

Present Newborn Screening for Phenylketonuria

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Newborn screening for phenylketonuria (PKU) began in the United States in the early 1960s following development of the Guthrie bacterial inhibition assay that allowed for the easy, rapid screening of elevated blood phenylalanine levels collected on newborn filter paper samples. Since that time, a number of other techniques that screen for PKU, other inborn errors of metabolism, and a variety of nonmetabolic disorders have been developed using newborn blood samples. The most advanced, comprehensive technique available today is tandem mass spectrometry (MS-MS), which simultaneously identifies and measures many compounds of varying structural classes allowing for concurrent screening of many disorders, including aminoacidemias, organic acidurias, and fatty acid disorders.

Despite this progress in presymptomatic neonatal detection of PKU, there are some difficulties with newborn screening. These include false-positive results, occasional missed diagnoses, and problems surrounding early discharge. In addition, not all elevated phenylalanine levels are a result of a deficiency of the liver enzyme phenylalanine hydroxylase (PAH). Some infants have transiently elevated levels. Others have defects in the synthesis or recycling of tetrahydrobiopterin (BH₄), the cofactor of PAH, and some have secondary phenylalanine elevations due to disorders that affect the liver, such as tyrosinemia or galactosemia. Several of these problems, including the number of false positives, may be eliminated by pattern identification via MS-MS.

Once identified with a significant or persistent elevation in phenylalanine, infants and their families are referred to a metabolic center for further evaluation, including repeat quantitative testing and, if necessary, dietary or cofactor therapy. © 1999 Wiley-Liss, Inc. MRDD Research Reviews 1999;5:144-149.

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HISTORICAL ASPECTS

In 1934, when Folling described the association of phenylketonuria (PKU) with mental retardation, PKU became the first metabolic disorder to be linked to mental retardation [Folling, 1934]. Twenty years later, Bickel and coworkers showed that a diet low in phenylalanine could control the increased phenylalanine-related biochemical abnormalities in PKU and could also reverse some of the neurological features of the disorder [Bickel et al., 1954]. Others subsequently found that the diet could prevent the mental retardation in PKU if it was initiated in early infancy [Armstrong and Tyler, 1955]. However, for early and presymptomatic diagnosis, a screening test was required and this had to be performed as early as possible, preferably in newborn infants. At that time, the only available

screening test for PKU was the urinary ferric chloride color test that Folling had originally used [Folling, 1934]. Unfortunately, that test was unreliable for detecting the presence of abnormal compounds found in PKU during the newborn period [Stephenson and McBean, 1967] and more complex and time-consuming assays [Dent, 1946] were not practical for mass screening. In 1962, Robert Guthrie, a microbiologist and physician at the Children's Hospital in Buffalo, solved this problem by developing a bacterial inhibition assay for phenylalanine that could test newborns for PKU in only a few drops of blood collected from the heel and dried on filter paper [Guthrie and Susi, 1963]. Robert MacCready, a physician who directed the Diagnostic Laboratories of the Massachusetts Department of Public Health, very soon began using the test on specimens collected from newborn infants when they were discharged from the nursery [MacCready, 1963]. After a successful trial in Massachusetts that resulted in the fortuitous detection of seven cases of PKU out of the first 26,955 samples tested [MacCready and Hussey, 1964], screening for this disorder by the Guthrie assay expanded widely in the United States and abroad.

Today, infants throughout much of the world are screened soon after birth, not only for increased phenylalanine to detect PKU but also for congenital hypothyroidism and other metabolic and nonmetabolic disorders. Newborn screening continues to expand and may soon include testing for many additional inborn errors of metabolism and endocrinopathies, for nonmetabolic genetic disorders, for certain infectious diseases, and even for childhood cancer.

DETECTION OF ELEVATED PHENYLALANINE

Specimen Collection

Blood dried on filter paper is the specimen universally used for PKU newborn screening. This specimen should always be collected in the nursery from the lanced heel of the infant before nursery discharge. Exceptions to this method include collection by venipuncture or using blood from an umbilical line [Lorey and Cunningham, 1994]. Designated circles on the filter paper card are saturated with drops of blood applied to only one side of

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the card. The specimen is air dried for at least three hours in the nursery and then placed in an envelope or plastic bag for transport to a central laboratory.

