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The importance of early molecular-genetic diagnosis of PAH gene mutations of phenylketonuria patients in the treatment of the disease

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Abstract
The reviewed texts provide valuable information on PKU and highlight the importance of early detection and prompt treatment to minimize the harmful effects of Phe accumulation in the body. They highlighted the need for effective communication between primary care providers, specialists, and families to ensure adequate access to necessary care. In addition, different methods of diagnosis and treatment have been presented, including the exclusion of Phe from the diet and the use of specialized formulas and foods. PKU management is based on personalized dietary approaches, regular monitoring of Phe levels, and the education of mothers and families. In addition, continued research, government subsidies, and social support are important to improve access to medical foods and cover the costs associated with treatment.
Recommendations derived from these texts include the implementation and promotion of neonatal screening programmes for PKU as well as the immediate initiation of treatment once a diagnosis is made. Effective communication and collaboration between health professionals and families are essential to ensuring timely access to necessary care.
This article highlights the significance of inborn errors of metabolism and focuses specifically on phenylketonuria (PKU), a well-known inheritance disorder caused by the deficiency or absence of phenylalanine hydroxylase (PAH). This review discusses associated mutations in the PAH gene and their impact on phenylalanine metabolism. This study emphasizes the importance of early diagnosis and highlights the ongoing need for advancements in screening methods and treatment approaches to optimize patient outcomes in PKU patients. This review provides valuable insights for healthcare professionals involved in the care of children with PKU and contributes to the enhancement of clinical practice in this field.

Keywords: Phenylketonuria, gene mutation, rare disorder, inherited metabolic disease.

History of phenylalanine and tyrosine metabolic measurements

Phenylalanine and tyrosine kinetics were not measured in humans until the 1970–1980s. The first application was for determination of the degree of blockage of phenylalanine hydroxylation in patients with hyperphenylalanemia and phenylketonuria, but this approach was expanded to determination of phenylalanine hydroxylation in normal subjects. Far more uses have been demonstrated for measuring rates of phenylalanine disposal and tyrosine production in relatively normal subjects than in patients with in-born errors of metabolism (1).
Hereditary metabolic diseases are rare disorders of single genes coding for enzymes that convert substrates into specific products. These diseases usually emerge with the accumulation of toxic substrates or the deficiency of essential metabolites. Despite their rare incidence, these disorders constitute an important public health problem, totally (2).
Prior to 1940, there was only circumstantial evidence that tyrosine was produced from phenylalanine. As reported by Moss and Schoenheimer (3), Embden and Blades had shown in 1913 that L-tyrosine was formed in livers perfused with DL-phenylalanine and assumed that phenylalanine metabolism proceeded by conversion to tyrosine. However, Shambaugh, Lewis and Tourtellotte in 1931 suggested that phenylalanine was not converted to tyrosine. Others in the 1920–30's had shown that hydroxylated metabolites of phenylalanine could be found in the urine of animals given a load of phenylalanine. The 1940 study of Moss and

Schoenheimer provided the key evidence and ended the speculation about the metabolism of phenylalanine. They added deuterated DL-phenylalanine to a casein-containing diet given to both growing and adult rats. Today we take these isotopic study results for granted, but in 1940 the use of a deuterated tracer by Moss and Schoenheimer was key in proving the metabolic link between phenylalanine and tyrosine and the ability of phenylalanine to substitute for tyrosine in the diet. Probably the most convincing evidence at that time came from the study of Womack and Rose showing that phenylalanine was essential to the diet of rats, but that tyrosine was not, arguing that tyrosine is formed from phenylalanine, but not the reverse (4).
What quickly became clear from studies in the 1960's was that measurement of whole-body protein synthesis using tracers is actually very difficult. In contrast, whole body protein breakdown is easy to measure using an indispensable amino acid tracer. The dilution of the indispensable amino acid tracer in blood occurs due to release of unlabeled indispensable amino acid from protein breakdown and entry from the diet (5).

