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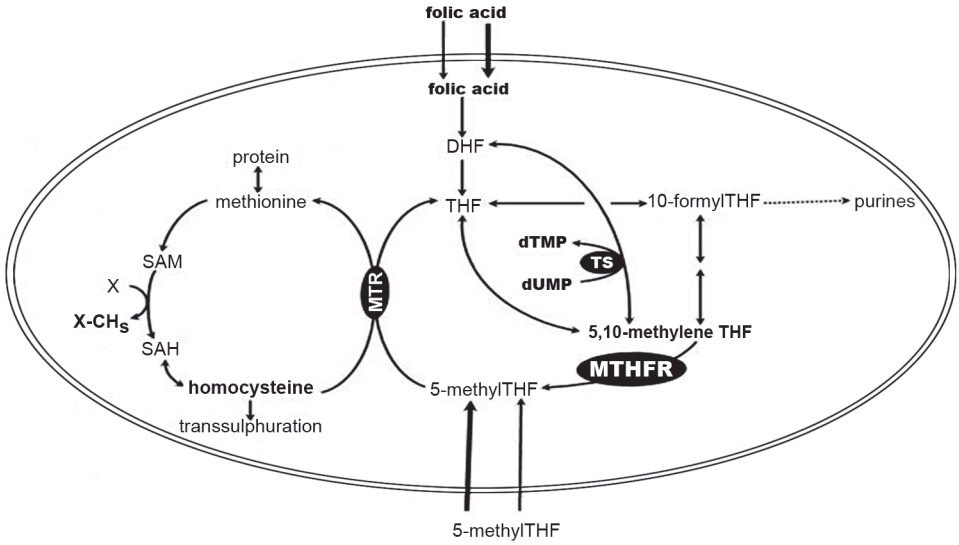
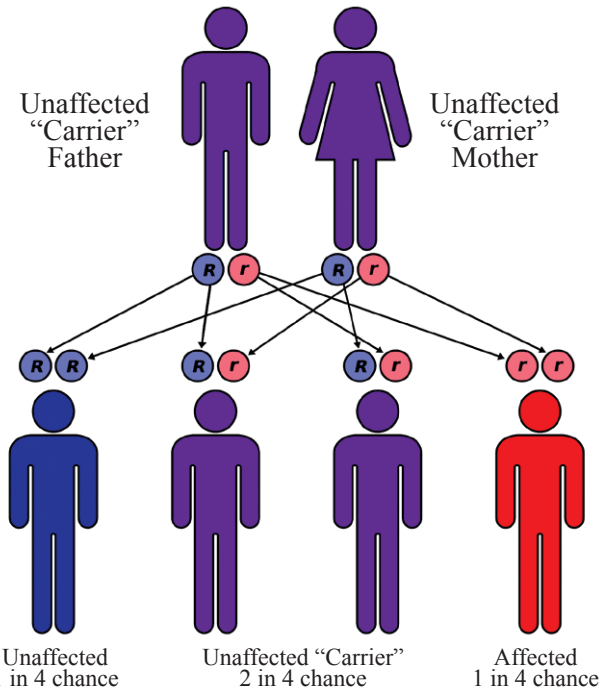
Inborn Errors of Metabolism





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From the Editor's Desk

Technological advances are revolutionizing the way in which we can diagnose and screen populations for inborn errors of metabolism (IEM). The current state of knowledge allows the diagnosis of many IEM by detection of the aberrant gene. However, as multiple genetic defects may result in the same biochemical phenotype, most diagnosis of genetic defects in a clinical setting are still accompanied by an abnormal accumulation of the metabolites of the affected pathways, or unusual metabolites from alternate pathways.

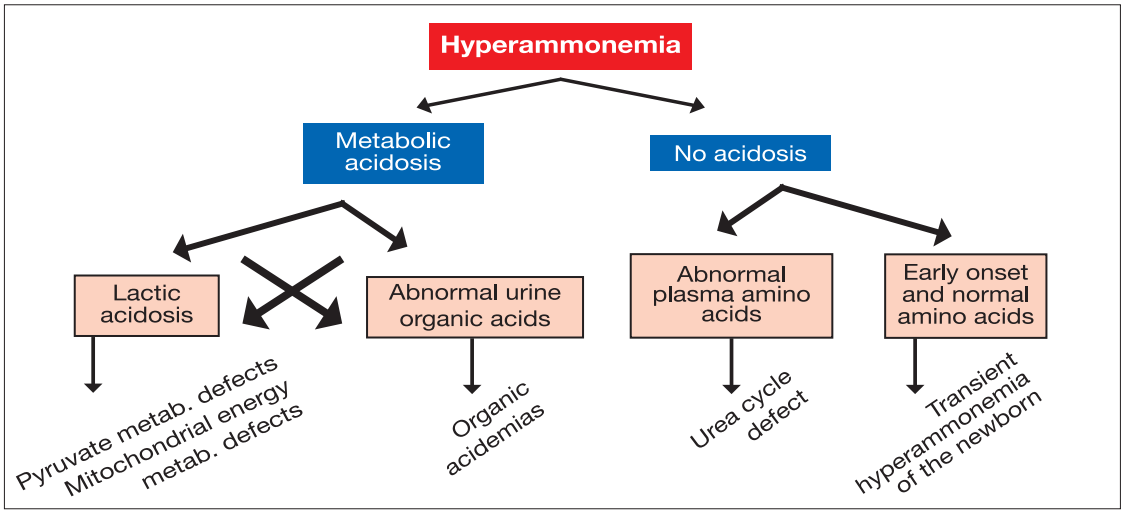
The advances made in the diagnosis of IEM in recent years have not only been in the field of discovering new genes, but in technical ability to detect intermediates of metabolism in smaller samples, with greater efficiency and with fewer tests.

Chromatographic techniques are ideal for separation of compounds out of a complex mixture in a biological matrix based on identification of compound on retention time. Plasma amino acids analysis by cation exchange chromatography and organic acids determination by gas chromatography mass spectrometry have been widely used for diagnosis of aminoacidopathies and organic acidemias.

In Pakistan, evidence on the underlying incidence on the genetic disorder is lacking mainly because of lack of diagnostic facilities. The population of Pakistan consists of a fine amalgamation of people from diverse tribal, cultural, religious and social backgrounds. Mortality and morbidity from a vast number of medical and surgical conditions is significant and well above compared to the developed countries. Although a large component of the human health problem in Pakistan is related to infection, malnutrition and other preventable causes, a significant proportion is linked to hereditary factors reflecting in the form of chromosomal, single gene and complex medical diseases.

This issue is focused on the newly introduced metabolic genetic testing services offered at AKUH clinical laboratory in the Section of Chemical Pathology for diagnosis of IEM. We share relevant information about the service offered and address the issues and challenges for pathologist and physicians in Pakistan. In addition the issue also includes tests that are being offered in other sections. It is hoped that with the introduction of these services, our efforts for awareness and education of our physicians will lead to substantial improvement in the diagnosis of IEM in Pakistan.

Dr Aysha Habib Khan
Chemical Pathologist



Approach to a Sick Neonate with Hyperammonemia

Diagnosis of Inborn Errors of Metabolism in Pakistan: Issues and Challenges

Dr Aysha Habib Khan
Chemical Pathology

The scientific concept of inborn errors of metabolism (IEM) was brought forward by Sir Archibald Edward Garrod in early 1900s. The term 'inborn errors of metabolism'' was used to describe the hereditary deficiency or alteration in enzyme reactions. Although individual IEM are relatively rare conditions; as a group they represent a vast and diverse collection of diseases that are a significant cause of morbidity and mortality worldwide. The reports in literature often quote a cumulative incidence varying between 1 in 1500 and 1 in 5000 live births.

In early 1960s, the concept of newborn screening (NBS) was evolved. NBS allows screening of newborns for disorders just after birth that can cause severe illness or death unless detected and treated early. For example phenylketonuria, congenital hypothyroidism, congenital adrenal hyperplasia (CAH), maple syrup urine disease (MSUD) etc. Programs are designed to provide early diagnosis and treatment before significant and irreversible damage occurs. A comprehensive NBS system/program consists of education, screening (specimen collection, transportation, and testing), follow-up of abnormal and unsatisfactory results, confirmatory testing and diagnosis, treatment and periodic outcome evaluation, quality assurance and program evaluation, validity of testing systems, efficiency of follow-up and intervention, and assessments of long-term benefits to individuals, families, and society. Public awareness coupled with professional training and family education is also a part of the complete NBS system. NBS has been demonstrated to save lives and prevent serious disability. Reports have shown its cost effectiveness and it represents a public health success.

The criteria for inclusion of a test/disorder into a newborn screening program continue to evolve and are given in Table 1 and the disorders included in NBS are given in Table 2. Pakistan has as yet no newborn screening programme at the national level. The health care system is mainly hospital-based and primary health care facilities are practically non-existent. A large proportion of

Table 1. Wilson and Jungner Criteria used to Justify Screening for a Specific Disorder*

1	The condition sought should be an important health problem
2	There should be an accepted treatment for patient with recognized disease
3	Facilities for diagnosis and treatment should be available
4	There should be a recognizable latent or early symptomatic stage
5	There should be a suitable test or examination
6	The test should be acceptable to the population
7	The natural history of the condition, including development from latent to declared disease should be adequately understood
8	There should be an agreed policy on whom to treat as patients
9	The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole
10	Case-finding should be a continuing process and not a 'once and for all' project

*Reproduced from Bulletin of the World Health Organization. Revisiting Wilson and Jugner in the genomic age: a review of screening criteria over the past years.