Analysis of Specimens

Although the blood samples can be analyzed by any one of a variety of techniques, each method is designed to detect an elevation of phenylalanine and not the enzymatic defect in PKU, which is a deficiency of phenylalanine hydroxylase (PAH) activity expressed only in the liver. The various methods used for PKU screening and the advantages and disadvantages of each technique are described below and summarized in Table 1.

Guthrie bacterial inhibition assay

This assay is the most commonly used method for detecting blood phenylalanine elevations in newborn screening. Small disks are punched from the blood-impregnated circles in the filter paper cards and placed on agar gels that contain a strain of *Bacillus subtilis* and B-2-thienylalanine, an inhibitor of bacterial growth but, as an analogue of phenylalanine, is counteracted by phenylalanine. Each plate holds 60–80 discs. The amount of bacterial growth around each disc is directly proportional to the amount of phenylalanine present in the blood, with actual concentrations estimated by comparison with a series of standards in the center of the plate. Sensitivity is relatively high, detecting phenylalanine elevations as low as 180–240 μM (3–4 mg/dl). However, this assay is only semiquantitative and its accuracy can be affected by factors such as the presence of antibiotics in the infant at the time of specimen collection.

McCaman-Robins fluorometric technique

In 1962, McCaman and Robins reported the use of a fluorometric method for phenylalanine measurement that utilized the principle of fluorescence enhancement of a phenylalanine-ninhydrin product by a dipeptide [McCaman and Robins, 1962]. This method allowed phenylalanine to be measured quantitatively in the presence or absence of other amino acids and was adapted to automation by Hill et al. [1965]. Several screening centers currently use more updated automated versions of the original McCaman-Robins technique [Blau, 1983; Gerasimova et al., 1989; Jew et al., 1994]. The reliability of this assay for newborn screening of PKU has been questioned [Ambrose, 1973; Wendel et al., 1990] but the experience of a number of large programs indicates that it is quite

reliable. In fact, it seems to be as reliable as the Guthrie bacterial inhibition assay and may be slightly more sensitive [Jew et al., 1994].

Chromatography (paper and thin layer)

The detection of PKU by paper chromatography using untreated plasma was described in 1962 [Culley et al., 1962]. A variation of that technique using either whole blood or urine impregnated and dried in filter paper was reported by Efron et al. in 1964 and could detect not only PKU but also a number of other aminoacidopathies [Efron et al., 1964]. Discs from the dried blood specimen are autoclaved (to prevent streaking in the presence of red blood cells), pressed into pre-punched holes in chromatographic paper, chromatographed overnight in solvent, then dried and stained with an isatin-ninhydrin mixture for the detection of amino acids. A similar method of paper chromatography, employing plasma collected and transported in heparinized capillary tubes and then spotted on chromatographic paper [Scriver et al., 1964], is still in use for newborn screening in Manchester, Great Britain. Although levels of phenylalanine below 480 μM (8 mg/dl) cannot be consistently detected using these techniques, sensitivity is improved by inspection of the developed chromatograms immediately after heating.

Thin layer chromatography provides more efficient recovery of a compound, more rapid analysis, increased sensitivity, and better resolution in many cases than paper chromatography. However, it is more expensive and is not currently used by any program for newborn PKU screening.

HPLC (high performance liquid chromatography)

HPLC provides a quantitative method for simultaneously determining both phenylalanine and tyrosine levels as well as several other amino acids but has also been too slow for large scale

newborn screening. In 1991, however, Qu et al. [1991] described an automated system for HPLC screening with a sample turnaround time of approximately three minutes. Because HPLC is highly sensitive and since both phenylalanine and tyrosine are quantitated so that the increased phenylalanine/tyrosine ratio in PKU can be determined, the investigators postulated that HPLC provides a more definitive screen for PKU and, thereby, a decrease in the number of repeat specimens required from infants who are discharged early or who have false positive results. This can reduce the cost in an otherwise expensive system. HPLC is presently used for confirmation of abnormal screening results found by other techniques, including the Guthrie bacterial inhibition assay, but not as a primary screening method.

