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Selective Screening for Inborn Errors of Metabolism: A Report of Six Years Experience

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Abstract

Background: Tandem MS analysis of dried blood spots is a widely used method for diagnosis of inborn errors of metabolism. Clinical laboratories performing this test for clinically suspected children at different ages are faced with the challenge of using appropriate reference ranges for the diagnostic markers.
Objectives: Retrospective evaluation of laboratory data was performed to establish the disease spectrum and clinically relevant reference ranges for the diagnostic markers.
Methods: The results of clinically suspected 4800 patients were extracted from laboratory information system and correlated with clinical data. Relevant reference ranges for the analytes in dried blood spots was determined using nonparametric statistical methods.
Results: Forty four patients were diagnosed with 12 different inborn errors of metabolism. There were 23 patients with organic acid disorder, 13 patients with amino acid or urea cycle disorder and 8 patients with fatty acid oxidation disorder. The reference ranges were significantly different between the children under and over 1 year of age for some acylcarnitines (C0, C2, C3, C5OH, C14, C16 and C18) and amino acids (citrulline, arginine, tyrosine, valine and leucine).
Conclusions: The interpretation of the Tandem MS analysis results showed that the difference in the reference ranges for children under or over one year of age did not affect the diagnosis for most frequent inborn errors of metabolism.

Keywords: Inborn Errors of Etabolism, Reference Values, Cutoff

1. Background

Inborn errors of metabolism (IEM) consist of a large class of genetic diseases that could lead to serious clinical consequences for affected individuals (1). The use of Tandem Mass Spectrometry (MS) is widely growing for implementation of newborn screening (NBS) programs for IEM as well as for selective screening of children at various ages. The results obtained from these expanded NBS programs have provided information about the prevalence of these disorders in the population in USA and also in some European countries (2-8).

In other parts of the world where there is no or a limited NBS program implemented, the laboratories performing Tandem MS analysis for clinically suspected patients, provide information regarding the frequency of these disorders (1, 9-14). The analysis of dried blood spot (DBS) samples by Tandem MS is increasingly used by many laboratories for selective screening of metabolic diseases considering the high frequency of IEMs mostly due to high consanguinity rate in Turkey. These test results of patients at different age groups are interpreted by comparison to reference values and/or cut off levels established for the newborns. However, it is widely accepted that children require the use of reference populations that reflect changes associated with growth and development. Although there is limited access to healthy control populations in the pediatric group, the laboratory data may provide information for estimation of reference ranges.

2. Objectives

In this study we report the disease spectrum of the clinically suspected patients referred to our laboratory for tandem MS analysis in association with the clinically relevant reference ranges obtained from the patient data of 6 years long experience in our laboratory.

3. Methods

Tandem MS analysis of amino acids and acylcarnitines was performed in DBS from clinically suspected patients referred to the metabolic diseases laboratory of Dokuz Eylul University Hospital, from January 2010 to December 2016. The study was reviewed and approved by the ethics committee of Dokuz Eylul University.

Blood samples were collected on Guthrie card filter papers (Whatman no. 903 TM cards, USA). The analytes were extracted with methanol containing isotopically labeled standards. The extract was dried and treated with n-butanol in acetylchloride to form butylester derivatives. Following evaporation, the residue was reconstituted with 100mL of acetonitrile: water (80:20 v/v).

The triple quadrupole MS/MS (API 2000) and the injection module was from Agilent Technologies (California, US). Electrospray ionization was used as the ion source. Data was analyzed by ChemoView for Analyst 1.5 software of AB/MDS Sciex.

Deuterated internal standards were from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, UK). Quality control samples were obtained from Recipe (Clin Chek, Munich, Germany) and external quality control program was obtained from Centers for Disease Control and Prevention (Atlanta, GA). Our laboratory was accredited for Tandem MS analysis for acylcarnitine profile and amino acids according to the ISO 15189 standard.

The diagnosis of the disorders was confirmed with clinical evidence, routine laboratory tests, amino acid analysis by ion exchange chromatography and organic acid analysis by GC-MS according to standard protocols and/or to professional guidelines. DNA analysis was also performed if necessary. Phenylketonuria (PKU) and hyperphenylalaninemia (HPA), the most frequent IEM both in the world and in our country is not included in this study because the national NBS in Turkey includes PKU.