Table 2. Recommended uniform newborn screening panel; Good Laboratory Practices for Biochemical Genetic Testing and Newborn Screening for Inherited Metabolic Disorders

Inborn errors of amino acid metabolism	***Endocrine disorders***
Argininosuccinic aciduria	Primary congenital hypothyroidism
Citrullinemia, type I	Congenital adrenal hyperplasia
Maple syrup urine disease	
Homocystinuria	
Classic phenylketonuria	
Tyrosinemia, type I	
Disorders of fatty acid oxidation	***Hemoglobin disorders***
Carnitine uptake defect (carnitine transport defect)	SS disease (sickle cell anemia)
Medium-chain acyl-CoA dehydrogenase deficiency	S, beta-thalassemia
Very long-chain acyl-CoA dehydrogenase deficiency	
Long-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency	
Trifunctional protein deficiency	
Disorders of organic acid metabolism	***Other conditions***
Propionic acidemia	Biotinidase deficiency
Methylmalonic acidemia (methylmalonyl-CoA mutase deficiency)	Cystic fibrosis
Methylmalonic acidemia (cobalamin disorders)	Classic galactosemia
Isovaleric acidemia	Hearing loss
3-Methylcrotonyl-CoA carboxylase deficiency	Severe combined immunodeficiencies
3-Hydroxy-3-methylglutaric aciduria	
Holocarboxylase synthase deficiency	
ß-Ketothiolase deficiency	
Glutaric acidemia type I	

deliveries occur at home and majority of these home deliveries are usually attended by unskilled birth attendants. Pakistan also has a very high consanguinity rate of 46-61 per cent due to strong cultural preferences. The burden of inherited disease is expected to be very high, and is considered to be a major contributor to the high infant mortality rate in Pakistan. Infants are not diagnosed early and die without the benefit of available treatment. In addition to this genetic counseling services are not available and appropriate genetic advice is not given to parents of these children so they often have the additional problems of further affected infants before the underlying disorder is detected.

There is a need to consider IEM as a potential cause of any severe illness and to have a systemic approach to diagnosis and management. However, this was not possible as the diagnostic facilities were non-existent. Due to the lack of diagnostic facilities and absence of national registries for disease prevalence of various inherited diseases are not known. Some of the IEM like beta thalassemia, G6PD, congenital hypothyroidism and CAH are widely prevalent in our society.

Despite undergoing major revolutions in the past years in the world, the field of inborn errors of metabolism has so far been neglected in Pakistan. The primary reason is non-availability of the diagnostic facility, lack of expertise both in terms of human resource as well as equipment needed to perform the analysis. The equipment's are not only technically demanding but are very expensive. In addition, there is no data to reflect the prevalence of the defects from Pakistan. However, the incidence appears to be high due to high degree of consanguineous marriages. Aga Khan University Hospital Clinical Laboratory has now successfully acquired this technique and has initiated the specialized testing for diagnosis of IEM. The tests will be performed on state of art equipment for diagnosis of amino acid disorders and organic academia. The two techniques together

can diagnose a number of disorders in a suspected patient. Table 3 shows the IEM that has been diagnosed from January to July 2013 in our lab.

Table 3. Aminoacidopathies and Organic acidemias Diagnosed at AKUH Clinical Laboratory (20th January 2013-3rd July 2013)

N= 306,	Aminoacidopathies/ Organic Acidemia
Total No. of Positive cases	25
Methylcobalmin defect or deficiency	7
2-methyl,-3-OH butryl Co A dehydrogenase deficiency	2
Hyperphenylealalinemia	1
Cystathionine beta synthetase deficiency	3
Propionic acidemia	2
Ethylmalonic aciduria	1
Urea cycle disorder	2
MSUD	1
Biotinidase or holocarboxylase deficiency	1
Isovaleric acidemia	2
Glutaric aciduria	1
2-methyl butyryl glycine aciduria	1
Lysinuric protein intolerence	1

Physicians play an important role in the recognition of infants who may have an IEM and in the initial evaluation and stabilization of these patients and specific treatment. At the same time Chemical Pathologist has a great responsibility of appropriately diagnosing these conditions. A very close liaison is required between chemical pathologist and treating physician. Clinicians should provide related history of the patient to the Clinical Laboratory that helps in appropriate processing of the specimen and analysis; hence report interpretation and correct diagnosis by Chemical Pathologist.

It is important to progressively bring the inborn errors of metabolism to a higher level among health priorities. Challenges to the progress of the IEM field include huge disparities in our ethnic populations, high prevalence of malnutrition and infections, co-existence of very different models of public health services, unstable socio-economic and political conditions, and difficulties in integrating the various stakeholders. There is a need to develop activities for provision of health services, education and research as an integrated package, increase training of human resources, expansion of access to diagnostic tests, and use of the neonatal screening framework to expand the provision of services. In a country with no IEM centers, there is a major need for such groups to work in collaboration, complementing each other's capabilities, providing training, and developing joint projects. The integration of these groups into a large transnational network of reference centers would be our major aim for the coming years.

Inherited Metabolic Disorders - Presenting as Metabolic Emergencies

Dr Bushra Afroze
Department of Paediatrics and Child Health

"When you hear hoofs, think of horses"…*but do not forget Zebras!*

Inborn errors of metabolism (IEM) comprise a large group of inherited disorders resulting from either an enzyme or a co-factor defect. Individual disorders in the large group of IEM are rare; however collective incidence of IEM is 1 in 5000 live birth, which is same as the incidence of congenital hypothyroidism. A significant proportion of IEM present during the neonatal or early childhood period with severe acute clinical manifestation. Unfortunately, for most

Table 1. Clinical Presentation of IEM

Acute IEM Presentation	Chronic IEM Presentation
• poor feeding	• developmental delay
• persistent vomiting	• seizures resistant to anticonvulsant therapy
• lethargy	• movement disorder
• convulsions	• peripheral muscle weakness
• hypotonia or spasticity	• cardiomyopathy
• tachypnea/ Kussmaul breathing/apnea	• hepatosplenomegaly
• failure to thrive	• hypoglycemia
• coma, or	• renal failure
• lack of improvement to any of the above with standard therapy	• cataracts
	• retinal abnormalities
	• macrocephaly
	• dysmorphic features
	• unusual body odors

disorders the early symptoms are non-specific like lethargy, refusal to feed, vomiting and tacypnea or apnea (Table 1). This often leads to consideration of more common causes like birth asphyxia, sepsis and pneumonia at the initial assessment of such patients. IEM are often either completely not considered or considered very late. This leads to delay in diagnosis and appropriate management and eventually result in devastating outcomes.

Inherited metabolic diseases with acute severe manifestations can be divided into five categories:

(1) Disorders of the intoxication type
(2) Disorder with reduced fasting tolerance
(3) Disorders with disturbed energy metabolism
(4) Disorders of neurotransmission
(5) Disorders in which no specific emergency treatment is available.

Diagnostic emergency laboratory evaluation should cover all differential diagnoses that are therapeutically relevant and should always include blood glucose, serum ammonia, serum lactate and acid base status, serum electrolytes for anion gap and urine for ketones. Results of these should be available to treating physician within 30 minutes. According to the clinical situation and biochemical derangement, special metabolic investigations must be initiated in parallel, which include acylcarnitine profiling with tandem mass spectrometry (in plasma or dried blood spots) and analysis of amino acids in plasma and of organic acids in urine. The results of all laboratory investigations relevant to the diagnosis of metabolic disorders for which specific emergency therapy exists should be available within 24 hour. Emergency treatment of metabolic disorders consists of symptomatic treatment, which is applied regardless of the underlying disorder, which does not require knowledge of the nature of the underlying disorder in the critical phase of stabilization. However, details of the diagnosis and in-depth knowledge of the disease in a particular patient is needed for the disease specific long-term treatment and better outcome. The long-term outcome is inversely related to the time between the onset of symptoms and the start of specific emergency treatment in the critical phase of stabilization. This often requires the initiation of therapy before the exact diagnosis is known and rapid initiation of specific investigations. Thus physicians having first encounter with such patients have an important role in recognizing the below-mentioned groups of IEM, (Table 2) getting appropriate labs and initiating right emergency treatment.

Table 2. Categories of IEM with few examples

Disorders of Acute or Progressive Intoxication or Encephalopathy	Disorders Associated with Energy Deficiency	Disorders of Biosynthesis and Breakdown of Complex Molecules
Amino acid disorders	***Glycogenolysis (glycogen storage diseases, GSD)***	***Mucopolysaccharidoses (MPS)***
Cystinuria **Non-Ketotic Hyperglycinemia** **HHH syndrome** **Homocystinuria** **Hyperprolinemia** **Lysinuric protein intolerance** **Maple syrup urine disease** **Phenylketonuria** **Tyrosinemia types I & II** **Vitamin B12 processing disorders**	Liver glycogen synthase deficiency (GSD 0) GSD I; von Gierke disease GSD II; Pompe disease GSD III; Cori/Forbes disease GSD IV; Andersen disease GSD V; McArdle disease GSD VI; Hers disease GSD VII; Tarui disease Phosphorylase b kinase deficiency	MPS I (Hurler, Hurler-Scheie, Scheie) MPS II (Hunter) MPS III (Sanfillippo) MPS IV (Morquio) MPS VII (Sly) MPS IX (Natowicz)
Organic acidemias	***Gluconeogenesis***	***Disorders with deficiency of a single peroxisomal enzyme***
Methylmalonic acidemia **Propionic acidemia** **Isovaleric acidemia** **Glutaric acidemia type I** **3-Methylglutaconic aciduria** **2-Hydroxyglutaric aciduria**	Fructose 1,6-biphosphatase deficiency Pyruvate carboxylase deficiency Phosphoenolpyruvate carboxykinase deficiency Pyruvate dehydrogenase deficiency	Hyperoxaluria type 1 (alanine glyoxylate aminotransferase deficiency) Refsum disease (phytanoyl CoA hydroxylase deficiency) 2-methylacyl-CoA racemase deficiency
Urea cycle disorders	***Fatty acid oxidation defects***	***Sphingolipidoses***
Argininosuccinicaciduria **Carbamyl phosphate synthetase deficiency** **Citrin deficiency** **Citrullinemia** **Ornithine transcarbamylase deficiency** **N-acetyl glutamate synthetase deficiency** **Arginase deficiency**	Short chain acyl-CoA dehydrogenase (SCAD) deficiency Short chain hydroxyacyl-CoA dehydrogenase (SCHAD) deficiency Medium chain acyl-CoA dehydrogenase (MCAD) deficiency Very long chain acyl-CoA dehydrogenase) deficiency Glutaric acidemia type II	Tay-Sachs Fabry disease Farber disease Gaucher disease Niemann-Pick disease
Disorders of carbohydrate intolerance	***Mitochondrial disorders***	***Mucolipidosis***
Galactosemia **Galactokinase deficiency** **Hereditary fructose intolerance** **UPD galactose epimerase deficiency**	Pyruvate carboxylase deficiency Phosphoenopyruvate carboxylase deficiency Freidrich ataxia Cytochrome C oxidase deficiency Pearson syndrome	Mucolipidosis type I (Sialidosis) Mucolipidosis type II (I-cell) Mucolipidosis type III (pseudo-Hurler) Mucolopidosis type IV (Sialolipidosis
		Disorders of peroxisome biogenesis
		Zellweger syndrome Neonatal adrenoleukodystrophy (NALD) Infantile Refsum disease

1. Disorders of the Intoxication Type

This group comprises urea cycle disorders, organic acidopathies, amino acidopathies, fatty acid oxidation defects, galactosaemia and hereditary fructose intolerance.

