoxine and was advised to continue on low-methionine and high-cysteine diet.[26] Likewise, another case diagnosed with glutaric aciduria was advised to be kept on low-protein and low-lysine diet along with carnitine nutritional supplementation with further advice to parents to ensure avoidance of prolonged fasting intervals. Two of the children diagnosed with MSUD expired during admission while the third 24-days-old baby was managed initially on antibiotics, intravenous (IV) fluids, multivitamin supplements and mechanical ventilation. He was started on leucine-free milk powder on discharge.

Conclusion

The 6% prevalence of IMDs was quite significant. NKH was the commonest IMD diagnosed. IMDs must be kept in mind while treating a sick child in parallel with sepsis and a properly worked out criteria will be quite useful in the selection of infants to undergo expensive and sophisticated investigations like molecular testing.

Disclaimer: None.
Conflict of interest: None.
Source of Funding: None.

References

11. Gregory M. Enns, Packman S. Diagnosing Inborn Errors of Metabolism in the Newborn: Laboratory Investigations. Neo Reviews; 2001.
2. National Institute of Population Studies (NIPS). Pakistan demographic and health survey 2006-07. Islamabad: National Institute of Population Studies and Macro International Inc; 2008.
3. Inherited Metabolic Disorders-General Information. In: Ijaz A, Aamir M, Hafeesz A, eds. Inherited Metabolic Disorders. 1st ed. Rawalpindi, Pakistan: Zaheer Art Press, 2016: pp. 1-10
4. Leonard JV, Morris AAM. Diagnosis and early management of inborn errors of metabolism presenting around the time of birth. Acta Paediatr 2006; 95: 6-14.
5. Hawkes CP, Walsh A, O'Sullivan S, Crushell E. Doctors' knowledge of the acute management of Inborn Errors of Metabolism. Acta Paediatr 2011; 100:461-463.
6. Ezgu F. Inborn Errors of Metabolism. Adv Clin Chem 2016; 73: 195-250.
7. Walter JH, Lee PJ, Burgard P. Hyperphenylalaninaemia. In: Fernandes J, Saudubray JM, van den Berghe, Walter JH, (Editors). Inborn Metabolic Diseases. 4th ed. Germany: Springer; 2006.
8. Howell RR. National Institutes of Health Consensus Development Conference Statement: phenylketonuria: screening and management, October 16-18, 2000. Pediatrics 2001; 108: 972-82.
9. Dulac O, Rolland MO. Non ketotichyperglycinemia (Glycine encephalopathy). In: Fernandes J, Saudubray JM, van den Berghe, Walter JH, (Editors). Inborn Metabolic Diseases. 4th ed. Germany: Springer; 2006.
10. Maple syrup urine disease. Genetics Home Reference. [Only] [Cited 2013 Dec 15]. Available from: URL: http://ghr.nlm.nih.gov/condition/maple-syrup-urine-disease
11. Sherazi NA, Khan AH, Jafri L, Jamil A, Khan NA, Afroze B. Selective Screening for Organic Acidurias and Amino Acidopathies in Pakistani Children. J Coll Physicians Surg Pak 2017; 27: 218-21.
12. Mufti PE, Ahmed IQ. Inherited Metabolic Disorders in Pakistan: Presentation, diagnosis and outcome of congenital hyperammonemias. J Pak Med Assoc 1994;44: 229-32.
13. Afroze B, Lakhani L, Naz F, Somani S, Yunus Z, Brown N. Challenges identified in the management of patients with inherited metabolic disorders-A five years experience from Pakistan. Egypt J Hum Med Genet 2016; 17: 259-64.
14. Ayub A, Hafeez A, Salman S, Ijaz A, Lodhi MA, Ali A. Late onset non-ketotichyperglycinemia - a rare presentation in children. Pak J Pathol 2016: 27: 136-9.
15. Applegarth DA, Toone JR. Nonketotic hyperglycinemia (glycine encephalopathy): laboratory diagnosis. Mol Genet Metab 2001; 74: 139-46.
16. Rashad MR, Nassar C. Tyrosinemia Type 1: A case report. Sudan J Paediatr 2011; 11: 64-7.
17. Afroze B, Fadoo Z, YunusZM. Hereditary Tyrosinemia type I presenting as multiple focal hepatic lesions. J Pak Med Assoc 2013; 63: 1044-6.
18. O Axler, Holmquist P. Intermittent Maple Syrup Urine Disease: Two Case Reports. Pediatrics 2014, 133: e458-60.
19. Choudhry S, Khan M, Rao HA, Jalan A, Khan EA. Etiology and outcome of inborn errors of metabolism. J Pak Med Assoc 2013; 63: 1112-6.
20. Cheema HA, Malik HS, Parkash A, Fayyaz Z. Spectrum of inherited metabolic disorders in Pakistani children presenting at a tertiary care centre. J Coll Physicians Surg Pak 2016; 26: 498-502.
21. Gul SS, Majeed AI, Talpur AF. Inborn Errors of Metabolism in Newborns: An Experience of Tertiary Care Hospital in Islamabad. Ann Pak Inst Med Sci 2017; 13: 213-6.
22. Saudubray JM, Desguerre I, Sedel F, Charpentier C. A Clinical Approach to Inherited Metabolic Diseases. In: Fernandes J, Saudubray JM, van den Berghe, Walter JH, (Editors). Inborn Metabolic Diseases. 4th ed. Germany: Springer; 2006
23. Takehana S, Yoshida H, Ozawa S, Yamazaki J, Shimbo K, Nakayama A, et al. The effects of pre-analysis sample handling on human plasma amino acid concentrations. Clin Chim Acta 2016; 455: 68-74.
24. Hafeez A, Fatima S, Ijaz A, Memon A, Waseem M. Bilirubin Interference in Plasma Amino Acid Analysis by Ion Exchange Chromatography. J Coll Physicians Surg Pak 2018; 28: 667-71.
25. Ayub A, Hafeez A, Salman S, Ijaz A, Lodhi MA. Late onset non-ketotichyperglycinemia - a rare presentation in children. Pak J Pathol 2016: 27; 136-9.
26. Fatima S, Hafeez A, ijaz A, Awan A, Sajid A. Classical Homocystinuria in a Juvenile patient. J Coll Physicians Surg Pak 2018, 28: 488-9.