For determination of reference intervals of the relevant parameters, the results of Tandem MS analysis were filtered from Laboratory Information System excluding the patients with confirmed diagnosis of IEM. Statistical analysis was performed using SPSS 15.0. Tests for normality of analyte distributions were carried out with Kolmogorov-Smirnov test. Because analyte distributions were universally skewed, reference intervals were determined nonparametrically and correspond to the 2.5^{th} - 97.5th percentiles of the experimental distribution. Comparison of analyte distributions across age groups was performed using the nonparametric Mann-Whitney U test. $P < 0.05$ was considered significant.

4. Results

Among 4800 clinically suspected patients, 44 were diagnosed with 12 different inborn errors of metabolism. Among these patients 16 were female and 28 were male. Median age was 2.5 years (range; 3 days to 28 years). The diagnosis of the patients with IEM and the levels of the related diagnostic metabolites and ratios for each disorder are listed in Table 1.

4.1. Amino Acid and Urea Cycle Disorders

These patients were aged between 3 days and 12 years. For 6 of 7 patients with citrullinemia, citrulline levels were nearly 30 folds higher than the upper reference limit while in only one patient citrulline showed a milder increase. Severe hyperammonemia, neurological manifestations of cerebral edema were common to all citrullinemia patients. The ratio of Cit/Arg were well above the upper reference limit. Two of the patients were siblings, the boy aged 2.6 years and the girl aged 3 days who died at 6 days of age. Maple syrup urine disease (MSUD) patients presented with poor feeding, failure to thrive and developmental delay. Valine level was in the reference range in one patient yet the other metabolite leucine-isoleucine was increased about two folds the upper limit. One of the patients with tyrosinemia had liver failure and diagnosed as type I, while the other was type 2.

4.2. Organic Acid Disorders

These patients were aged between 4 days and 14 years. Among the 23 patients with organic acid disorders (OAD), there were only two patients who had the primary marker level in the reference range. One of them was a girl aged 2.5 years who was admitted with attacks of vomiting and seizures and her C3 level was in the reference range while C3/C2 ratio, the secondary marker for methylmalonic acidemia (MMA) was slightly above the upper reference limit. The 3 months old sister of this patient had similar clinical symptoms and the C3 level was at the upper reference limit while the C3/C2 ratio was in the reference range. Both sisters were diagnosed as MMA by urinary organic acid analysis. The other patient also had C3 level in the reference range yet the C3/C2 ratio was well above the upper reference limit and this patient was diagnosed as propionic acidemia (PA) with urinary organic acid analysis.

4.3. Fatty Acid Oxidation Defects

This group of patients were between 1.4 and 15.5 years of age and one of the patients with carnitine palmitoyltransferase II (CPT II) deficiency was an adult at 28 years of

Table 1. Concentrations of Primary and Secondary Markers for Patients with IEMs

Metabolic Disorder		Number of Positive Cases	Mean Age at Diagnosis, y	Metabolites and Ratios	Mean, μmol/L	Range, μmol/L
Amino acids and urea cycle disorders	CIT	7	1.3 years	Cit	1114	234 - 2723
				Cit/Arg	83.3	21.8 - 197
	MSUD	4	5.8 years	Val	379	242 - 516
				Xle	616	331 - 952
	TYR	2	2.3 years	Tyr	387	296 - 478
Organic acid disorders	MMA	11	3.0 years	C3	21.9	3.17 - 44.7
				C3/C2	1.26	0.16 - 2.32
	GA I	6	3.9 years	C5DC	1.91	1 - 2.66
				C5DC/C8	64.4	20 - 182
	PA	2	5 days	C3	35.1	3.65 - 66.5
				C3/C2	1.21	0.93 - 1.49
	IVA	3	6.9 years	C5	7.5	5.05 - 12.1
				C5/C2	0.58	0.39 - 0.73
	3MCC	1	3.7 years	C5OH	29.4	
				C5OH/C8	978	
	CPT II	4	16.7 years	C16	1.74	0.56 - 3.33
				C18	0.87	0.3 - 1.91
				C0/(C16 + C18)	27	1.4 - 66.3
				(C16 + C18)/C2	0.23	0.08 - 0.33
Fatty acid oxidation defects	PCD	2	7.8 years	C0	1.6	1.56 - 1.64
	MCAD	1	1.4 years	C8	4.43	
				C8/C2	0.42	
				C8/C10	13.4	
	VLCAD	1	5.2 years	C14	0.43	
				C14-1	0.98	