Clinical presentation: Children with disorders of the intoxication type are typically born after an uneventful pregnancy and show a symptom-free period after birth. The patient with a disorder of protein or fat catabolism typically develops symptoms in the first few days of life during the naturally occurring catabolic period. Symptoms include poor sucking, vomiting, and lethargy progressing to coma. Sepsis is a common diagnosis considered in this situation. After the neonatal period, catabolism is rare in the first months of life. Towards the end of the first year of life, however the risk of metabolic decompensation increases as a consequence of episodes of infection and protein-rich meals.

Principles of therapy: The oral intake of toxic precursors (protein in cases of disorders involving protein metabolism and fat in disorders involving fat metabolism) must be stopped. The endogenous catabolism of protein or fat must be reversed in disorders of protein or fat degradation. Specific detoxification measures should be instituted if available. However, neither protein nor fat can be stopped from diet for more than 48 hours. After initial 48 hours, only the involved amino acid in protein metabolic disorders and long chain length fatty acids in fatty acid oxidation defects are restricted based on the diagnosis established based on acylcarniitne, plasma amino acid and urine organic acids results.

2. Disorders with Reduced Fasting Tolerance

This group includes disorders of glucose homeostasis (glycogen storage diseases, disorders of gluconeogenesis, congenital hyperinsulinism) and disorders in which ketone bodies cannot be synthesized for use as alternative substrates once glycogen stores are exhausted (fatty acid oxidation defects, disorders of ketogenesis and ketolysis).

Clinical presentation: These disorders become symptomatic when the intervals between meals become longer or in the case of limited glucose supply, for example when vomiting or anorexia result from inter-current infection. A few patients may present in the neonatal period, however most present after infancy when nocturnal feeding is usually not as frequently given as in early infancy.

Principles of therapy: Glucose administration at the rate of the hepatic glucose production is basically sufficient to meet caloric demands in these disorders. Patients with congenital hyperinsulinism may need glucose much higher than the hepatic glucose production, which needs to be adjusted based on individual patient's requirement. Forced anabolism is not needed in these disorders, as there is no intoxication due to catabolism.

3. Disorders with Disturbed Mitochondrial Energy Metabolism

This group comprises defects of the pyruvate dehydrogenase complex and the respiratory chain.

Clinical presentation: Generalized lack of energy often impairs intrauterine growth and development. Due to presence of prominent lactic acidosis, perinatal asphyxia is often considered as the initial diagnosis in affected neonates.

Principles of therapy: Correction of acidosis is a major goal. Glucose supply has to be limited, especially in defects of the pyruvate dehydrogenase complex, because a high glucose supply may exacerbate lactic acidosis.

4. Disorders of Neurotransmission

Acute emergency treatment is available in two disorders of neurotransmission: vitamin B6- and folinic acid-responsive seizures.

Clinical presentation: present immediately after birth with an epileptic encephalopathy, which is unresponsive to conventional anti-convulstants therapy.

Principles of therapy: In every newborn with epileptic encephalopathy a trial with intravenous pyridoxine and folinic acid should be performed.

5. Disorders with No Specific Emergency Treatment available

There are many other metabolic disorders that may present with acute, often encephalopathic, manifestations (nonketotic hyperglycinaemia, sulphite oxidase deficiency) or that usually follow a chronic progressive course but may worsen during episodes of acute illness (congenital disorders of glycosylation, peroxisomal disorders).

Role of Biochemical Genetics Laboratory in Evaluation of IEM

Dr Farhan Javed Dar
Chemical Pathology

Laboratory evaluation for inborn errors of metabolism (IEM) should be undertaken in all patients with suggestive history examination, and/or abnormalities of routine laboratory tests. Patients with life-threatening illness should undergo concurrent evaluation for other conditions in the differential diagnosis such as sepsis, cardiac disease, etc. An optimal outcome of a child with IEM depends upon early recognition of the signs and symptoms of metabolic disease followed by prompt laboratory evaluation and referral to a center familiar with the management of these disorders. Delay in diagnosis may result in acute metabolic decompensation, progressive neurologic injury, or death.

Laboratory investigations can be tiered up to three levels which help in making diagnosis of IEM in a patient.

1. Metabolic screening panel consisting of initial laboratory tests
2. Specialized testing consisting of qualitative detection of urine organic acids and quantification of amino acids in plasma, urine, CSF, etc
3. Enzyme assay

1. Metabolic Screening Panel: First Line Investigations for Diagnosis of IEM

An abnormal laboratory value may be the first finding noted that suggests an IEM. The first line investigations are very important diagnostic clue for further confirmatory laboratory investigations in suspected patient of IEM. However, in some disorders, these laboratory abnormalities are only present during the episode of metabolic decompensation. If possible, blood and urine samples should be obtained for both the initial and specialized tests at the time of presentation. Samples for specialized tests should be processed and stored appropriately for further testing if indicated.

In suspected patient of IEM, following are the first line investigations offered at Clinical Laboratory of Aga Khan University Hospital. These tests provide a valuable clue of establishing a differential diagnosis as shown in Table 1.

Table 1. Distinguishing Biochemical Findings of IEM

Findings	MSUD	OA	UCD	DCM	FAO
Metabolic Acidosis	±	++	-	±	±
Respiratory Alkalosis	-	-	+	-	-
Hyperammonemia	±	+	++	-	±
Hypoglycemia	±	±	-	+	+
Ketones	A/ H	H	A	A/ H	A/ L
Lactic Acidosis	±	±	-	+	±

MSUD:	Maple syrup urine disease,	-	usually absent
OA:	Organic acidemia,	±	sometimes present
UCD:	Urea cycle disorders,	+	usually present
DCM:	Disorders of carbohydrate metabolism,	++	always present
FAO:	Fatty acid oxidation	A H L	appropriate inappropriately high inappropriately low

Complete Blood Count: provides a clue to sepsis, which may be the trigger for a metabolic crisis. IEM patient may have anemia, thrombocytopenia, or pancytopenia.

Arterial blood: is used to detect acid-base disturbances. Metabolic acidosis with an increased anion gap is commonly associated with organic acidemias. It may also may be present in amino

acid disorders, disorders of pyruvate metabolism, mitochondrial disorders, and disorders of carbohydrate metabolism. Respiratory alkalosis is commonly seen in urea cycle disorders as a result of hyperammonemia.

Serum Electrolytes: Measurement of serum electrolytes is necessary to calculate the anion gap. A metabolic acidosis with an increased anion gap is commonly seen in organic acidemias.
Plasma lactate: Lactic acidosis caused by abnormal oxidative metabolism is a frequent finding in mitochondrial disorders, glycogen storage disorders, and disorders of gluconeogenesis.
Plasma ammonia: Significant elevations in ammonia are most commonly associated with urea cycle disorders and certain organic acidemias (particularly propionic and methylmalonic acidemias). High blood levels of ammonia may also be found in other amino acid disorder and other fatty acid oxidation defects.

Plasma Glucose: Hypoglycemia is typical of disorders of ketogenesis e.g. fatty acid oxidation disorders, glycogen storage disorders, and disorders of fructose metabolism. It also may occur in amino acid disorders, organic acidemias, and mitochondrial disorders.

Urine detail report: Urine detail report gives clue to certain IEM disorders e.g. urine specific gravity lowers down in number of IEM disorders having renal tubular dysfunction. The urine pH is also helpful in determining the cause of metabolic acidosis. Presence of ketones may also suggest IEM. Ketones are usually presents in patients with hypoglycemia and glycogen storage disease, organic academia.

Urine reducing substances: Reducing substances in the urine is a clue to certain IEM if the urine dipstick is negative for glucose. Positive result may indicate carbohydrate intolerance disorder (e.g. galactosemia, hereditary fructose intolerance) or an amino acid disorder. However, the absence of reducing substances in the urine does not exclude these disorders.

2. Second Line Tests:

Specialized tests are performed on the basis of clinical context and first line of investigations. These tests include quantitation of amino acids, organic acids, carbohydrate and other metabolites, mucopolysaccharide separation and speciation, etc.

Few frontline laboratory tests in this tier are:
Gas chromatography mass spectrometry (GCMS): of urine for diagnosis of organic acidemias Plasma amino acids and acyl-carnitine profile: by tandem mass spectrometry (TMS) for diagnosis of organic acidemias, urea cycle defects, aminoacidopathies and fatty acid oxidation defects.

High performance liquid chromatography (HPLC): for quantitative analysis of amino acids in blood and urine; required for diagnosis of organic acidemias and aminoacidopathies

CSF aminoacid analysis: CSF Glycine levels elevated in non-ketotic hyperglycinemia
Urinary orotic acid- in cases with hyperammonemia for classification of urea cycle defect
Urinary Succinylacetone in cases of tyrosinemia.

Plasma very long chain fatty acid (VLCFA) levels: elevated in peroxisomal disorders
Urine chromatography by thin layer chromatography and liquid chromatography mass spectrometry (LCMS); for qualitative and quantitative detection of sugars.

Urine purines and pyrimidines analysis by Gas chromatography mass spectrometry.

3. Definitive Diagnostic Tests; Enzyme Assay:

To confirm the disorder detected on specialized tests, a specific enzyme assays in leucocytes, plasma/serum or red cells, immunoassays and DNA/ Mutation based analysis is required. Utility of enzyme assay in establishing diagnosis of IEM is confirmative. These tests are available at highly specialized centers for IEM.