Metabolic role of tyrosine

Metabolic products of tyrosine are tyrosine-O-sulfate, phenol, tyramine, melanin pigment, catecholamine, tri-diiodotyrosine and thyroxine. The most obvious application of determination of phenylalanine and tyrosine kinetics is in patients with impaired ability to metabolize phenylalanine or tyrosine. However, the degree of impairment of phenylalanine hydroxylation is usually so large in PKU patients that the whole-body tracer approach has not been terribly effective in defining hydroxylation rate differences due to the difficulty of measuring small enrichments of the phenylalanine tracer in tyrosine. The tracer method has been far more effective when applied to normal subjects. Obvious applications are in subjects whose phenylalanine or tyrosine kinetics are altered, as in the case when tyrosine is limited in the diet or in patients who have mild but significant alterations in phenylalanine-tyrosine metabolism. A key to an accurate picture of whole-body phenylalanine-tyrosine metabolism requires determination of intracellular enrichments at the site of phenylalanine hydroxylation (6). Determination of enrichments of faster turnover proteins secreted by liver is an obvious approach to get information about intracellular hepatic enrichments. Apo-B from VLDL has been demonstrated to be useful for phenylalanine in this regard and should be applicable to measurement of tyrosine as well. Because phenylalanine is an indispensable amino acid, its flux provides a good representation of whole-body protein breakdown. One of the more consistent uses of phenylalanine tracers has been measurement or rates of protein breakdown in the whole body and in tissues that do not hydroxylate phenylalanine, e.g., muscle. There are a limited number of indispensable amino acids whose metabolism and availability of tracers coincide for use of determining protein kinetics in humans, and phenylalanine and phenylalanine tracers are important in this regard (7)
Homogentisic acid is a metabolite in the breakdown process of amino acids such as tyrosine and phenylalanine. In normal condition, it is not detected in urine and blood. In deficiency of homogentisic acid dioxygenase, homogentisic acid builds up in the blood and excretes in urine. When come in contact with air, homogentisic acid reacts with oxygen and cause the urine to become black. This is because of black pigment knows as alkapton and termed as alkaptonuria. This same black pigment in a procedure known as ochronotic causes bone and tissue to darken and degenerate. This causes disabling and painful joint disease called as osteoarthritis (8)

Role of phenylalanine in the body

This supplement is necessary to the usual working of the central nervous system; particularly regarding manifestations like chronic pain and depression along with numerous other disorders that have been associated with the nervous system malfunction. It is involved in formation of neurotransmitters such as nor-epinephrine, epinephrine, and dopamine. Nervous system requires all these chemicals for proper functioning. As a nootropic, phenylalanine has numerous valuable properties improved motivation, increased concentration and focus, anxiety relief and mood enhancement (9)
Diseases associated with an abnormal metabolism of phenylalanine and tyrosine. It is likewise called phenyl pyruvic oligophrenia. It is because of absence of the chemical phenylalanine hydroxylase, which changes over phenylalanine to tyrosine. Phenylalanine is redirected to its ordinarily minor metabolic pathway framing para

hydroxy phenyl pyruvic corrosive, para-hydroxy phenyl lactic corrosive, para-hydroxy phenyl acidic corrosive, and phenyl acetyl glutamine all of which gather in the body alongside phenylalanine. These are discharged in pee in vast sums, which causes mental hindrance. The infection ought to be determined early because to have appropriate treatment (low phenylalanine abstains from food) the impediment of mental improvement can be halted. The best test is finding a raised blood level of phenylalanine. Nonetheless, it can likewise be analyzed prenatally (before birth) by DNA ponders as the quality for phenylalanine hydroxylase has been cloned. The name of the illness phenylketonuria is because of the discharge of para hydroxyphenyl pyruvic corrosive, which is a keto corrosive. This infection is currently gathered under the term hyperphenylalanemia of which there are numerous assortments (6,10).