Phenylalanine dehydrogenase assay

Automated methods of detecting elevated phenylalanine by an enzyme assay have recently been developed [Wendel et al., 1990; Keffler et al., 1994]. The enzyme assay uses phenylalanine dehydrogenase derived from *Rhodococcus* sp. M4 which catalyzes the nicotinamide adenine dinucleotide (NAD) dependent oxidative deamination of phenylalanine to phenylpyruvate ammonia, and NADH. Phenylalanine is quantitated by NADH measurement, either directly or by coupling the reaction with an intermediate electron acceptor. However, there are concerns about the reliability and sensitivity of this assay.

Tandem mass spectrometry

This is the most exciting new development in newborn screening and offers many advantages over current screening methods. It is discussed elsewhere in this issue.

Summary of Techniques

Many factors are involved in a newborn screening program's decision to select one screening technique over

Table 1. Methods for PKU Detection

Testing	Sensitivity	Specificity	Range of Screening Coverage	Complexity of Testing
Guthrie	Moderate	Moderate	Low	Low
Fluorometric (McCaman-Robins)	High	High	Low	Moderate
Phenylalanine dehydrogenase enzyme assay	Moderate	High	Low	Moderate
Paper and thin layer chromatography	Low	Moderate	Moderate	Low
HPLC	High	High	Moderate	High
Tandem mass spectrometry	High	High	High	High

others. These factors include cost and complexity of the methodology. Although the Guthrie bacterial inhibition assay is technically easy and the most cost efficient of the available methods for PKU screening, its usefulness in screening for other disorders is limited. This is also true for the other screening methods described, with the single exception of the tandem mass spectrometry methodology. When making decisions about screening methods, the cost of repeat sampling for false positive results and, most importantly, the human cost sustained from missed diagnoses in the less complex systems must be considered when comparing costs to those of instrumentation and personnel training in the more complex systems.

POTENTIAL PROBLEMS IN NEWBORN SCREENING

Several important potential problems can affect the specificity and sensitivity of the screening tests and should be considered when faced with abnormal results.

False-Positive Results

Most positive results in newborn screening are transient aberrations not caused by a metabolic disorder or any other pathologic state. In some cases, the transient abnormality is associated with an identifiable temporary influence, such as prematurity, postprandial status, or contamination of the blood filter paper specimen. More often, however, no cause is identified. These transient abnormalities are usually mild. The cut-off level for the purpose of suspecting a disorder is set at the value at which affected and unaffected infants begin to overlap to avoid missing truly affected infants whose screening assay results are only mildly abnormal. As a result, repeat specimens will be requested from normal infants.

The number of false-positive to true-positive results is about 10:1 or higher. Not only do false-positive results produce anxiety in families, sometimes very severe anxiety, but they also very substantially increase the expense and workload of newborn screening. Studies on parental stress generated by positive results indicate that the stress can be reduced, although not eliminated. Reducing the anxiety requires communicating to the parents clear and simple explanations of the test results and the reasons for repeat sample collections [Bodegard et al., 1983; Sorenson et al., 1984]. Some newborn screening programs distribute written explanations in advance to physi-

cians or include them with the requests for follow-up specimens. The number of false-positive results would decline if more programs substitute sensitive and specific systems such as tandem mass spectrometry for currently used methods.

Early Newborn Screening

Almost all newborn screening programs insist upon collection of the blood specimen before the neonate is discharged from the hospital to guarantee screening of all newborns. Until recently in the United States, this meant that the specimen would be obtained no earlier than the second or third day of life. However, the practice of discharging infants at or before 24 hours of age now means that some neonates are being screened "early". This had led to concern that an affected infant may be missed because of screening before the metabolic abnormality has become detectable. To prevent this, most programs recommend collection of a second blood specimen at one or two weeks of age in infants tested at or before 24 hours of age [Naruse and Levy, 1994]. Although it has been shown that classical PKU can be identified before 24 hours of age [Doherty et al., 1991; Jew et al., 1994], important variant PKU could be missed and other disorders, including homocystinuria [Whiteman et al., 1979], are even more likely to be missed unless a second specimen is collected from these infants.