Biochemical Metabolic Testing At Clinical Laboratory AKUH:

Other than first line investigations (metabolic screening panel) specialized tests have been introduced at Clinical Laboratory AKUH. These tests are useful in diagnosis of disorders of amino acids, urine organic acids and some fatty acid oxidation defects. The specialized tests for IEM available are:

Plasma, Urine and CSF Amino Acids (Quantitative, Method: cation-exchange HPLC)
Urine Organic Acid (Qualitative, Method: GCMS)
Urine Succinylacetone (Quantitative, Method: GCMS)

Amino Acid Chromatography for the Diagnosis of Inborn Errors of Metabolism

Azeema Jamil
Chemical Pathology

Amino acids are the building blocks of proteins and have many functions in the body. Amino acid disorders are a group of inherited metabolic conditions, each associated with a specific enzyme deficiency involved with protein metabolism that causes the accumulation of amino acids or metabolites in blood and urine. The accumulated compounds and metabolites are toxic, resulting in the clinical features of these disorders.

Using amino acid chromatography 22 amino acids can be quantified. Of these nine are essential amino acids. Amino acids eluted on chromatogram after analysis of standard prepared are listed in Table 1.

Table 1. List of amino acids quantified in Clinical Laboratory of AKUH

Amino Acid Quantification	
Taurine	Cystine
Aspartate	Methionine
Threonine	Isoleucine
Serine	Leucine
Aspargine	Tyrosine
Glutamate	Phenylalanine
Glutamine	Ornithine
Glycine	Lysine
Alanine	Histidine
Citrulline	Arginine
Valine	Proline

Principle of Amino Acid Quantification:

The amino acid analyzer (Shown in Fig. 1) is designed to provide accurate quantitative analysis of amino acid mixture. It gives fully automatic and high speed analysis which is very sensitive. Basic principle of instrument is cation-ion exchange high performance liquid chromatography (HPLC).

Fig. 1. Analysis of Amino Acids is in progress
Courtesy of Section of Biochemical Genetics Laboratory, Aga Khan University

The sample containing a mixture of amino acids is loaded into a column of cation-exchange resin. The sample is moved down in the controlled column temperature along with buffer of required pH which is programmed to produce the required separation. At high temperature of reaction coil, ninhydrin reacts with amino acids present in the eluent to form colored compound. In elute, the amount of colored compound produced is directly proportional to the quantity of amino acids present. The amount of each colored compound is determined at two different wavelengths, 440nm and 570nm. At 440 nm hydroxproline and proline is measured, the rest are estimated at 570 nm.

Estimation of one sample takes almost 3 hours to complete. The unit of reporting is µmol/l (Table 2) and age specific reference ranges are in used. The standard chromatogram of plasma amino acid is shown.

Table 2: Marked elevation of phenylalanine is noted in the amino acid quantification report below which is suggestive of hyperphenylalaninemia.

Plasma Amino Acid Quantification

Test	Result	Unit	Normal Range
Taurine	36	µmol/L	(19 139)
Aspartate	1	µmol/L	(0 - 16)
Threonine	43	µmol/L	(36 - 136)
Serine	108	µmol/L	(42 - 174)
Aspargine	43	µmol/L	(26 - 70)
Glutamate	18	µmol/L	(24 - 68)
Glutamine	299	µmol/L	(337 - 709)
Glycine	153	µmol/L	(74 - 290)
Alanine	109	µmol/L	(144 - 348)
Citrulline	20	µmol/L	(6 - 34)
Valine	92	µmol/L	(79 - 267)
Cystine	28	µmol/L	(43 - 67)
Methionine	5	µmol/L	(9 - 29)
Isoleucine	28	µmol/L	(32 - 92)
Leucine	63	µmol/L	(53 - 149)
Tyrosine	35	µmol/L	(14 - 114)
Phenylalanine	1201	µmol/L	(26 - 70)
Ornithine	36	µmol/L	(9 - 105)
Lysine	192	µmol/L	(56 - 146)
Histidine	40	µmol/L	(30 - 110)
Arginine	26	µmol/L	(18 - 78)
Proline	99	µmol/L	(53 - 201)

Organic Acid Chromatography for the Diagnosis of Inborn Errors of Metabolism

Farhat Jahan
Chemical Pathology

Organic acids are compounds with acidic properties and are intermediary or end product of metabolism Fig. 1. An enzyme defect in a metabolic pathway can lead to accumulation

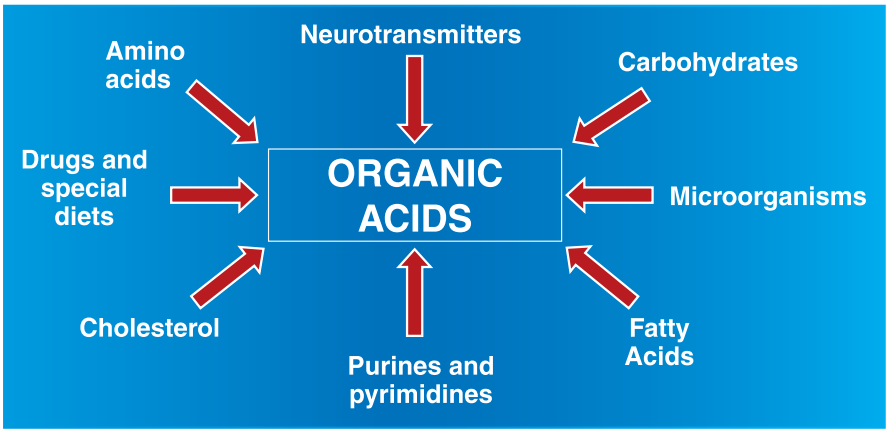


Fig. 1. Wide range of Compounds produce Organic Acids during their Metabolism

and increased excretion of one or more of organic acids in urine resulting in Organic Acidurias, that may lead to acute life-threatening illness, developmental delays, and metabolic decompensation. Urine organic acid analysis is an essential component of the workup of the patient suspected to have an inborn error of metabolism.

Principle of Detection of Urine Organic Acids:

Organic acid analysis by Gas Chromatography-Mass Spectrometry (GC-MS) is critical for the diagnosis of organic acidurias. This technique is a combination of two powerful techniques: Gas chromatography and Mass spectrometry. GC can separate volatile and semi-volatile compounds with great resolution whereas MS can provide detailed structural information on most compounds such that they can be exactly identified.

The process involves processing of sample followed by injection into the column via injector (Fig. 2). The sample is then carried into the column by carrier gas 'helium' and the compounds in sample are separated on the basis on their boiling points and chemical structure as the column is heated. Effluent from GC passes through transfer line into the Ion trap/ Ion source where molecules then undergo electron ionisation (EI). These ions are then analysed according to their mass to charge ratio and are detected by electron multiplier which produces a signal proportional to

Fig. 2. Analysis of Urine Organic Acid is in Progress
Courtesy of Section of Biochemical Genetics Laboratory, Aga Khan University

ions detected resulting in a unique spectrum. Urine organic acid chromatogram achieved in a patient with 'hyperphenylalaninemia' is shown in Fig. 3.

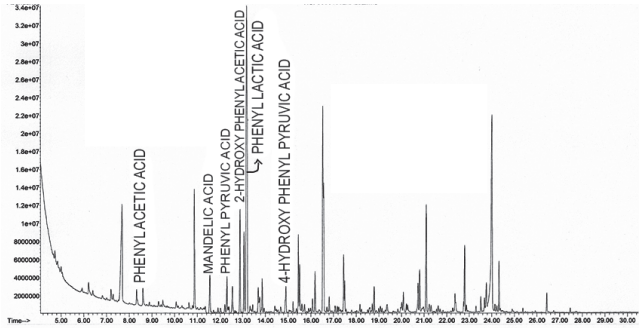


Fig. 3. Urine Organic Acid Chromatogram showing Markers of Hyperphenylalaninemia Courtesy of Section of Biochemical Genetics Laboratory, Aga Khan University

Sample preparation is done in batches which take almost 3.5 hours and the sample analysis time on instrument is 35 minutes. All the peaks in chromatograms are matched with available organic acid libraries. After skillful interpretation the accurate diagnosis is made.

Polymorphism in Methylenetetrahydrofolate Reductase (MTHFR) Gene

Zahida Amin Mir and Lamia Altaf
Molecular Pathology

Methylenetetrahydrofolate Reductase (MTHFR) is a rate-limiting enzyme in the methyl cycle. This enzyme is encoded by the MTHFR gene on chromosome 1 location p 36.3. It plays a vital role in folate metabolism, by catalyzing reduction of 5,10- methylenetetrahydrofolate to 5-methylenetetrehydrofolate (Fig. 1), which is

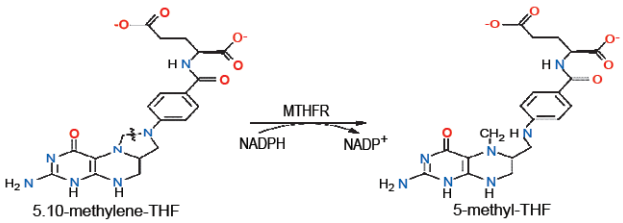


Fig. 1. Schematic diagram of the Reductive Carbon-nitrogen Bond Cleavage (represented by wavy line) Catalyzed by Methylenetetrahydrofolate Reductase

required for the multistep process that converts the amino acid homocysteine to another amino acid, methionine (Fig. 2). As human body requires methionine for protein synthesis, this is a critical enzyme. Mild to severe loss of enzyme activity results in elevated levels of homocysteine in

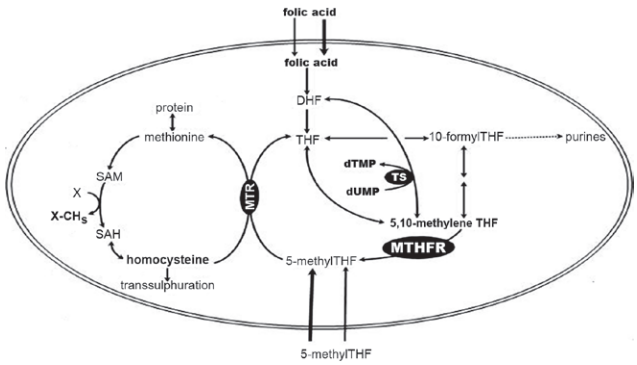


Fig. 2. Overview of the Human Folic Acid Metabolic Pathway and the role of MTHFR S-adenosyl Methionine (SAM), S-adenosyl Homocysteine (SAH), Dihydrofolic Acid (DHF), Tetrahydrofolic Acid (THF), Dihydro-Folate (DHF), Methionine Synthase (MTR), Thymidylate Synthase (TS)

blood and/or urine, leading to development delays and mental retardation. Elevated homocysteine levels may also cause irritation of blood vessels and clotting of blood in the veins. As a result of which, coronary artery disease leading to a heart attack or stroke may occur. In addition due to MTHFR deficiency body is unable to absorb folic acid which is essential for the development and health of the fetus.