Symptoms of Phenylketonuria

Newborns with PKU initially don't have any symptoms. However, without treatment, babies usually develop signs of PKU within a few months. Signs and symptoms of untreated PKU can be mild or severe and may include: A musty odor in the breath, skin or urine, caused by too much phenylalanine in the body; Nervous system (neurological) problems that may include seizures; Skin rashes, such as eczema; Lighter skin, hair and eye color than family members, because phenylalanine can't transform into melanin — the pigment responsible for hair and skin tone;Unusually small head size (microcephaly); Hyperactivity; Intellectual disability; Delayed development; Behavioral, emotional and social problems; Mental health disorders (11).

The severity of PKU depends on the type: Classic PKU-The most severe form of the disorder is called classic PKU. The enzyme needed to break down phenylalanine is missing or severely reduced. This results in high levels of phenylalanine that can cause severe brain damage.

Less severe forms of PKU-In mild or moderate forms, the enzyme still has some function, so phenylalanine levels are not as high, resulting in a smaller risk of significant brain damage (12)

Regardless of the form, most infants, children and adults with the disorder still require a special PKU diet to prevent intellectual disability and other complications (13).

Women who have PKU and become pregnant are at risk of another form of the condition called maternal PKU. If women don't follow the special PKU diet before and during pregnancy, blood phenylalanine levels can become high and harm the developing baby (14)

Even women with less severe forms of PKU may place their unborn children at risk by not following the PKU diet (15).

Babies born to women with high phenylalanine levels don't often inherit PKU. But a child can have serious problems if the level of phenylalanine is high in the mother's blood during pregnancy. At birth, the baby may have: Low birth weight; Unusually small head; Problems with the heart (13).

In addition, maternal PKU can cause the child to have delayed development, intellectual disability and problems with behavior (16).

Inheritance

For a child to inherit PKU, both the mother and father must have and pass on the changed gene. This pattern of inheritance is called autosomal recessive. It's possible for a parent to be a carrier-to have the changed gene that causes PKU, but not have the disease. If only one parent has the changed gene, there's no risk of passing PKU to a child, but it's possible for the child to be a carrier (17).

Most often, PKU is passed to children by two parents who are both carriers of the changed gene, but don't know it (15).

Risk factors for inheriting PKU include: Having both parents with a gene change that causes PKU. Two parents must pass along a copy of the changed gene for their child to develop the condition; Being of a certain racial or ethnic descent. PKU affects people from most ethnic backgrounds worldwide. But in the United States, it's most common in people of European ancestry and much less common in people of African ancestry (18).

Untreated PKU can lead to complications in infants, children and adults with the disorder. When women with PKU have high blood phenylalanine levels during pregnancy, it can harm their unborn baby (19)

Untreated PKU can lead to: Irreversible brain damage and marked intellectual disability beginning within the first few months of life; Neurological problems such as seizures and tremors; Behavioral, emotional and social problems in older children and adults; Major health and developmental problems (20).

Clinical manifestations

Manifestations include psychiatric disorders, behavioral problems, delayed development, seizures, and intellectual disability, lighter hair, skin, musty, or mouse like odor, microcephaly. Studies propose that untreated phenylketonuria in pregnancy is linked to attention deficit hyperactivity disorder, intellectual disability, and microcephaly. If PKU is untreated, patients can experience severe intellectual disability, epilepsy, seizures, psychiatric movement behaviors, microcephaly, generalized hypopigmentation of skin (including eyes and hair), eczema, and a musty sweat odor (21). However, with early intervention after birth, dietary treatment can prevent sequelae. Late diagnosed or untreated PKU may be due to newborn screening failures and is most common in countries without newborn screening protocols or treatment. If treatment is not adequate, clinical signs can include lower extremity spasticity and cerebellar ataxia, tremor, encephalopathy, and visual abnormalities. Some cases may not be diagnosed until adulthood, presenting with mild-to-moderate neurological complications related to PKU. Since brain damage is one of the greatest risks for PKU patients, early detection and assessment of neural activity are important for patient health. Sometimes, dementia may be associated with PKU in adulthood. However, treatment helps prevent major neurological deficits, cognitive abnormalities, and specific learning disabilities immediately after birth (14-16, 22)

