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Inborn errors of metabolism revealed by organic acid profile analysis in high risk Egyptian patients: Six years experience

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ABSTRACT

Objective: To determine the prevalence and types of inborn errors of amino acid or organic acid metabolism in a group of high risk Egyptian children with clinical signs and symptoms suggestive of inherited metabolic diseases.
Subjects and Methods: 117 (79 males = 67.5 % and 38 females = 32.5 %) high risk patients with signs and symptoms of a metabolic disorder were studied, their ages ranged from 3 days to 12 years.
Analysis of urine organic acids by gas chromatography/mass spectrometry (GC/MS) was performed to all patients.
Results: 22(18.8 % of the total) cases were diagnosed with different types of aminoacidopathies or organic acidurias. The disease profile showed increased lactate in 12 cases (54 %), glutaric aciduria type I 3cases (13 %), phenylketonuria 2 cases (9 %), maple syrup urine disease 1 case (4.5 %), glutaric aciduria type II 1 case (4.5 %), methylmalonic aciduria 1 case (4.5 %), Canavan disease 1 case (4.5 %) and non ketotic hyperglycemia 1 case (4.5 %).
Conclusion: The results demonstrate the importance of the organic acid profile in the diagnosis of high risk patients. The diagnosed organic acid pattern in this study showed that 10.2 % of the patients had a mitochondrial energy defect.

Key Words:

Organicacidurias, organicacidemias, gas chromatography/mass spectrometry, organic acid profile analysis.

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INTRODUCTION

The term “organic acidemia” or “organic aciduria” applies to a diverse group of disorders characterized by the excretion of non-amino organic acids in urine. The organic acidemias share many clinical similarities. Most organic acidemias result from deficiencies of specific enzymes in the breakdown pathways of amino acids or from enzyme deficiencies in beta oxidation of fatty acids or carbohydrate metabolism. Organic acids also are found in the urine of some patients with mitochondrial disease. The pathophysiology results from accumulation of precursors and deficiency of products of that affected pathway. The accumulated

precursors are themselves toxic or metabolized to produce toxic compounds.[1]

Inherited disorders of organic acid metabolism have a high cumulative frequency, and despite heterogeneous etiology and varying clinical presentation, the manifestation of neurological disease is common. It has been demonstrated for some of these diseases that accumulating pathological metabolites are directly involved in the manifestation of neurological disease. Various pathomechanisms have been suggested in different models in vitro and in vivo including an impairment of brain energy metabolism, an imbalance of excitatory and inhibitory neurotransmission, altered transport across the blood-brain barrier and between glial cells and neurons, impairment of myelination and disturbed neuronal efflux of metabolic water.[2]

Many of the organic aciduria responds to treatment, especially in the neonatal period, they demand urgent diagnosis and management. The aim of therapy is to restore biochemical and physiological homeostasis. The treatments, while similar in principle, depend on the specific biochemical lesion and are based on the position of the metabolic block and the effects of toxic compounds. Treatment strategies include:

1. Dietary restriction of the accumulated precursor.

2. Use of adjunctive compounds to dispose toxic metabolites.

3. Use of adjunctive compounds to increase activity of deficient enzymes.

Despite appropriate management, patients with organic aciduria have a greater risk of infection and a higher incidence of pancreatitis, which can be fatal.[3]

Clinical and laboratory findings that should suggest an organic aciduria include acidosis, ketosis, hyperammonemia, abnormal liver function tests, hypoglycemia and neutropenia.[4]

SUBJECTS AND METHODS

Subjects:

Random urine samples were collected from 117 Egyptian children referred to our lab (Biochemical Genetics) from different hospitals and clinics with suspicion of an inborn error of metabolism from 2002-2008.

Blood samples were also obtained and the plasma immediately separated.