MTHFR Gene Polymorphism

The two major single nucleotide polymorphisms (SNP) associated with MTHFR are C677T and A1298C.

C677T SNP (Ala222Val)

The MTHFR nucleotide at position 677 in the gene has two possibilities, C (cytosine) or T (thymine). C at position 677 (leading to an alanine at amino acid 222) is the normal allele. The 677T allele (leading to a valine substitution at amino acid 222) encodes a thermolabile enzyme with reduced activity. Individual with two copies of 677C (677CC) have the "normal" or "wildtype" genotype. 677TT individuals (homozygous) are said to have mild MTHFR deficiency. 677CT individuals (heterozygotes) are almost the same as normal individuals because the normal MTHFR compensate for the thermolabile MTHFR.

A1298C SNP (Glu429 Ala)

At nucleotide 1298 of the MTHFR, there are two possibilities: A or C. 1298A (leading to a Glu at amino acid 429) is the most common while 1298C (leading to an Ala substitution at amino acid 429) is less common. 1298AA is the "normal" homozygous, 1298AC the heterozygous and 1298CC the homozygous for the "variant". The protein encoded by 1298C cannot be distinguished from 1298A in terms of activity, thermolability, flavin adenine dinucleotide release, or the protective effect of 5-methyl-tetra hydro folic acid. The C mutation does not appear to affect the MTHFR protein. It does not result in thermolabile MTHFR and does not appear to affect homocysteine levels.

MTHFR Genotyping

DNA is isolated from blood and subjected to in vitro amplification for the desired regions of the MTHFR gene codons 677 and 1298 using sequence specific primers. The amplified products are analyzed by gel electrophoresis followed by ethidium bromide stainingThe amplified PCR products (MTHFR) are subjected to Hinf I for codon 677 and Mbo II for codon 1298 restriction enzyme digestion at 37°C overnight. The PCR products subjected to enzyme digestion are visualized on 3% agarose gel stained with ethidium bromide.

For MTHFR 677, the PCR yielded a 198 bp product, which on digestion with Hinf I produced a 175 and 23 bp fragments for TT condition (homozygous polymorphic) and a 198,175 and 23 bp fragments for CT condition (heterozygous polymorphic). An undigested product length of 198 bp was retained by the wild types (Fig. 3A). For MTHFR 1298 genotyping PCR conditions were same as that of MTHFR 677 and the product yielded a 241bp fragment. AA genotype (wild type) on digestion with Mbo II, yield two fragments of sizes 204 and 37, CC genotype (homozygous polymorphic) after digestion retained the 241 fragment size and AC genotype (heterozygous polymorphic) produced three fragments of size 241,204 and 37bp (Fig. 3B).

Fig. 3A. Gel Electrophoresis showing C677T Single Nucleotide Polymorphism

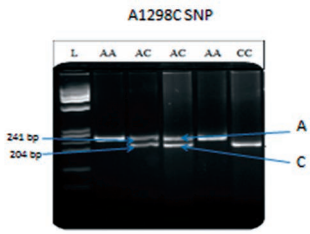


Fig. 3B. Gel Electrophoresis showing A1298C Single Nucleotide Polymorphism

Diagnosis of Tyrosinemia Type I

Nasir Ali
Chemical Pathology

Tyrosinemia type I is an autosomal recessive disorder characterized by lack of the enzyme fumarylacetoacetate hydrolase (FAH), which is needed to break down the amino acid tyrosine. Failure to properly break down tyrosine leads to abnormal accumulation of tyrosine and its metabolites; succinylacetone and succinylacetoacetate in the liver, resulting in severe liver disease. (Fig. 1) Tyrosine may also accumulate in the kidneys and central nervous system.

Others types of tyrosinemia includes type II

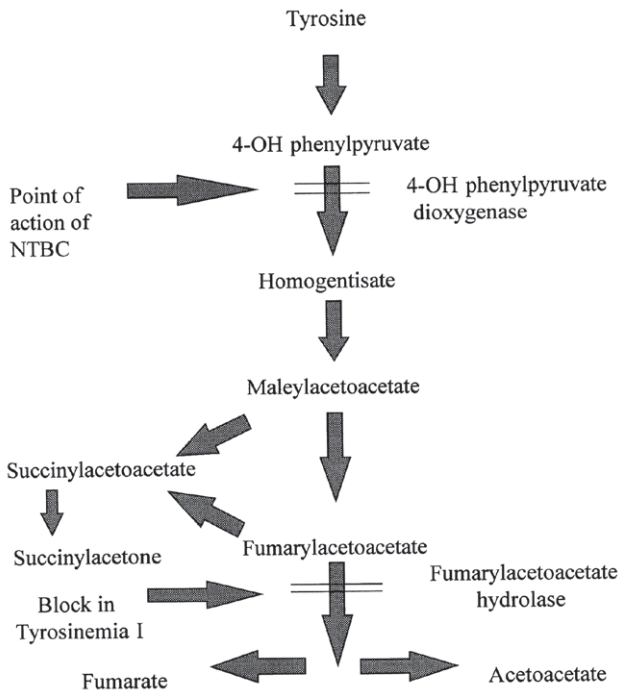


Fig. 1. Pathway for Degradation of Tyrosine showing the Defect in Tyrosinemia Type 1 and the point of Action of NTBC

and III. Tyrosinemia type II, also known as oculocutaneous tyrosinemia, characteristically effects the cornea and skin, and is caused by deficiency of tyrosine aminotransferase, which acts at the first step in tyrosine catabolism. Tyrosinemia type III is a rare disorder caused by deficiency of 4-hydroxyphenylpyruvate dioxygenase (4HPPD). In addition to the three inherited disorders, transient tyrosinemia of the newborn is the major cause of tyrosine elevations detected on newborn screening. Patient with tyrosinemia may present with symptoms within the first few months of life and may include diarrhea, bloody stools, failure to thrive, vomiting, lethargy, irritability, and a "cabbage-like" odor to the skin or urine. If untreated, liver problems such as hepatomegaly jaundice, easy bleeding/bruising, and swelling of the legs/abdomen are common. Kidney problems can cause rickets and delays in walking. Without treatment, liver and kidney problems usually lead to death. Periodic episodes of pain/weakness (particularly in the legs), tachycardia, breathing problems, seizures, and coma may occur.

The diagnosis of tyrosinemia is confirmed by measuring the elevated levels of tyrosine often with methionine and perhaps generalized aminoacids in the blood and organic acids along with 'succinylacetone' in the urine. Enzyme testing and genetic testing of the FAH gene may also be used to confirm the diagnosis.

Only 2-5 ml spot (untimed) urine is required for analysis of succinylacetone. Succinylacetone is photosensitive therefore protection of urine from light is advised. It can easily be done by wrapping the container with dark plastic bag. Urine succinylacetone is reported in mmol/mol creatinine and is normally not detected in urine.

Once diagnosed, children with tyrosinemia are treated with a medication called nitisinone (previously called NTBC). A diet low in tyrosine, phenylalanine, and methionine, and a special medical formula is often recommended. In the long term, individuals with liver damage or liver cancer may require a liver transplant. Treatment can prevent liver disease, kidney problems, and the neurological problems that can be associated with tyrosinemia.

Highlights of Metabolic Services at Clinical Laboratory of AKUH

Azeema Jamil, Rakshanda Mahar, Farhat Jahan, Nasir Ali
Chemical Pathology

Specimen Collection and Submission

Three types of specimens can be submitted at AKUH Clinical Laboratory to perform specialized tests related to amino acid, urine organic and fatty acid oxidation disorders. For plasma amino acid detection in blood, only a few millilitres, 2-4 ml of venous blood in tube containing lithium heparin (green top) is required. Fasting specimen (at least two hours for infants and four hours for children) is preferred to avoid the increase of most amino acids after a meal intake.

Only 2-5 ml of urine collected in a clean sterile bottle is required for amino acid quantification in urine, qualitative detection of organic acids and quantification of succinylacetone. Urine can be collected anytime during the day. However, an early morning sample of urine is preferred.

The third specimen type in which quantification of amino acid is performed is Cerebrospinal fluid (CSF). The CSF is collected by the patient's concerned doctor in a sterile container/ micro-tube. Only 0.5 ml clear CSF is required for the analysis along with 2 ml whole blood collected in lithium heparinized tube. Plasma amino acid quantification is required to calculate the ratio between CSF and plasma amino acids. For analysis minimum acceptable volume of separated plasma, urine and CSF is 0.2 ml (200 µl). It is advisable to collect sample for laboratory tests related to IEM when symptoms are most pronounced in patient and before start of any treatment.

Specimen Transportation

For transportation of above specimens few important things must be followed. The most important one for plasma amino acid is that blood should be transported to laboratory on ice within four hours where it should be centrifuged and separated plasma should be stored at minus -20°C. If the sample is collected outside AKUH then separated plasma should be transported to laboratory in dry ice. Freezing of whole blood collected in tube containing lithium heparin is prohibited.

Urine sample should also be frozen if the transportation time of more than four hours is required. For succinylacetone one more thing should be taken care of that is to avoid any light exposure.

CSF is very precious sample to analyze and it should be sent on ice immediately as collected. If CSF has to be transported from outside AKUH it should be transported in dry ice.