Genetic etiology

PAH gene associated with PKU. Pathogenic variants most often cause PKU in the *PAH* gene (OMIM 612,349) inherited in an autosomal recessive pattern. The *PAH* gene, mapped to chromosome 12q23.2, spans 90 kb and consists of 13 exons that are not equally distributed, as the exons are more condensed in the second moiety of the gene. The *PAH* gene's coding sequence is 1359 base pairs, which encode 452 amino acid polypeptides with a molecular weight of ~ 52 kDa. The absence of TATA boxes characterizes the promoter region of the PAH gene. However, GC boxes, CCAAT boxes, CACCC boxes, two activator protein sites, partial glucocorticoid response elements, and partial cyclic AMP response elements are present (12).

Diagnosis

Phenylketonuria is analyzed by examining the amino acids in the plasma. Screening programs have been introduced in numerous countries that permit identifying the illness in neonates within the first few days of birth. The objective of these recognition programs is to manage the babies prior to the initiation of exhibiting manifestations of the illness. Once identified, the children will be referred to a reference hospital for differential diagnosis with other less frequent forms of diseases, which can cause a rise of blood phenylalanine levels and initiate the management. The analysis of the PAH gene mutations approves the diagnosis (23).

Children and young kids with phenylketonuria require having consistent blood tests for measurement of phenylalanine levels. If there is too much or too little phenylalanine in the blood, the formula and diet may require to be attuned (24).

The consequence is predictable to be very good if the food is carefully monitored, beginning soon after the birth of child. If management is late or the disorder remains untreated, damage of brain will occur. School working may be slightly reduced. If proteins comprising phenylalanine are not evaded, phenylketonuria can lead to intellectual incapacity by the completion of the first year of life (25).

Material and methods

Amino acid analysis by LC-MS/MS

In the newborn screening program blood specimens were collected by trained nurses via heel-stick and spotted on filter paper cards. Amino acid metabolism disorders in dried blood spots (DBS) was measured on liquid chromatography with tandem mass spectrometry (LC-MS/MS) method (Shimadzu LCMS-8040, Kyoto, Japan) by the use of a multiple reaction monitoring (MRM) approach. Patients who were evaluated with a pre-

diagnosis of amino acid metabolism disease and whose results were suspicious after three measurements were further examined by genetic analysis.

MLPA analysis

MLPA was carried out for PKU patients who lacked PAH mutations on one or both alleles, based on previous sequencing analyses. The SALSA PO55 PAH MLPA kit (MRC Holland, Amsterdam, The Netherlands) contains 25 sets of probes: 13 PAH-specific sets, and the remainder comprises control standard probes from other human genes. The assay was carried out in 200-µl tubes in a thermal cycler (Model 9700; Applied Biosystems) according to the manufacturer's instructions. Briefly, a total of 200 ng of genomic DNA from each subject was diluted in 5 µl of TE buffer and denatured at 98°C for 5 min. MLPA buffer and probe mix (1.5 µl of each) were then added, and the probes were annealed to the target genomic DNA by heating at 95°C for 1 min, followed by incubation at 60°C for 16 h. Thirty-two microliters of Ligase-65 mix were added to each sample, and the annealed probes were ligated at 54°C for 15 min, followed by inactivation at 98°C for 5 min. Ten microliters of the ligation reaction were removed for multiplex amplification using a pair of common primers, of which one was labeled with the fluorescent dye 6-FAM. *Taq* polymerase was added to the PCR (total volume of 50 µl) at 60°C, followed by 36 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 20 min. Between 0.5 µl and 0.75 µl of each reaction was mixed with 0.5 µl of TAMRA-labeled internal size standard and 12.5 µl deionized formamide, and used for fragment analysis on the ABI-3100 capillary sequencer (Applied Biosystems). The conditions used for the fragment analysis were: polymer POP-4 in a 36-cm capillary; run temperature, 60°C; injection voltage, 15 kV; injection time, 3-5 s; run voltage, 15 kV; run time, 24 min. The obtained data were analyzed using the Genescan 3.1.2 software. The peak heights were normalized, and exon deletions were adjudged when the sample peak height was less than 65% of the control peak height.