Methods:

All samples were screened by the following tests: Ferric chloride test for phenylketones, 2- 4- dinitrophenylhydrazin (DNPH) test for α-ketoacids, nitrosonaphthol test for increased tyrosine and its derivatives, Benedict test for reducing substances, cyanide-nitroprusside test for sulfur containing amino acids. Thin layer chromatographic separation of blood and urine amino acids was performed according to the method of Borden.[5]

Ammonia and plasma lactate were determined for some samples as requested by the physician. Ammonia enzymatic assay was detected according to the method of Ishihara[6]. Lactate was detected colorimetric according to the method of Artuch.[7]

Urine sample preparation and organic acid analysis:

Organic acid analysis was performed according to Sweetman[8]. Urine samples with volumes corresponding to a creatinine of 1mmol/L were made up to 2ml with distilled water acidified to PH 1.5 and mixed with 100µl of decanoic acid 5 mM/L, 100µl of Hepatodecanoic acid 75 mg/200ml and 100µl of Hepatoglycine 299µmpl/L (internal standards). Solid sodium chloride was added and the organic acids were extracted twice with 2ml ethylacetate.

The organic acids were pooled and evaporated under nitrogen at 37˚C in dry block heater.

Derivatization was then performed with 75µl of BSTFA (bis-trimethylsylil triflouracetamide + 1 % trimethyl chlorosilane) and 20µl of pyridine in a fume cupboard to each dried extract, the extract was heated for 30 minuets at 80˚C and then transferred into GC/MS vials.

The metabolites were chromatographically analyzed as trimethylsilyl (TMS) compounds. Half micro liter of each derivatized sample was then injected into Helwatt Packed 5890 equipment with a CP-sil 8 CB capillary column.

The GC/MS temperatures were as follows: Injector 250˚C, column 90˚C to 280˚C with an increment of 3˚C per min, ion source 150˚C and mass analyzer 35˚C. The total run time was 75 min. Finally, the mass spectrometer was programmed from m/z 10-650 at the rate of 0.6 Hz.

RESULTS

The present study included 117 patients, the distribution of these patients according to sex and age are represented in Tables 1 and 2, respectively.

Table 1: Sex distribution in the studied Patients.

Total numbers of patients	Male (M)	Female (F)	M/F
117	79 (67.5 %)	38 (32.5 %)	2/1

Table 2: Age groups of the studied patients.

Age	Number of patients	Percentage
Group I 1-50 days	12	10.3 %
Group II 2-12 months	43	36.8 %
Group III 1-4 years	45	38.4 %
Group VI 4-12 years	17	14.5 %

Table 3: Results of screening chemical tests.

Test	Number of patients tested	Positive results
Ferric chloride ($FeCl_3$)	117 (100 %)	3 cases were positive (2.61 %)
DNPH	117 (100 %)	8 cases were positive (6.8 %)
Benedict	117 (100 %)	7 cases were positive (6 %)
Cyanide-nitroprusside	117 (100 %)	Negative (0 %)
Nitrosonaphthol	117 (100 %)	Negative (0 %)

Table 4: Results of TLC of Plasma and Urine.

Test	Number of patients tested	Positive results
Amino acids (plasma)	117 (100 %)	- 25 cases increased glutamic acid & glutamine - 2 cases increased phenylalanine - 1 case increased valine, leucine, isoleucine - 2 case increased glycine - 13 cases increased alanine
Amino acids (urine)	117 (100 %)	- 25 cases increased glutamic acid & glutamine - 2 cases increased phenylalanine - 1 case increased valine, leucine, isoleucine - 1 case increased glycine

Ammonia level was measured in plasma of 43 cases only as well as plasma lactate was measured in plasma of 29 cases.

Table 5: Plasma ammonia and lactate results.

Test	Number of patients tested	Positive results
Plasma Ammonia	43 (36.8 %)	25 cases (58.1 %) had high NH_3 level (>47 µmol/L)
Plasma Lactate	29 (24.8 %)	12 cases (41.4 %) had high lactate level (> 12mg/dl)

Table 6: Organic acid disorders detected in this study.