[z]Abbreviations: CIT, citrullinemia; CPT II, carnitine palmitoyl transferase deficiency type II; GA I, glutaric acidemia type I; IVA, isovaleric acidemia; MCAD, medium chain fatty acid dehydrogenase deficiency; 3- MCC, 3-methycrotonyl CoA carboxylase deficiency; MGA, 3-methylglutaconic aciduria; MMA, methylmalonic acidemia; MSUD, maple syrup urine disease; PA, propionic acidemia; PCD, primary carnitine deficiency; TYR, tyrosinemia; VLCAD, very long chain acyl CoA dehydrogenase deficiency; Xle, Leu-Ile.

age. Among the four patients with CPT II deficiency, two had C16 levels above the upper reference limit. The other two patients were sisters at the ages of 13 and 10 who was admitted with recurrent attacks of rhabdomyolysis. Both of these sisters had normal levels of C16, C18 and also the ratio of C0/(C16 + C18); one of them could be diagnosed with the elevated (C16 + C18:1)/C2 ratio. One patient with CPT II deficiency could not be diagnosed by Tandem MS results of the DBS sample.

4.4. Reference Ranges

Reference ranges for acylcarnitines and amino acids as well as the ratios related to the diseases diagnosed in our patient population are presented in Table 2 and Table 3 respectively. The reference ranges were significantly different between the children under and above 1 year of age for some acylcarnitines (C0, C2, C3, C5OH, C14, C16 and C18) and amino acids (citrulline, arginine, tyrosine, valine and leucine) ($P < 0.05$).

In our patient population, 15 patients were under and 29 patients were over one year of age. The interpretation of the Tandem MS analysis results showed no difference related to the change in reference limits according to age in 28 out of 29 patients over one year of age diagnosed as IEM. For one patient with CPT II at 15.5 years of age, a false negative result would be obtained if not for age related refer-

Table 2. Reference Ranges of Acylcarnitines and Ratios

Parameter[a]	Under 1 year		Above 1 year	
	Percentiles		Percentiles	
	2.5	97.5	2.5	97.5
C0	10.9	73.7	12.2	57.3
C2	6.8	46.2	6.0	38.4
C3	0.39	4.5	0.52	3.5
C5	0.05	0.33	0.04	0.27
C5OH	0.05	0.26	0.06	0.4
C5DC	0.0	0.21	0.0	0.20
C8	0.02	0.18	0.02	0.19
C10	0.02	0.28	0.02	0.28
C14	0.05	0.38	0.03	0.24
C14:1	0.02	0.19	0.02	0.22
C16	0.31	2.9	0.25	1.6
C18	0.13	1.2	0.17	1.2
C3/C2	0.03	0.22	0.03	0.22
C5DC/C8	0.0	4.5	0.0	4.6
C5/C2	0.0	0.02	0.0	0.03
C5OH/C8	0.55	6.7	0.5	10.7
C0/C16 + C18	6.4	73.3	10.8	53.5
(C16 + C18)/C2	0.03	0.20	0.03	0.20
C8/C2	0.0	0.01	0.0	0.02
C8/C10	0.29	2.5	0.25	2.3

[a]Values in μmol/L.

Table 3. Reference Ranges of Amino Acids and Ratios

Parameter[a]	Under 1 year		Over 1 year	
	Percentiles		Percentiles	
	2.5	97.5	2.5	97.5
CIT	8.64	42.7	9.9	46.1
ARG	5.4	53.9	5.7	73.6
TYR	27.6	196	29.5	143
VAL	64.2	280	81.8	274
XLE	67.5	249	63.9	227
CIT/ARG	0.35	3.2	0.36	3.9

[a]Values in μmol/L; Xle, Leu-Ile.

ence range.