Transferred and Transfused Infants

Failure to collect the newborn screening specimen is not uncommon among infants transferred to a special care nursery, especially when this transfer is to another hospital. To safeguard against this omission, the initial screening specimen should be collected before transfer and a second screening specimen should be obtained at the time of discharge.

The infant who receives a blood transfusion is also vulnerable to missed diagnosis in newborn screening. Although the detection of PKU is not affected by transfusion because of rapid equilibration of accumulated phenylalanine between the intracellular (tissue) and extracellular (blood) spaces, detection of galactosemia [Sokol et al., 1989] and sickle cell disease always relies on the presence of red blood cell markers (i.e., lack of galactose-1-phosphate uridylyltransferase enzyme activity and presence of hemoglobin S, respectively). These abnormalities can be masked by the presence of donor erythrocytes. The initial screening specimen should be collected before any transfusion and a second sample obtained

at about two months of age when the infant has replaced most of the donor erythrocytes with new red blood cells.

Missed and Uncovered Disorders

Although the vast majority of infants with disorders detectable by newborn screening are identified, an occasional infant with PKU or another screened disorder is missed [McCabe, 1992]. While this may be secondary to a delay in appearance of a marker, e.g., lack of elevated blood methionine in homocystinuria [Whiteman et al., 1979], the most common reason an infant is missed is laboratory error [Holtzman et al., 1986]. Regardless of the reason, it is important to consider the possibility that an infant with a clinical phenotype compatible with a particular metabolic disorder could indeed have this or a related disorder despite the presumption of a normal newborn screen or even the presence of a normal newborn screening report. There is also the misconception that newborn screening includes testing for the full spectrum of metabolic disorders. To cover the possibility of a newborn screening miss and incomplete coverage of metabolic disorders, evaluation of clinically abnormal infants and children should include tests for all relevant metabolic diseases, including PKU and others covered by neonatal screening.

Routine Follow-Up Screening

Some screening programs advocate routine collection of a second sample from all neonates at two to four weeks of age. Advocates justify this practice on the basis of avoiding missing an infant because of delay in appearance of the abnormality or because of program error in conjunction with the initial newborn specimen. A study published in 1979, however, found that the frequency of PKU detected solely by the follow-up blood specimen was only one in 596,000, making routine second sample collection extremely unproductive [Sepe et al., 1979]. Currently, only a few screening programs include routine follow-up testing.

BIOCHEMISTRY OF PHENYLALANINE METABOLISM

Phenylalanine is an essential amino acid, normally converted to tyrosine by the liver enzyme phenylalanine hydroxylase (PAH). A cofactor, tetrahydrobiopterin (BH₄), is necessary for this conversion and is also required for the activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TrH), which catalyze the syntheses of the neurotransmitters,

dopamine and norepinephrine, and serotonin, respectively (Fig. 1). BH₄ is formed by a series of reactions involving multiple enzymes beginning with GTP cyclohydrolase I and including 6-pyruvoyltetrahydropterin synthase (6-PTS). BH₄ is also recycled from quinonoid dihydrobiopterin (qBH₂) by the enzyme dihydropteridine reductase (DHPR). The body normally maintains a blood phenylalanine level of less than 120 μM (2 mg/dl).

Causes of Elevated Phenylalanine

There are several causes of elevated phenylalanine (hyperphenylalaninemia) [Scriver et al., 1995]. Consequently, investigation of this abnormality requires careful consideration before assigning a specific diagnosis to the infant and embarking on therapy. These etiologies include:

- 1) Phenylalanine hydroxylase (PAH) deficiency
- 2) Disorders of tetrahydrobiopterin (BH₄) deficiency
 - a) GTP cyclohydrolase I deficiency
 - b) 6-PTS deficiency (biopterin synthesis defect)
 - c) Dihydropteridine reductase (DHPR) deficiency
- 3) Transient hyperphenylalaninemia
- 4) Tyrosinemia with secondary hyperphenylalaninemia
- 5) Galactosemia and other causes of liver dysfunction with secondary hyperphenylalaninemia

Table 2 lists these causes and the biochemical findings that allow for their differentiation.