Diseases	Number of patients diagnosed	Percent from total number (117)	Percent from diagnosed cases (22)
Lactic acidemias	12	10.2 %	54.5%
Glutaric aciduria type I	3	2.6 %	13.6%
Glutaric aciduria type II	1	0.9 %	4.5 %
Phenylketonuria (PKU)	2	1.7 %	9 %
Maple syrup urine disease	1	0.9 %	4.5%
Methylmalonic aciduria	1	0.9 %	4.5%
Non ketotic hyperglycemia	1	0.9 %	4.5%
Canavan disease	1	0.9 %	4.5%

Table 7: Other findings in the organic acid profile.

Other finding	Number of patients	Diagnosis
Hippuric acid	4	Na benzoate intake
Valproate	7	Antiepileptic drugs

The 22 diagnosed cases were further tabulated according to age groups as shown in (Table 8).

Table 8: Diagnosed cases according to age groups.

Group / Age	Number	Diagnosed	Percentage	Diseases
Group I 1-50 days	12	3	25%	PKU (1) Glutaric aciduria type I (1) Lactic acidemias (1)
Group II 2-12 month	43	10	23.3%	PKU (1) Nonketotic hyperglycemia (1) Glutaric aciduria type I (1) Lactic acidemias (5) MSUD (1) Glutaric aciduria type II (1)
Group III 1-4 years	45	7	15.6%	Glutaric aciduria type I (1) Lactic acidemias (5) Canavan disease (1)
Group IV 4-12 years	17	2	11.8 %	Lactic acidemias (1) Methylmalonic acidemia (1)

DISCUSSION

Organic acidemias or organic aciduria are a group of inborn error of metabolism generally caused by a severe deficiency of one enzyme activity of the amino acid, carbohydrate or lipid metabolism[9,10] and spans a wide range of presentations involving multiple body systems with the predominance of the central nervous system.

Disorders of organic acid metabolism are currently detected by GC analysis of urinary organic acids. Since 1966 by Tanaka who was the first one to use GC to identify isovaleric acidemia.[11]

Many reports have shown that organic acidemias are relatively common disorders among the inherited errors of metabolism. [12-16]

Organic acidemias are frequent inherited metabolic disorders among severely-ill children and constitute, together with aminoacidopathies, the most prevalent group of inborn errors of metabolism in high-risk patients.[17]

It is therefore essential to detect the affected children early in order to institute treatment that is available for some of these disorders. The diagnosis of these disturbances requires identification of the abnormal patterns of organic acids in biological fluids, especially urine by gas chromatography coupled to mass spectrometry.[8]

The present study is an extended and complete study for the diagnosis of primary disorders of organic acid metabolism in high-risk Egyptian patients using GC/MS. 117 patients with suspi-

cion of inherited error of metabolism especially organic acidemias had their urine specimen analyzed for organic acids pattern.

Blood and urine specimens from these patients were also screened for the detection of other inborn errors of metabolism. Plasma ammonia and lactate were determined for some of them as requested by the pediatrician; although plasma NH3 and lactate should be determined to all high risk children at the time of obtaining the urine for the organic acids analysis.

The present study included 79 males (67.5 %) and 38 females (32.5 %) (Table 1). Their age ranged from 2 days to 12 years (Table 8). Although organic acids are mostly inherited autosomal recessive disorder; the male predominance reflects the importance of males to our population like most eastern communities[18]. This male predominance is observed in other studies in our population done to diagnose other metabolic disorders. All these previous studies showed male predominance inspite of the autosomal recessive mode of inheritance of the investigated disorders.