Obtaining DNA extraction

Material used was venous blood with anticoagulant of 633 patients. Genome DNA was obtained by automatic isolation from 200 ml of venous blood. The DNA concentration was measured by the Digital spectrometer. The integrity of the isolated genomic DNA was detected in a 2% agarose gel. The venous blood for research was drawn into a tube containing EDTA or heparin. Genomic DNA and RNA kits made by Qiagen GmbH (Hilden, Germany) were used for analysis.

Gel electrophoresis

Integrity and quantity of genomic DNA and polymerase chain reaction (PCR) products were identified by electrophoresis on 2% agarose gel (PowerPacBasicGelDoc™ EZ; Bio-Rad Laboratories, Hercules, CA, USA). The genome DNA underwent the PCR procedure for every protein-encoding exon of the *PAH* gene. Positive PCR samples that were checked by electrophoresis in agarose gel were purified by an enzymatic method.

Polymerase chain reaction

Polymerase chain reaction was carried out in a following conditions: denaturation at 96 ^{0}C for 30 seconds; annealing at 55 ^{0}C for 30 seconds; extension at 75 ^{0}C for 1 min. This cycle was repeated 25 times, 72 ^{0}C for 10 min. and 4 ^{0}C pause. The PCR was carried out on a Professional Thermocycler Biometra system (BiometraBiomedizinischeAnalytik GmbH, Göttingen, Germany). A pair of for-ward and reverse primers was used for each genomic fragment. For the purification of DNA fragments after the first stage of PCR, a set of magnets was used: AgencourtAMPure XP PCR purification and SPRIPlate 96 Super Magnet Plate (Beckman Coulter Inc., Beverly, CA, USA). The second amplification of the purified DNA fragments was carried out in the following condition: denaturation at 95 ^{0}C for 30 seconds; annealing at 55 ^{0}C for 30 seconds; extension at 77 for 2 min. This cycle was repeated 25 times, and 72 ^{0}C for 10 min. and 4 ^{0}C pause (12).

Sequencing

The nucleotide sequence of purified fragments was studied in GENOME Lab GeXP™ Sequencing (SCIEX, Brea, CA, USA). Purified product was dyed with fluorescent dye by BiqDye Terminator V.3.1. (Applied Biosystems, Foster City, CA, USA) and processed by Cycle Sequencing PCR. Positive Cycle Sequencing PCR samples, controlled by electrophoresis in agarose gel, were extracted from the BiqDye XT (Applied Biosystems with dye-purifying agent.

The obtained nucleotide chains were identified through SeqScape® version 2.7 software program (Applied Biosystems, Foster City, CA, USA; http:/tools.thermofisher. com/content/sfs/manuals/4401740.pdf), then

compared by means of the National Center for Biotechnology Information (NCBI) Blast Ce, to normal PAH nucleotide chains, and only then were the substitutions and mutations identified. All PCR fragments were sequenced using the same primers as used for PCR amplification. All identified mutations were confirmed using a new PCR product of the abnormal fragment (forward and reverse).