Specimen

There is a sample rejection policy at AKUH clinical laboratory where hemolyzed blood sample, stained CSF and unfrozen specimen received at laboratory are rejected. All specimens received at the processing bench in the Section of Chemical Pathology are checked and received as per specimen collection policies. If any specimen is rejected such as specimen collected in wrong tube, a telephone call is made to the provided contact number given at time of specimen deposition for request of fresh sample.

Specimen Reporting

The turnaround time for amino acid quantification is 10 days and for urine organic acid is seven days after receiving the sample at Clinical Laboratory of AKUH. However, the stat testing of these tests within three days can be accomplished on physician request. Urine succinylacetone is performed on every Wednesday and the reporting is available on next Wednesday.

Coordination between Laboratory and treating Physician

All positive cases of IEM are discussed and reported to concerned physicians.

Filling of History Form

Physicians need to fill the patient's history form for all the tests being performed at the biochemical genetic lab for the diagnosis of IEM. This history form contains information regarding patient's current health sign and symptoms along with patient's detailed clinical history.

This history form is available at the Clinical Laboratory reception within AKUH as well as in all; Pediatric Ward, NICU, ER, etc in AKUH campus. It is also available at all AKUH phelebotomy centers within and outside Karachi.

This is the sample copy of the form.

AGA KHAN UNIVERSITY
CLINICAL LABORATORY
BIOCHEMICAL GENETICS SECTION

Important Note:
Please fill up the entire form to ensure correct interpretation of given results.
Tick the appropriate one (√)

Patient Information:

Name: _____

Sex: _____ Age: _____ MR#/L#: _____ Hospital: _____

Home address: _____

Tel #: _____ Cell #: _____

Patient's Doctor Name: _____ Doctor's contact #: _____

SIGN/ SYMPTOMS OF CURRENT ILLNESS: (tick the appropriate one, √)

Fever	Yes	No	Poor sucking/ feeding	Yes	No
Jaundice	Yes	No	Vomiting	Yes	No
Hypotonia/ floppy	Yes	No	Mental retardation	Yes	No
Lethargy/ drowsy	Yes	No	Developmental delay	Yes	No
Coma	Yes	No	Failure to thrive	Yes	No
Septicemia like illness	Yes	No	Colored urine	Yes	No
Smelly urine	Yes	No			

If yes, tick the appropriate one (√):

Sweaty | Dirty socks/ smelly | Rotten vegetable | Caramel syrup/ burnt

Skin lesion Yes No Eye lesion Yes No

Other Sign/ symptoms:

_____

FEEDING HISTORY:

Breast / formula / Mixed/Any special Diet/Milk _____

NPO (tick the appropriate one, √) Yes No If Yes, how many days _____

Solid diet Yes No

SIGNIFICANT ANTENATAL HISTORY:

Gestation: _____ weeks

SIGNIFICANT BIRTH HISTORY: _____

SIGNIFICANT PAST HISTORY: _____

FAMILY HISTORY:

Consanguinity Yes No

PHYSICAL EXAMINATION:

Evidence of failure to thrive Yes No Dysmorphic features Yes No

Involuntary movement Yes No

CVS: _____

Abdomen: _____ Liver: _____ Spleen: _____ Skin: _____

Fundoscopy: Tick the appropriate one (√) Normal Abnormal

TREATMENT GIVEN: Drug therapy. Tick the appropriate one (√)

Antibiotic Yes No Anticonvulsant Yes No Steroid Yes No

Other drugs: _____

LABORATORY RESULTS (last available results, please mention dates available)

Blood Biochemistry:

	Results	**Units**		**Results**	**Units**
Arterial blood gases			Total bilirubin		
pH			Direct bilirubin		
PCO_2			Indirect bilirubin		
PO_2			SGPT (ALT)		
SO_2			SGOT (AST)		
HCO^-_3			GGT		
			Alkaine phosphatase		
Serum sodium			Plasma glucose		
Serum potassium			Plasma ammonia		
Serum chloride			Plasma lactate		
Serum bicarbonate					

Urine Biochemistry:

Urine pH: _____ Urine protein: _____

Tick the appropriate one (√)

Ketones Positive Negative Reducing sugars Positive Negative

CSF Biochemistry:

	Results	Units
Appearance		
CSF Protein		
CSF Glucose		
TLC counts		

Microbiology:

Blood culture Positive No growth CSF culture Positive No growth

Tick the appropriate one (√)

C.T SCAN/MRI Brain:

_____

_____

PROVISIONAL DIAGNOSIS:

_____

_____

Date of form filled: _____

History form filled by: _____

History form received by: _____

Congenital Hypothyroidism

Dr Noreen Sherazi
Chemical Pathology

Congenital hypothyroidism (CH) is one of the most common preventable causes of mental retardation that occurs due to thyroid hormones deficiency at birth. In most cases, the disorder is permanent. Less commonly, the altered neonatal thyroid function is transient, attributable to the transplacental passage of maternal medication, maternal blocking antibodies, or iodine deficiency or excess.

CH is caused by an absent or defective thyroid gland classified into agenesis (22-42 per cent), ectopy (35-42 per cent) and gland in place defects (24-36 per cent). There could be genetic defects of thyroxine or triiodothyronine synthesis within a structurally normal gland. In a small proportion of cases of CH, the defect is due to a deficiency of thyroid stimulating hormone (TSH), either isolated or as part of CH. (Fig. 1)

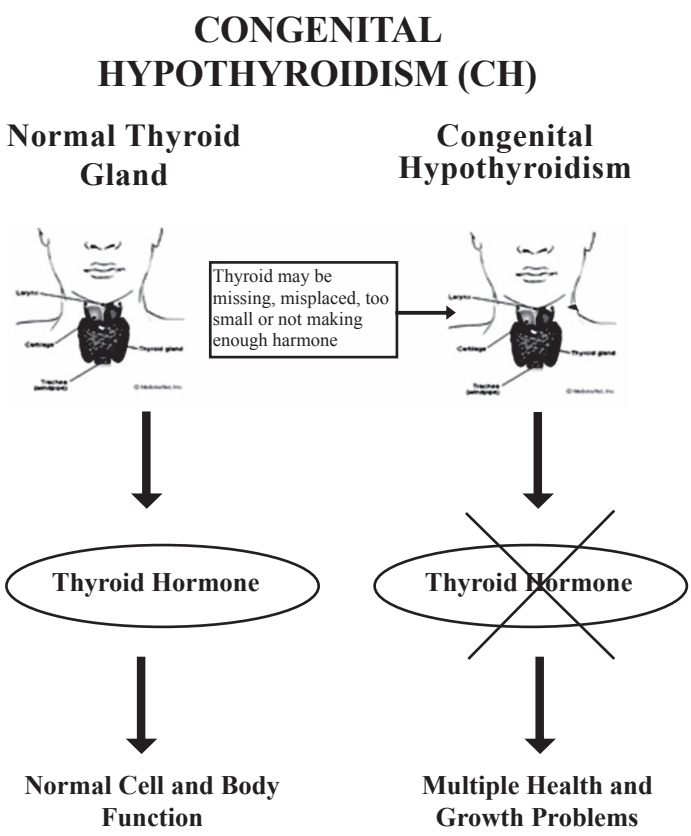


Fig. 1. Metabolic Defect in Congenital Hypothyroidism

The signs and symptoms in CH may be so subtle that they can be easily missed. Excessive sleeping, reduced interest in nursing, poor muscle tone, low or hoarse cry, infrequent bowel movements, exaggerated jaundice, and low body temperature If fetal deficiency was severe because of complete

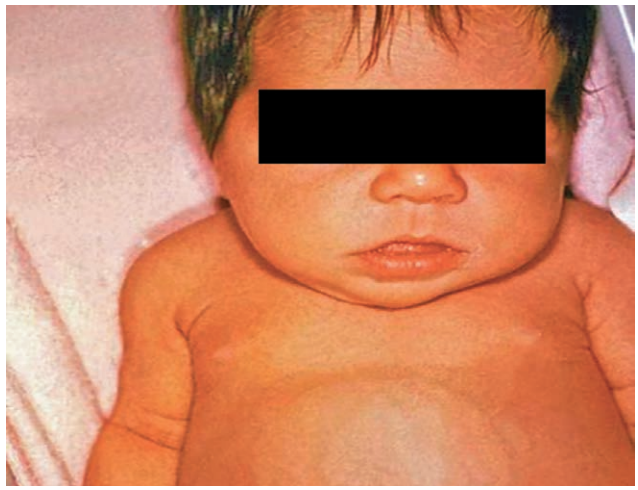


Fig. 2. Features of congenital hypothyroidism

absence (athyreosis) of the gland, physical features may include a larger anterior fontanel, persistence of a posterior fontanel, an umbilical hernia, and a large tongue (macroglossia) (Fig. 2).

In the developed world, nearly all cases of CH are detected by the newborn screening program. It is based on measurement of TSH or thyroxine (T4) on the second or third day of life. There could be high TSH or low T4 in individuals with CH. Two screening strategies for the detection of CH have evolved, a primary TSH/backup T4 method and a primary T4/backup TSH method. In addition, an increasing number of programs use a combined primary TSH plus T4 approach. This represents the ideal screening approach, especially once it is possible for FT4 to be measured accurately and cost-effectively in the eluates from filter-paper blood spots. Every infant should be tested before discharge from the nursery, optimally by 48 hours to four days of age. Specimens collected in the first 24 to 48 hours of life may lead to false-positive TSH elevations when using any screening test approach. Newborn screening test results must be

communicated rapidly back to the physician or hospital identified on the screening filter-paper card.

Most of the hereditary types of CH are inherited in an autosomal recessive manner with an incidence of 1:3000 to 1:4000 according to international data and mostly found to be of increased association with female gender and gestational age >40 weeks. Despite absence of a national program in Pakistan, individual hospitals like Aga Khan University Hospital (AKUH) and Shifa International have made an attempt to screen for CH. In the first two years of screening all newborns delivered at AKUH and four other maternity homes working under umbrella of AKUH were targeted, which included 5,000 births. The first report that was published in JPMA 1989 covers the initial 5000 children screened at AKUH and 997 babies screened at Shifa international over a three year period showing an incidence of 1:1000 for CH. Further in 2008, an audit of CH screening results was published from AKUH covering the data on 53,619 total births where TSH was done on 41,816 newborns out of which 10 cases were detected to be suffering from CH and an incidence of 1:16000 was reported.