The patients were divided into 4 age groups (Table 2). Group I from 1-50 days included 12 patients (10.3 %), Group II from 2-12 months included 43 patients (36.8 %), Group III from 1-4 years included 45 patients (38.4 %) and Group IV from 4-12 years included 17 patients (14.5 %). The significant differences in the concentrations of the metabolites between different age groups show the necessity of using age related reference ranges for the diagnosis of metabolic diseases[19]. Most of organic acidemias present early in life, still Group III (age 1-4 years) was the largest one. But according to table (8) the diagnosed cases within this group represented 15.6 % only, while 25 % of Group I was diseased. Group I (age 1-50 days) represent the vulnerable age and unfortunately most of them die from acute life threatening crises very early without being diagnosed. This might be the cause that this group constituted only 10.3 % of total studied cases.

This situation shows the importance of early screening of this group by urine analysis of organic acids profile. These cases are mostly misdiagnosed with infectious diseases or remain undiagnosed due to lack of awareness by the physician by the metabolic disorders. As seen from our study Group II and Group III comprise 36.8 % and 38.4 % of the cases, respectively. These age groups are relatively high for the diagnosis of organic aciduria. This could be tentatively attributed to lack of awareness of these genetic diseases by the Egyptian physicians especially in the villages together with the lack of their awareness with the presence of a metabolic lab in Cairo- Egypt for diagnosis of such cases. This leads to the high mortality of patients affected by these diseases without being diagnosed. History taking and the presence of a previously affected child in a consanguineous marriage should draw the attention of the physician to a metabolic disease.

The chemical tests and qualitative amino acids chromatography were done to all patients (Tables 3&4, respectively). 3 cases had positive ferric chloride test (2.61 %), 8 cases had positive DNPH (6.8 %) and plasma and urine thin layer chromatography showed increased phenylalanine in plasma and urine of two patients diagnosed as PKU and increased branched chain amino acids in

plasma and urine of one patient, diagnosed as MSUD.

Synchronously the organic acid profile was analyzed and confirmed the diagnosis of Phenylketonuria and MSUD in these patients. This proves that simple tests are still very helpful to diagnose some diseases without the need of more sophisticated techniques, which need more time, skills and are costier. In our lab and in many other labs around the world; these tests are still used as a first screening strategy before using a higher one. Organic acid analysis is expensive and this is the setback especially for developing countries with limited resources for diagnosing rare disorders. Thin layer chromatographic separation of amino acids showed increased glutamic acid and glutamine in the urine and plasma of 25 patients (Table 4). This is secondary to the high plasma NH3 not tolerated by these cases. Plasma NH3 levels were measured to 43 cases (36.8 %) (Table 5) according to the pediatrician request. 25 cases had high NH3 level (58.1 %). High plasma NH3 level is recorded in many metabolic disorders and must be determined early with the first screening tests to institute treatment before any brain damage is caused by the high NH3 levels not tolerated by the patients as seen by the increased glutamic acid and glutamine in the plasma and urine of them. The abnormal laboratory findings in cases with organic aciduria beside the hyperammonemia are metabolic acidosis, hypoglycemia, ketosis/ketonuria and pancytopenia.

Plasma lactate was measured for 29 cases according to the pediatrician request and 12 cases showed high plasma lactate levels (41.4 %) (Table 5). Primary lactic acidemia was the most frequent disorder diagnosed among the Brazilian patients in a study done on 234 cases in a study like ours on high-risk children[16]. Further more, despite the absence of suspicion by referral physician, the presence of ketolactic acidosis with or without hyperammonemia is an indication for organic acid analysis. Accumulation of lactic acid is found in various disorders especially those with energy defects i.e. mitochondrial disorders including fatty acids oxidation defects.

Out of the 117 samples analyzed for organic acid profile, 22 patients (18.8 %) showed abnormal urinary excretion of organic acids. The most frequently diagnosed disorders in this high-risk group (Table 6), were lactic acidemia 12 cases (10.2 %) of the total cases and 54 % of the diagnosed cases.