Results

This study was conducted in the patients diagnosed with phenylketonuria who presented to Scientific Research Pediatric Institute of the Ministry of Health of Azerbaijan and hospitals of different regions of Azerbaijan. Exons 6, 7, 8, 11 and 12 of the PAH gene from 30 with PKU were identificated with DNA sequence. They were from different parts of Azerbaijan. At present, more than 600 mutations of *PAH* have been reported worldwide, mostly on exon 6th, 7th, 8th, 11th, and 12th exon. Among them, the 18 mutations are the most common types of PAH mutations in Azerbaijan population. The rate of consanguine marriage was 50%, and most of the parents were cousins. A total of 18 different mutations (V245V, R261Q, Q232Q, V245V, P281L, R241C, L385L, V399V, E280K, R261X,A434D, R176X, Ex6–96A > G, R241C, R243Q, R252Q, Y356X, R413P) were characterized in Azerbaijanian patients.

The most obvious reason to measure rates of phenylalanine and tyrosine kinetics in humans is to understand the derangements of the metabolism of these amino acids that occurs in patients with the in-born error phenylketonuria (PKU) and in diseases that affect their metabolism, such as in liver or renal disease. However, phenylalanine and tyrosine also have specific characteristics as amino acids that make them useful as markers of protein metabolism. Firstly, both phenylalanine and tyrosine are indispensable amino acids that are essential to our diet. In the postabsorptive state there is no entry of amino acids from dietary sources, and the flux of phenylalanine in the body is derived from entry of phenylalanine released from protein breakdown. That input is matched by phenylalanine removal via protein synthesis and via metabolic disposal by conversion to tyrosine. Therefore, the measurement of the whole-body rate of appearance of phenylalanine in the postabsorptive state is a measure of the whole body rate of proteolysis. Additional reasons for determining phenylalanine and tyrosine kinetics are the determination of dietary requirements of phenylalanine and tyrosine and measurement of the production of tyrosine from dietary phenylalanine.

Phenylketonuria (PKU) is an autosomal recessive genetic disease, caused by the phenylalanine hydroxylase (PAH) deficiency in the metabolic pathway, which prevents phenylalanine from being converted into tyrosine, leading to a large amount of phenylalanine discharged from the urine. Therefore, it is necessary to establish a simple, fast, accurate and reliable PKU molecular diagnostic method for clinical diagnosis.

Phenylalanine-restricted diet

Dietary control of PKU is challenging but possible. As Phe is an essential amino acid, patients with PKU must use a diet containing low-Phe concentrations to maintain blood Phe at 2–6 mg/dL (120–360 μmol/L) throughout the life span as recommended by the US National Institutes of Health. The European and US guidelines recommend treating individuals with PKU when Phe levels exceed 360 mmol/L. Long-standing dietary deficiency in protein leads to a decrease in vitamin B12 (found in meat, poultry, and fish), as well as a decrease in calcium and vitamin D. Thus, supplements rich in minerals and vitamins must be taken to avoid growth retardation and osteoporosis (25,26).

The dietary treatment comprises three aspects: restricting natural protein intake, supplementing with a low-Phe or Phe-free amino acid mixture, and consuming low-protein food products. Phe restriction can only be performed by restricting the intake of natural protein. The extent of natural protein (Phe) restriction is based on the amount of Phe required for net protein synthesis (e.g., age-dependent growth and balance between anabolism and catabolism in periods of illness) and the severity of the PAH deficiency. During restricted Phe consumption, the intake of other essential amino acids, vitamins, minerals, and carnitine should be balanced. However, natural protein can be replaced with an amino acid mixture that lacks Phe but is enriched in Tyr. Moreover, intake of low-protein foods containing carbohydrates and fats may replace basic foods such as bread and pasta to supply energy. Enormous improvements in intellectual and cognitive outcomes have been observed in PKU patients when dietary Phe is restricted before considerable damage has occurred (16,27,28).

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Conflicts of interest
There is no conflict of interest.

Availability of data and materials
Not applicable

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Author Contributions
L.H. study director, conducting experiments, collecting and analyzing data. A.A and G.V. are collecting and analyzing data.

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