FOLLOW-UP OF ELEVATED PHENYLALANINE LEVELS

Most, if not all, newborn screening laboratories have a standardized protocol for follow-up of elevated phenylalanine

levels. Figure 2 is an algorithm for follow-up of neonatal hyperphenylalaninemia. In the New England Newborn Screening Program, phenylalanine levels below 180 μM (3 mg/dl) are considered normal. If the phenylalanine level in a specimen collected after 24 hours of age is 180–300 μM (3–5 mg/dl), the attending physician is faxed and mailed a letter for a repeat blood filter paper sample. If, upon repeat testing, the blood phenylalanine level is again in that range, the diagnosis of non-PKU mild hyperphenylalaninemia (MHP) is suggested and the infant is

referred to a metabolic center for evaluation. A newborn screening blood phenylalanine level of 360 μM (6 mg/dl) or greater in a specimen collected after 24 hours of age or above 240 μM (4 mg/dl) in a specimen collected within the first 24 hours of life prompts an immediate telephone call to the attending physician and recommendation of referral to a metabolic center. There is a high likelihood that these infants have PKU.

Obviously, there is considerable parental anxiety concerning an abnormal screening result. Written explanatory

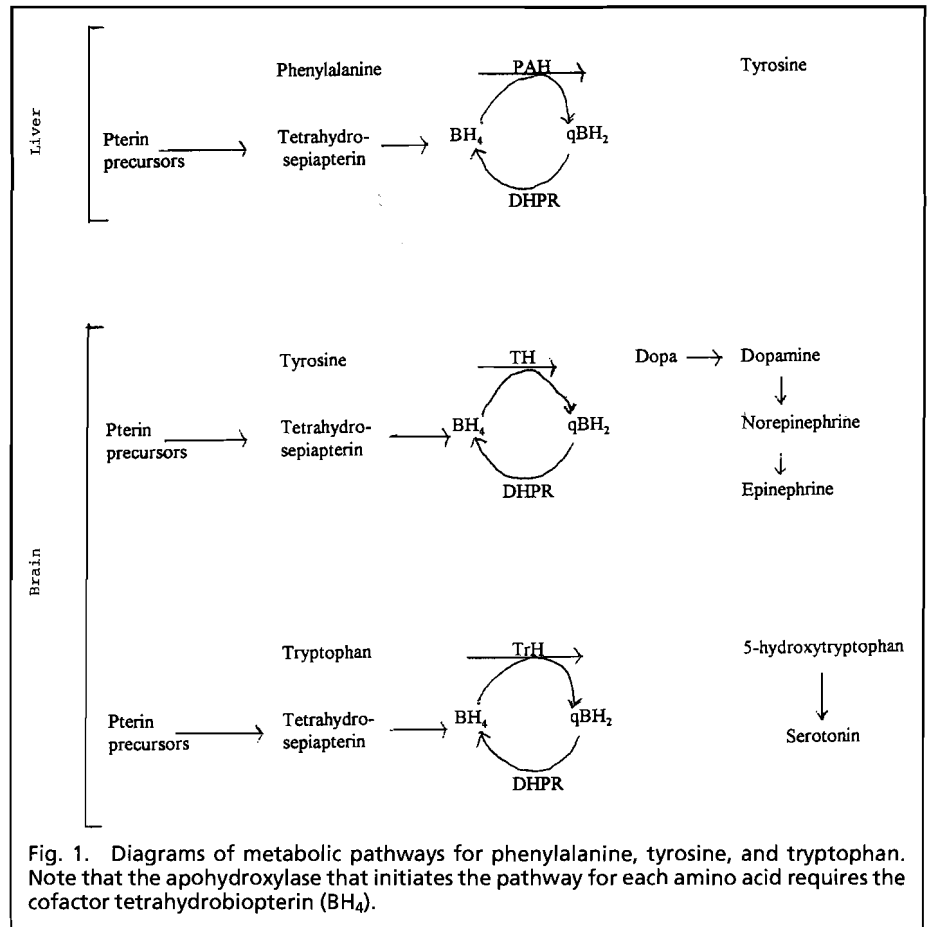
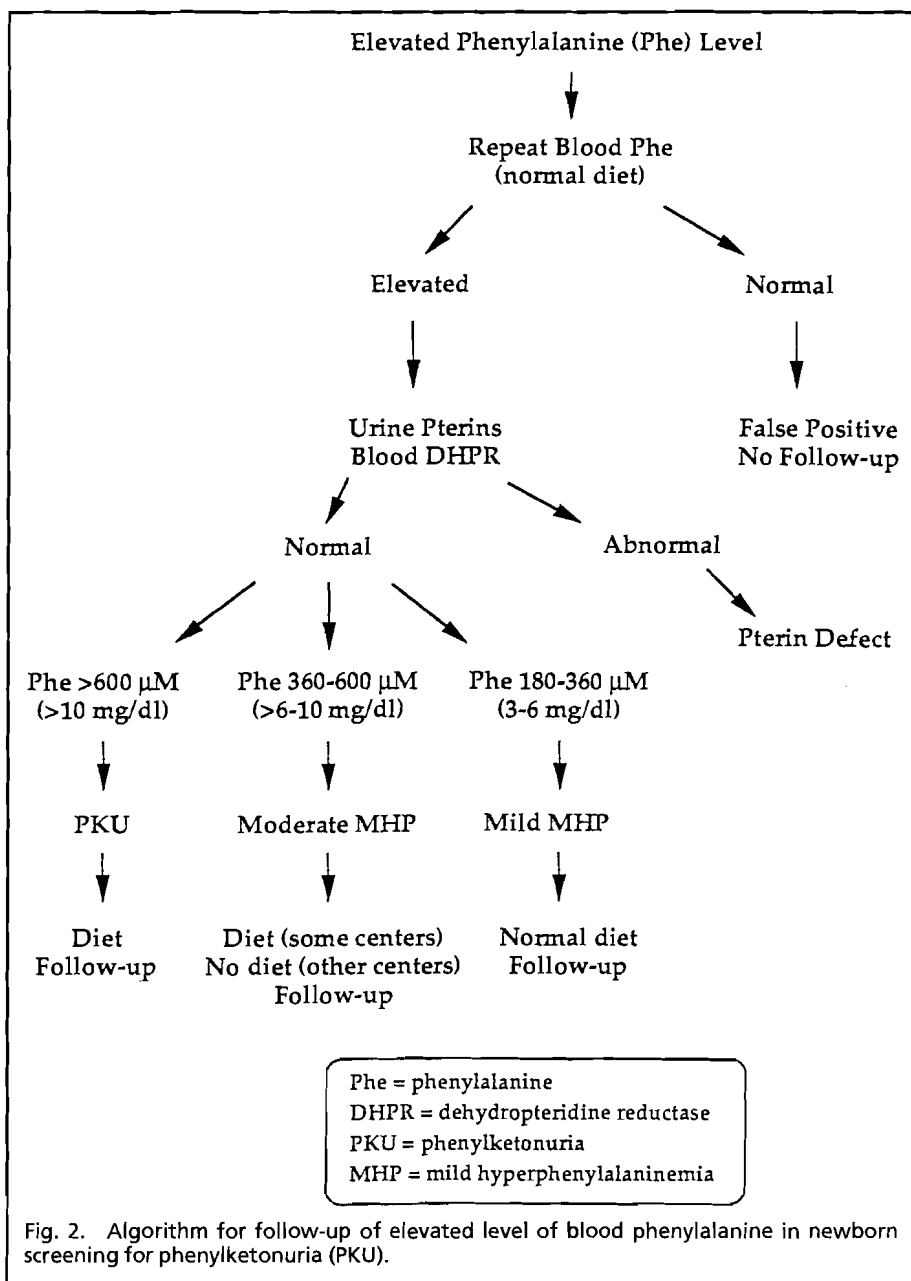


Fig. 1. Diagrams of metabolic pathways for phenylalanine, tyrosine, and tryptophan. Note that the apohydroxylase that initiates the pathway for each amino acid requires the cofactor tetrahydrobiopterin (BH₄).