Primary lactic academia presented 54 % of the diagnosed cases, reflecting that over 50 % of the high-risk diagnosed patients have an energy defect; which needs further enzymatic and molecular studies to reach a final diagnosis. No MCAD deficiency was diagnosed among them; although in a previous study by the same group of researchers who found 82 % MCAD deficiency among high-risk patients with nonketotic hypoglycemic attacks[15]. This is attributed to the selection criteria of that study which is totally different from this one.

The second frequent diagnosed disease in this study is glutaric aciduria type I presenting 13.6 % of the abnormal organic acid profile detected. This is a surprisingly high frequency of the disorder, which can not reflect its frequency among our population. Two patients were diagnosed in the second and the third age groups when the macrocepha-

lly was very obvious. The third case was diagnosed relatively early in group I because he was a brother of one of the already diagnosed patients.

A larger study of the Egyptian population must be done to find out the real prevalence of this disorder.

In the age Group I (1-50 days) 3 cases from 12 (25 %) were diseased, in the age group II (2-12 months) 10 cases from 43 (23.3 %) were diagnosed, in the age Group III (1-4 years) only 7 cases from 45 (15.6 %) were also diseased and in the age Group IV (4-12 years) 2 cases from 17 (11.8 %) were diagnosed.

The highest frequency was found in the age group I (1-50 days) 25 %. Still this group is undiagnosed due to several factors discussed before like the high mortality rate among this Group of disorders if not diagnosed and treated very early in life. Add to this factor the lack of awareness of such genetic disorders among the physicians in Egypt.

Two cases of phenylketonuria (9 % of diagnosed cases) were diagnosed in age Group I and II this is due to the lack of neonatal screening program for PKU in Egypt. The two cases were diagnosed by positive FeCl3 and positive DNPH tests (Table 3) and the presence of phenylalanine bands in the TLC (Thin Layer Chromatography) of their plasma and urine. Organic acid profile confirmed the diagnosis.

This draws the attention to the importance of adding PKU to the existing national neonatal screening program for TSH. Our findings also show the importance of simple tests in diagnosing this disorder without delay and performing higher technical and more expensive tests.

One case of MSUD (4.5 %) was also diagnosed by positive FeCl3 and positive DNPH tests with the presence of a branched chain amino acids band in the TLC of plasma and urine of the affected patient. The organic acid profile confirmed the diagnosis by the presence of the 2OH isovaleric peak. MSUD can be simply diagnosed by the screening chemical tests and TLC; which can be life saving to such cases during crisis.

A case of nonketotic hyperglycemia was also detected by the presence of excessive glycine band in the TLC and negative DNPH test. The diagnosis was also confirmed by abnormal organic acid profile.

Both cases of MSUD and nonketotic hyperglycemia were diagnosed in age group II (2-12 month), which is not too late for MSUD. Regarding nonketotic hyperglycemia, it is usually an aggressive disorder leading to acute encephalopathy in most of the affected neonates. Although it can be simply diagnosed early by TLC of the patients plasma and urine[20] without the presence of ketone bodies unlike the excretion of glycine observed with cases of organic aciduria, where ketosis is a main laboratory finding.

Our results regarding these 4 cases (18.1%) diagnosed with aminoacidopathies plus the 5 cases (22 %) diagnosed with primary organic acid disorders (both 40.1%) coincide with the results of the Brazilian experience[21]. Both studies agree that organic acids are frequent inherited disorders among severely ill children and constitute, together with

the aminoacidopathies, the most prevalent group of in born error of metabolism in high-risk populations. Symptoms are usually severe and the outcome is often bad. Once they become symptomatic, leading to a considerable number of patients to death. Those who survive present a variable degree of physical and/or mental disability. Early detection and treatment are essential to save and for better outcome of these patients. Abnormal patterns of organic acids in the biological fluids especially urine, provide a diagnostic clue to IEM including amino acids disorders, urea cycle, disorders of carbohydrate metabolism and mitochondrial fatty acid oxidation defects.