The goal of newborn screening programs is to detect and start treatment within the first 1–2 weeks of life. Treatment consists of a daily dose of thyroxine, available as a small tablet. The tablet is crushed and given to the infant with a small amount of water or milk. The most commonly recommended dose range is 10-15 µg/kg daily, typically 37.5 or 44 µg (Table 1). Within a few weeks, the T4 and TSH levels are rechecked to confirm that they are being normalized by treatment. As the child grows up, these levels are checked regularly to maintain the right dose. Persistence of severe, untreated hypothyroidism resulted in severe mental impairment, with an intelligence quotient (IQ) below 80 in the majority.

Most children born with CH and correctly treated with thyroxine grow and develop normally in all respects. Even most of those with athyreosis and undetectable T4 levels at birth develop with normal intelligence, although as a population, academic performance tends to be below that of siblings and mild learning problems occur in some.

Table 1. Management of Congenital Hypothyrodism

Initial workup Detailed history and physical examination Referral to pediatric endocrinologist Recheck serum TSH and FT4 Thyroid ultrasonography and/or thyroid scan
Medications L-T4: 10-15 ug/kg by mouth once daily
Monitoring Recheck T4, TSH 2-4 wk after initial treatment is begun Every 1-2 mo in the first 6 mo Every 3-4 mo between 6 mo and 3 y of age Every 6-12 mo from 3 y of age to end of growth
Goal of therapy Normalize TSH and maintain T4 and FT4 in upper half of reference range
Assess permanence of CH If initial thyroid scan shows ectopic/absent gland, CH is permanent If initial TSH is < 50 mU/L and there is no increase in TSH after newborn period, then trial off therapy at three y of age If TSH increases off therapy, consider permanent CH

Congenital Adrenal Hyperplasia, an Insight of Laboratory Data and Frequency Status

Dr Sahar Iqbal and Dr Aysha Habib Khan
Chemical Pathology
Reproduced from JCPSP

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Congenital adrenal hyperplasia (CAH) refers to autosomal recessive diseases resulting from deficiency of enzymes involved in the production of cortisol by the adrenal glands. More than 90 per cent of cases of CAH are caused by 21-hydroxylase enzyme deficiency (21OHD). Severe deficiency produces classical form of CAH with ambiguous genetalia and salt wasting. However, mild deficiency of enzyme causes hidden late onset or non-classical CAH with non-specific presentation including hirsutism, acne, amenorrhea or infertility at later childhood, adolescence or after puberty.

Neonatal screening program for blood 17 OHP levels are intended to identify CAH cases of classical and non-classical forms in affected children before complication of salt wasting crises arises and for early diagnosis of late non-specific presentation, respectively. Screening of non-classical CAH through testing blood 17 OHP levels is corner stone for early diagnosis as disease presentation is non-specific as well as late in onset. Levels of 17 OHP >4 ng/ml is characteristic feature for disease suspicion in non-classical form in contrast to the diagnostic levels of 200 ng/ml in classical form of CAH.

The non-classic form is one of the most common autosomal recessive diseases in some ethnic groups and one of the reasons of high prevalence of CAH is high rates of inter-marriages among same ethnic groups. Frequency of CAH is not known in Pakistan but expected to be high due to increase rates of consanguineous marriages. The rationale of this is to determine the frequency of CAH among different age groups, gender and distribution in four provinces of Pakistan by evaluating the laboratory data of baseline blood 17-hydroxyprogesterone (17 OHP) levels. The findings of the report are presented in Table 1.

In a retrospective analysis, laboratory data of 5853 samples tested for blood 17 OHP levels was examined. The samples were collected between period of 2007 to 2010, at the main laboratory of AKUH in Karachi and its 188 phlebotomy centers located in four provinces of Pakistan. Subjects of all ages and gender were included with first time and baseline 17 OHP blood levels performed at clinical laboratory. Those were excluded with any follow up blood 17 OHP levels or stimulated with adrenocorticotropic hormone. On the basis of exclusion criteria data was edited for subject's replication and a total of 2282 subjects were recruited with non-probability purposive sampling technique for final analysis. 17OHP results were grouped on the basis of locations of subjects, from where the test was ordered into Sindh, Baluchistan, Punjab and Khyber Pakhtunkhwan (KPK). Subjects were further categorized into four different age groups and three gender groups and frequency was observed for each age group and gender.

Our data showed an annual increasing frequency of blood 17 OHP levels screening (16.3 per cent, 16.9 per cent, 20.4 per cent and 46.4 per cent through the years from 2007 to 2010 respectively). Similarly an increasing trend was also seen annually, for frequency of subjects with suspected CAH (blood 17 OHP levels $\geq$ 4ng/ml) as 5.1 per cent, 6.5 per cent, 7.1 per cent and 15.7 per cent respectively through the years 2007 to 2010. In general, screening for CAH was observed to perform for different age groups and genders. However, first time screening was predominantly performed in female population and after 15 years.

Overall, 34.4 per cent subjects were observed to have 17 OHP blood levels $\geq$ 4ng/ml with suspicion of CAH. Majority of subjects with 17 OHP levels $\geq$ 4ng/ml were from infant age group ($\geq$1 month to $\leq$ 1 year). With respect to gender, mostly females were found positive for suspicion of CAH in comparison with other genders ($p = 0.0001$). Variable percentages were observed for referrals of 17 OHP levels from different provinces but majority were referred from Sindh (73.4 per cent). The demographic details of the subjects are shown in Table 1.

Table 1. Demographic features and Distribution of subjects among different Provinces of Pakistan Tested for Blood 17 OHP levels at Aga Khan University Clinical Laboratory during 2007 - 2010 (n=2282)

Variables	Total Subjects	17 OHP levels ≥ 4 ng/ml	*P - value
	n=2282(%)	n=784(34.4%)	
Age groups			
Up to 1 month	32 (1.4)	28 (1.2)	
>1 month to ? 1 yr	477 (20.9)	328 (14.4)	0.0001
>1yr to ?15 yrs	758 (33.2)	278 (12.2)	
>15 yrs	1015 (44.5)	150 (6.6)	
Gender			
Male	756 (33.1)	363 (15.9)	
Female	1520 (66.6)	416 (18.2)	0.0001
Ambiguous	6 (0.3)	5 (0.2)	
Subjects distribution within country			
Sindh	1676 (73.4)	541 (23.7)	
Punjab	524 (23.0)	209 (9.2)	0.108
Baluchistan	9 (0.4)	3 (0.1)	
KPK	73 (3.2)	31 (1.4)	
***P-value <0.05 is considered as significant**			

As discussed earlier from positive cases (33.4 per cent), most of the suspected cases were infants (14.4 per cent), reflecting the importance of neonatal screening. However we observed that CAH screening was most commonly ordered in elder age group (after 15 years). This late screening may be explained by the late emergence of non-classical CAH signs and symptoms.

Variable frequency of referrals from different provinces of Pakistan was observed as shown in Table 1. Higher frequency of cases with suspected CAH was observed from Sindh (23.7 per cent) as compared to other provinces. The most likely reason of this higher frequency from Sindh may be dependent on numbers of referrals from the Province (73.4 per cent). In Sindh we observed that from Karachi, 68 per cent of the samples were collected from a large number of (78) phlebotomy centers and main AKU campus. We also received 5.4 per cent of samples from others phlebotomy centers located in different cities of Sindh other than Karachi, but we found Karachi city as the main hub for 17 OHP referrals from Sindh province, which may be due to large number of phlebotomy centers. However, beside AKU clinical laboratory, the blood testing of 17 OHP is also offered by other laboratories in Punjab and Sindh. From this laboratory data we could not conclude about the ethnicity of subjects with blood 17 OHP ≥ 4 ng/dl, but it could be presumed that CAH exists throughout the country with variable frequency. This finding was also supported by the results of a previous study from Pakistan where Khan et al also reported presence of CAH in different ethnic groups.

The importance of establishing neonatal screening programme for CAH is enhanced by our present data. However, the benefits of screening for any disease in a state are dependent on the existing healthcare system. The treatment or management of inborn disorders has not been acknowledged appropriately in Pakistan. Additionally the existing healthcare system generally does not address complex issues commonly seen in genetic disorders including CAH patients.

In conclusion, the present laboratory data first time determine the frequency of suspected CAH (33.4 per cent) in Pakistan. However, incidence of CAH remains unidentified due to absence of new born screening program, diagnostic testing, and lack of disease awareness in the society and failure of case identification by primary physician. The need of developing a team of expertise is crucial for early identification and management of CAH to reduce the morbidity and mortality. A well-structured and appropriate screening program should be instituted. The establishment of CAH registry is also critical at regional or national levels for the identified cases and respective prenatal treatment.

About Cystic Fibrosis

Dr Zeeshan Ansar Ahmed and Dr Lena Jafri
Molecular Pathology and Chemical Pathology

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that affects lungs, pancreas, liver, and intestine. CF is caused by a mutation in the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR). This protein is required to regulate the components of sweat, digestive fluids, and mucus. CFTR regulates the movement of chloride and sodium ions across epithelial membranes, such as the alveolar epithelia located in the lungs.

Discovery of CFTR Gene

This gene localized to 7q21-34 on long arm chromosome 7, was discovered in 1985 and its gene sequencing was done in 1989. The gene shown to encode a 1480 amino acid protein, CFTR. This protein has long linkage of amino acids, where they play the role of a brick/block. If any of these bricks are missing or abnormal, then the protein may not work properly. More than 1500 CF mutations have been reported, many of which are rare and some of which may not result in clinical signs or symptoms. The various mutations affect the function of CFTR in different ways.