Table 2. Causes of Elevated Phenylalanine

	Blood			Pterins	
	Phenylalanine (μM)	Tyrosine	Other Amino Acids	Urine	Blood DHPR
PKU	>1200	<100	Normal	Normal	Normal
Mild PKU	600–1200	<100	Normal	Normal	Normal
Mild hyperphe	180–600	<100	Normal	Normal	Normal
Pterin defects					
GTP cyclohydrolase deficiency	180–1200	<100	Normal	Abnormal	Normal
6-PTS def	180–1200	<100	Normal	Abnormal	Normal
DHPR def	180–1200	<100	Normal	Abnormal	Abnormal
Transient Hyperphe	180–840	<250	Normal	Normal	Normal
Tyrosinemia I, Galactosemia, Liver Disease	180–600	>250	Increased methionine	Normal	Normal



information regarding the reason for repeat sampling should accompany requests for repeat specimens when the phenylalanine level is only mildly elevated. This has been successful in reducing anxiety in these and other situations [Bodegard et al., 1983; Sorenson et al; 1984]. Higher phenylalanine levels require more immediate referral and the initial burden of relaying adequate and accurate information rests with the pediatrician or family physician. The screening laboratory should provide information to the health providers, but primary care physicians should also be familiar with the screening program, including knowledge of which disorders to screen for and general current information about the treatment and long-term outcome of PKU and other screened disorders. Such

knowledge will prove invaluable in allowing the physician to support the infant's family through the initial, often difficult, stages of diagnosis.

Upon referral to a metabolic specialist or center, an infant undergoes an extensive evaluation that includes quantitative plasma or serum amino acid levels, with particular attention to phenylalanine and tyrosine, and collection of additional blood and urine samples for pterin studies. A discussion of PKU and related disorders and genetic counseling should also be provided for the family. In certain cases, especially when dietary tolerance for phenylalanine is higher than expected from the initial high blood phenylalanine level, obtaining a PAH genotype may be very helpful in assuring families of diagnostic accuracy and the need for

ongoing medical management. Measurement of the blood phenylalanine level in each parent should be obtained at the initial evaluation of the infant. A rare parent could himself or herself have an elevated level and be a compound heterozygote for two PAH mutations. This will mean that the couple could have future children with a milder or more severe form of hyperphenylalaninemia than that in the immediate infant, in contrast to the usual recurrence risk of another child with the same degree of hyperphenylalaninemia as the proband [Ledley et al., 1986; Guldberg et al., 1994].

Once it has been determined that the infant's phenylalanine level is sufficiently elevated to begin therapy (greater than 480 μ M or 8 mg/dl in our clinic), the phenylalanine restricted diet is initiated. If the pterin studies are found to be abnormal, the addition of supplemental BH_4 and neurotransmitter enhancing medications may be required [Scriver et al., 1995].

CONCLUSIONS

Newborn screening for PKU began in the United States in the early 1960s following development of the Guthrie bacterial inhibition assay that allowed for the easy, rapid screening of elevated blood phenylalanine levels collected on newborn filter paper samples. Since that time, a number of other techniques that screen for PKU, other inborn errors of metabolism, and a variety of nonmetabolic disorders have been developed using newborn blood samples. There is now mandatory testing throughout the United States for PKU and congenital hypothyroidism, both treatable forms of mental retardation.

Future expansion using tandem mass spectrometry (MS-MS) as the newborn screening method of choice would not only greatly reduce or eliminate many of the inherent problems of present screening techniques such as false positive results but would allow for the concurrent screening and potentially effective early treatment of a broader spectrum of metabolic disorders. ■

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