The organic acid profile also showed increased excretion of Hippuric acid in 4 cases. Hippuric acid is the end metabolite of Na-benzoate used in the treatment of increased NH_3 levels in these high-risk children.

This shows the importance of NH_3 monitoring and the adjustment of the Na-benzoate dose accordingly. 7 cases showed increased excretion of Valproates. Valproates are a group of antiepileptic drugs, which should not be given to neonates with metabolic disorders as they aggravate the condition. This shows the lack of awareness about the management of metabolic disorders by many physicians. Valproates should not be prescribed by them to any condition with suspicion of a metabolic disorder.

Methylmalonic acidemia (MMA) is the most common organic acidemia in many studies[13,24]. In our study only one case with MMA was diagnosed; representing 4.5 % of the 22 abnormal organic acid profiles. If we say that this disorder is equal to 4.5 % of the abnormal pattern, this is more acceptable. Strange enough it was diagnosed in age group IV (4-12 years).

This is most probably a vitamin B_{12} responsive case of MMA, diagnosed late due to the abundance of vitamin B_{12} in the food of many Egyptians due to our nutritional habits.

Still a larger study is needed to know the real prevalence of MMA in our population, bearing in mind that the study represents the high-risk patients only and not a mass screening one.

Fifteen years experiment selective screening for organic acids in Brazil on the other hand diagnosed 34 cases with MMA from 866 Brazilian children representing 0.49 % but 14.5 % of the 234 abnormal organic acids diagnosed in their study. This incidence among the diagnosed cases is 10 % higher than our findings.

Of course their diagnosed cases were also ten times our 22 positive. Also, Brazil is a totally different population in origin.[16]

One case with glutaric aciduria type II was also diagnosed in age group II. It also represents 4.5 % of the diagnosed cases. The Brazilian study diagnosed 8 cases of glutaric aciduria type II presenting 3.4 % of their diagnosed cases. These rare disorders need very large studies to find out their frequency in a certain population.

Glutaric aciduria type II is due to deficient electron transfer from the FAD-dependant dehydrogenases to the respi-

ratory chain due to genetic defects of the electron transfer flavoprotein (ETF) or ETF-coenzyme Q oxidoreductase (ETF-QO). Neonatal presentation is usually fatal in the first weeks of life. This case was diagnosed in age group II. The case was well managed till the age of four years, when she died during an acute crisis.

One case of Canavan disease was diagnosed in group III (age 1-4 years). This is the age when the full clinical picture of the disease becomes obvious with macrocephally, leukodystrophy and optic atrophy. The high peak of N-acetylaspartic acid in the organic acid profile was characteristic of the disorder. This abnormality presented 4.5 % of the abnormal organic acid profiles detected in this study.

In a previous study in Egypt of 50 high risk Egyptian children by El Gammal[27], 25 cases (50%) were found to have different amino acid and organic acid disorders excluding phenylketonuria. Fourteen cases had organic acidemias and 4 cases had amino acid disorders.

CONCLUSION

The organic acid profile detection by GC/MS is a key to the diagnosis of many metabolic disorders. This needs a lot of experience and training by experts like our coauthors Prof. Dr. Boehles and Prof. Dr. Sewell.

This technology must be available in developing countries, with high consanguinity rates, to help in the early diagnosis and treatment of such cases. Still simple screening tests can help in the diagnosis of some of these disorders especially aminoacidopathies.

Also, the measuring of NH3 and lactate levels and early management of both items till a final diagnosis is reached; can rescue many newborns and prevent many of the undesired sequalae of these disorders.

Finally, a larger epidemiological study is needed to find out the frequency of each of the diagnosed disorders among our population. Because of the high rate of consanguineous marriages which leads to a high prevalence of autosomal recessive inherited disorders, with the use of new laboratory techniques, we expect a high frequency of inherited metabolic diseases in our country.

Recommendation: A larger study is recommended to determine the prevalence of the different inborn errors of metabolism among our patients.

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