Mode of inheritance and Risk of Passing on the CF Gene

CF has a simple Mendelian autosomal recessive inheritance pattern (Fig. 1). This shows that people

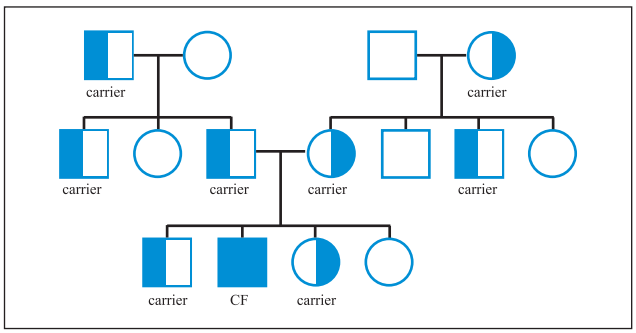

Fig. 1. Simple Mendelian Autosomal Recessive Inheritance. Affected individuals will have two copies of the Mutant CFTR Gene, one Inherited from each parent.

with CF have two copies of the mutant CFTR gene, one inherited from each parent. Carriers have one normal and one mutant CFTR gene and their health is not affected because the normal CFTR gene ensures production of enough protein to allow normal cellular function. Carriers have a 50 per cent chance of passing their mutant CFTR gene onto their children. When both parents are carriers there is a 25 per cent chance in every pregnancy that the child will have CF, a 25 per cent chance that the child will have two normal CFTR genes and a 50 per cent chance that the child will be a carrier.

At Aga Khan University Hospital Clinical Laboratory have the facility of Delta F508 mutation and sweat conductivity tests for cystic fibrosis.

Sweat Testing

It is recommended that sweat conductivity, which is related to the concentration of all ions, not just chloride, should only be used as a screening test. Several recent studies have demonstrated that measurement of sweat conductivity can reliably differentiate patients with CF from those without the condition.

Precautions: Because of a report of transient elevations in sweat electrolyte concentrations in the first 24 hours after birth, it is recommended that patients be at least 48 hours old before undergoing a sweat test. Before starting the procedure it should be made sure that the child is physiologically and nutritionally stable, well hydrated, is not suffering from any acute illness and is not on mineralocorticoids. The skin should be free of cuts, rashes and inflammation.

Technique: It occurs in three phases: sweat stimulation, sweat collection and sweat analysis. Localized sweating is produced by iontophoresis of the cholinergic drug into an area of skin (usually forearm). Macroduct sweat collector is placed over the stimulated area and firmly strapped to the forearm for at least thirty minutes. After attachment, sweat becomes visible in the spiral tube of Macroduct within one to four minutes, depending on the relative elasticity of the skin and the subjects sweating rate. The emergent sweat is turned blue by contact with a small amount of blue water soluble dye applied to the Macroduct collection surface. Sweat collected is then analyzed in the Wescor's conductivity cell.

Interpretation: 0-60 mmol/L - normal
60-80 mmol/L - a possible indication of CF
>80 mmol/L - definitive indication of CF

Failure to produce sweat: An insufficient sweat sample is not uncommon. It can be due to several factors such as dehydration, illness, age, race, skin condition, and issues in collection system. Differences in skin resistance because of ethnicity or individual patient variability may lead to insufficient sample volume. For example, in the United States a higher prevalence of 'quantity not sufficient' samples have been reported with black patients than with white patients.

Significance of Δ F508

In all CF mutations, the most common worldwide mutation is delta F508, which is around 70-82 per cent. it is more common in western countries but also seen in other ethnic groups but the range of mutations are different e.g. in Ashkenazi Jews delta F508 accounts for 27 per cent and W1282X for 51per cent of the mutations. Delta F508 mutation is a three base-pair deletion in exon 10 of the CFTR gene results in the omission of phenylalanine (one of the bricks) at position 508 in the 1480 amino acid chain. A mutant CFTR protein which cannot be folded into its proper shape is produced. The quality control mechanisms within the cell destroy this abnormal protein before it can reach the cell surface where its major normal function is to act as a channel through which chloride ions can pass in and out of the cell.

Detection of Mutation, Delta F508 in CF

DNA is extracted from blood and amplified by ARMS PCR. ARMS assay comprises two reaction, that is identification of specific mutation by two primers, one complementary to normal and other to the mutant DNA sequences. The amplified product is separated by agarose gel electrophoresis Fig. 2.

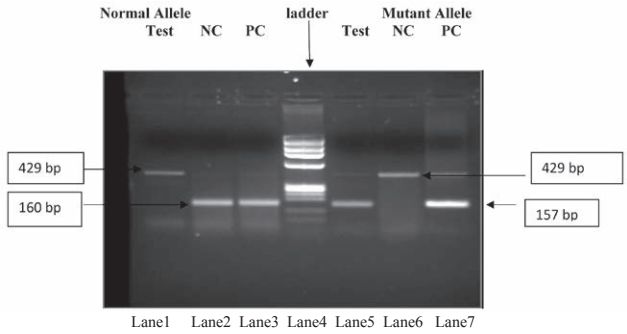

Fig. 2. Detection of Mutation, Delta F508

Normal allele contain 160 base pairs (bp) band with internal control (429 bp), Homozygous case shows mutant alleles (157bp) but in Heterozygous case in both normal and mutant allele seen.(Lane one & five are patient, two & six negative control , three & seven positive control for both normal & mutant allele and lane four is band size marker).

Inauguration of Biochemical Genetics Laboratory: A Step to Strengthen Diagnostic Facilities

Dr Farhan Javed Dar
Chemical Pathology

Aga Khan University Hospital (AKUH) always strives to introduce new Service Excellence projects to improve quality care. Introducing the laboratory diagnostic services for Inborn Error of Metabolism (IEM) in Pakistan is one of the major achievement of Clinical Laboratory in the current year (2013). State-of-the-art techniques such as high performance liquid chromatography (HPLC) and gas chromatography and mass spectrometry (GC-MS) are introduced for detection of IEM disorders.

To make it happen, in the year 2011, a team of Chemical Pathologists comprising of Dr Farooq

Group photo during training at IMR Malaysia; Sep' 2012
Courtesy of Dr Aysha Habib Khan

Ghani and Dr Imran Siddiqui collaborated with Institute of Malaysia Research (IMR). This research institute is a specialized center for IEM testing, headed by Dr Zabedah Md Yunus, a Chemical Pathologist of Malaysia.

In the first phase, two senior technologists and one pathologist from the section of Chemical Pathology visited IMR for four weeks training in October 2011 on amino acids and organic acids chromatograms. In the next phase, Dr Aysha Habib Khan, went to IMR, Malaysia for training in September 2012 to improve and strengthen this service.

On March 20th 2013 an official inauguration ceremony of section of Biochemical Genetics Laboratory was held.

Mr Nadeem Mustafa Khan Regional CEO, Health Services, Asia (AKUH, Pakistan and FMIC, Afghanistan) and Dr Mohammad Khurshid, Professor and Founding Chair Department of

Cake cutting ceremony

Ribbon cutting ceremony by Mr Nadeem Mustafa Khan, Dr Mohammad Khurshid and Dr Naila Kayani

Oncology inaugurated Section of Biochemical Genetics laboratory followed by a cake cutting ceremony. The inauguration ceremony was attended by Dr Naila Kyani and renowned pediatricians Drs Iqtidar Khan, Shahnaz Ibrahim, Bushra Afroze as well as other key officials of AKUH including Dr Nadeem Ahmed, Dr Zafar Sajjad, Mr Sohail Baloch at the Section of Chemical Pathology, Department of Pathology and Microbiology.

The inauguration ceremony was followed by Continuing Medical Education Seminar at the Auditorium of AKU. Dr Farooq Ghani emphasized during the CME seminar that the introduction of highly sophisticated chromatographic techniques has now made it possible to test a newborn for several genetic metabolic disorders simultaneously and informed the audiences how prompt detection substantially improves the prognosis for many of these conditions. Dr Bushra Afroze, metabolic physician at the AKUH was of the view that an

Dr Bushra Afroze, Consultant Clinical Geneticist, Assistant Professor, Department of Paediatics and Child Health

Dr Anita Zaidi, Professor of Paediatics and Child Health

Dr Naila Kayani, Chair Department of Pathology and Microbiology and Director Clinical Laboratory

affected baby could be treated and had every chance of growing normally. Dr Aysha Habib, the Head of the Section of Chemical Pathology at the AKUH, highlighted the issues and challenges and stressed on need for groups to work collaboratively, complementing each other's capabilities and sharing training resources. The CME was inspired by the words of encouragement by the chief guest Professor of Pediatiorcs and Child Health, Dr Anita Zaidi.

The ceremony and seminar ended with closing remarks of Professor Dr Naila Kayani, chairperson of the Department of Pathology and Microbiology and Director AKUH Clinical Laboratories. She lauded AKUH Clinical Laboratories for successfully initiating testing for diagnosis of IEM on state-of-the-art equipment, adding that this was another milestone after the launch of Pakistan's first fully automated biochemistry laboratory at AKUH, Karachi in 2012.

Workshop on "Inborn Errors of Metabolism" Problem Based Learning

'Nutricia Advanced Medical Nutrition' conducted full day workshops on Inborn Errors of Metabolism (IEM) in Karachi and Islamabad on 30th November and 2nd December 2013. Young pediatricians were welcomed by Global Product Manager of Nutricia Turkey, Mr A. Cem Atey. Then Ms Negar Esmail-zadeh, Medical Affairs Manager of Nutricia, Turkey provided a global perspective of IEM followed by an interactive overview by Dr. Bushra Afroze, Consultant Clinical Geneticist and Assistant Professor Department of Pediatrics and Child Health, AKUH. Dr. Lena Jafri, Instructor Department of Pathology and Microbiology, AKUH educated the participants on collection of critical samples in suspected IEM disorders. This was further elaborated by Dr Bushra Afroze who conducted a session on 'How to Deal with Metabolic Emergencies'. The second half of the conference commenced with Dr. Lena Jafri's session on 'What Pediatricians Need to Know about Urine Organic Acid and Plasma Amino Acid Analysis'.

The latter half of the session was dedicated to group sessions on real life IEM cases. The objective of the conference, to create awareness and build capacity in young doctors of the two metropolitan cities concerning the diagnosis and management of IEM was effectively achieved.

Group picture of Workshop Facilitators and Nutricia representatives

hospitals.aku.edu/Karachi/clinical-laboratories