5. Discussion

The laboratory diagnosis of metabolic disorders is challenging due to low incidence of these disorders and

lack of experience (15). In our study, the detection rate of patients with IEM among the suspected patients (44/4800) during 72 months was 0.92%. The results of similar studies on high risk patients from Egypt (6.8%), from Oman (10.8%), from Korea (0.29%), from China (6.2%), and from India (3.2%) show wide variation (12, 13, 16-18). This could be explained by the different diagnostic strategies and also by the prevalence of these disorders in different populations from diverse geographical locations. Another important factor is the difference in rate of consanguinity between countries. It should be noticed that PKU, the most frequent IEM, is not included in our patient group since PKU and HPA are part of the national NBS program.

Our spectrum for amino acid disorders shows similarity to reports from China (9, 10, 12) and Saudi Arabia (14). Yet in most studies from Arab and Mediterranean countries MSUD is the second most frequent amino acid disorder.

Organic acid disorders constitute a large group of disorders in which acyl-CoA esters accumulate in the mitochondria (13, 19). In our patient group, MMA accounted for 47.8% of all organic acidemia patients. It is interesting that in all studies conducted either as a selective screening or newborn screening, MMA is found to be the most common OAD regardless of the geographical/ethnic background of the population (1, 9, 10, 12-14, 16). Different from previous reports the second most common OAD in our patient group was glutaric acidemia (GA) type I.

In the MMA group all the patients had C3 levels as well as C3/C2 levels well above the upper reference limit except for two patients who were sisters. In these patients diagnosis could be achieved by detection of high MMA in urine by GC-MS. One of our patients with PA had C3 levels higher than all MMA patients while the other had a C3 value lower than the upper reference limit and for this patient C3/C2 ratio proved to be a useful secondary marker for decision making.

Fatty acid oxidation disorders refer to disorders of energy metabolism that represent impaired metabolic response to increased energy demands. There was only one patient with medium chain fatty acid dehydrogenase deficiency (MCAD), a disorder which is reported to be the most common in Europe and North America (2-5, 7). The rarity of MCAD is in accordance with Egypt, other Arab countries and also China (9, 14, 16).

For diagnosis of CPT II deficiency, the primary markers in DBS are C16 and C18. Two of the four patients were diagnosed with increased levels of C16. The most sensitive indicator for CPT II deficiency is reported to be an elevated (C16 + C18)/C2 ratio. The use of this ratio enabled us to diagnose one more patient. Yet one patient could not be diagnosed and this brings the argument whether DBS may not be the preferred sample for CPT II deficiency. It is suggested that elevations in long chain acylcarnitines may be more reliably detected in DBS since long chain acylcarnitines are absorbed on the surface of red blood cells (20). On the contrary, high endogenous levels of long-chain acylcarnitines in normal erythrocytes is suspected to reduce the diagnostic specificity in DBS compared to plasma samples (21).

In this study, the laboratory data which accumulated over a period of six years was also used for estimation of reference values for acylcarnitines and amino acids in DBS. For the diagnosis of IEM, the interpretation of the results of Tandem MS may be cumbersome if there is controversy about the appropriateness of the reference ranges for the patient population regarding age. Since it is difficult to collect samples from the healthy pediatric group, this indirect sampling technique has been applied as also recommended by NCCLS (22). It should be stressed that although there was statistically significant difference in reference ranges for some metabolites, the use of age related reference ranges prevented false negative interpretation for only one patient with CPT II deficiency in the patient group over 1 year of age. The increase in diagnostic metabolites was very marked for most patients. Thus, it may be concluded that the use of age related reference ranges could be meaningful in metabolic disorders which may occasionally present with only slight increases in acylcarnitine levels.

As to our knowledge, this is the first study from Turkey which focuses on the disease spectrum as well as on the laboratory diagnosis based on appropriate reference ranges. The main limitation of this study is that it includes only 12 different metabolic diseases.

Monitorization and reevaluation of reference values based on diagnostic experience is beneficial for clinical laboratories to achieve a better understanding and interpretation of amino acid and acylcarnitine analysis in DBS samples for suspected metabolic disease considering the great variety of IEM.

In conclusion, this study has demonstrated that the use of different reference ranges for children under or above one year of age did not affect the diagnosis based on Tandem MS analysis of DBS for most frequent IEM.

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