JJBS
Jordan Journal of Biological Sciences

Volume 13, Number 2, June 2020
ISSN 1995-6673
Pages 177 - 181

Establishment of a Cutoff Value of Phenylalanine for Phenylketonuria among West Bank-Palestinian Neonates

Ibrahim A. Ghannam*, Akram T. Kharroubi, Renad M. Mustafa, Sumaia M. Jamous, Shahd M. Almufreh and Nelly M. Othman

Medical Laboratory Sciences Department, Al-Quds University, P.O. Box:66, Abu Dies, East Jerusalem, Palestine

Received June 24, 2019; Revised July 10, 2019; Accepted July 12, 2019

Abstract

Phenylketonuria (PKU) is an autosomal recessive disease due to a deficiency in phenylalanine hydroxylase that converts phenylalanine to tyrosine; this deficiency will lead to serious consequences like mental retardation. The purpose of this study was to establish a cutoff value of phenylalanine for the Palestinian neonates and to determine the incidence of PKU. A retrospective cohort study was carried out using data obtained from the Palestinian Central Public Health Laboratory (CPHL). During 2017, a total of 75,744 dried blood specimens had been tested for PKU using IBL international Kit with an enzymatic colorimetric principle. The data was analyzed using Receiver Operating Characteristics (ROC) curve to obtain the cutoff value of phenylalanine for PKU. Descriptive statistics and independent sample T-test were also used in the statistical analysis. ROC curve analysis revealed blood phenylalanine cutoff value of 3.3 mg/dL with 100% sensitivity and 99.۸% specificity. Among the 75,744 tested, 27 were positive (incidence rate of 35.6 per 10^5 newborns per year), 16 (59.3%) males, and 11 (40.7%) females. The cut off value (3.3 mg/dL) established in this study can be used with strong confidence, so we recommend that this value be adopted and used in Palestine.

Keywords: PKU, ROC Curve, Phenylalanine, Cutoff Value

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive disease due to a mutation in the gene responsible for the production of phenylalanine hydroxylase (PAH) found on chromosome number 12. This enzyme converts phenylalanine (which is an essential amino acid important in the protein synthesis in the body) into Tyrosine (which is also an amino acid produced by the body used to support energy and motivation, promotes focus and mental clarity and improves mood and stress response) (Schuck *et al.*, 2015). Tyrosine is also the precursor of catecholamine, melanin and thyroid hormones (Figure 1). So, deficiency in PAH enzyme or its absence will cause phenylalanine build up in the infant's body leading to mental retardation, organ damage, abnormal posture and pale skin (Schlegel *et al.*, 2016). The damage is unfortunately irreversible, and these dangerous symptoms will become apparent in the first weeks of the infant's life (van Wegberg *et al.*, 2017).

This disease is considered an inborn error of metabolism where blockage of phenylalanine metabolism leads to the alternative pathway where phenylalanine is converted into phenylpyruvate and phenylacetate which are excreted by the kidney and seen in the urine, which is why it is called Phenylketonuria (PKU) (Williams *et al.*, 2008) and was first discovered by Asbjorn Folling (Folling, 1994).

There are 3 types of PKU disorders based on phenylalanine concentration in the infant's body: classic-type 1 with a concentration of >20 mg/dL, type 2 with a concentration of 10-20 mg/dL, and benign-type 3 with a concentration of <10 mg/dL (Hanley, 2004; Williams *et al.*, 2008).

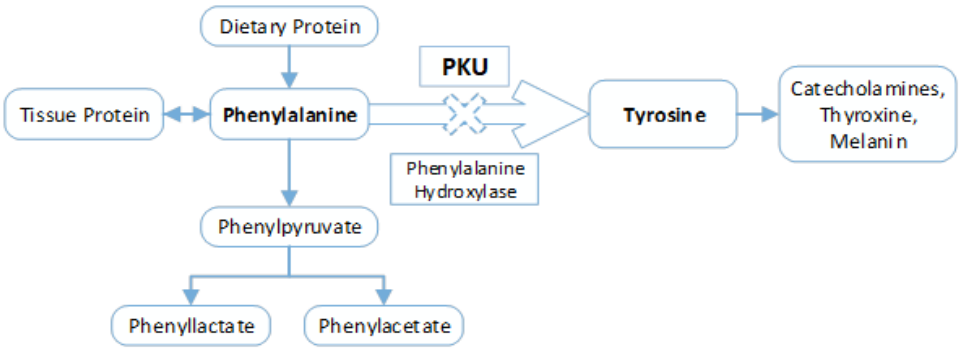

Figure 1. Phenylalanine metabolism pathway in Phenylketonuria.

Sample collection time for PKU screening differs depending on the method used, but most methods agree that it should not be less than 24-hours in order to differentiate between phenylalanine levels in those who are healthy and those who potentially have the disorder when quantitative methods are used, and 72-hour is believed to be the best time for testing because the baby will have got the appropriate amount of phenylalanine from the breastfeeding (van Wegberg *et al.*, 2017). Several methods can be used in PKU screening, such as Bacterial Inhibition Assay (Guthrie method) (Guthrie & Susi, 1963), Enzymatic Colorimetric method, Tandem Mass Spectrometry, enzymatic Fluorometric method, and newly emerging Molecular Methods (Blau *et al.*, 2014; Borrajo, 2016; Williams *et al.*, 2008).

This disease can be controlled by following a low phenylalanine diet; a restricted diet composed of less than usual breastfeeding along with formula milk free of Phenylalanine should be used to make sure the infant gets sufficient amount of Phenylalanine required for normal growth and development without causing harm (Kose *et al.*, 2018), or a milk formula with a little amount of Phenylalanine could be used alone but never breastfeeding alone (NIH, 2019).

According to a meta-analysis study done in European countries, patients with Phenylalanine concentration below 5.95 mg/dL should remain untreated as this concentration will not cause any symptoms to appear and has no effect on neurons. However, patients with Phenylalanine concentration below 5.95 mg/dL should be followed up and monitored during the first year of life because of the possibility of blood phenylalanine concentration increasing with age. So, according to this study, 5.95 mg/dL is considered as the upper target phenylalanine concentration (van Wegberg *et al.*, 2017). Some studies recommended a restricted diet for life (Guest *et al.*, 2013; Merrick *et al.*, 2003), while another study recommended a restricted diet until adulthood (Williams *et al.*, 2008). Mothers having PKU should follow a restricted diet during pregnancy in order to prevent building up and accumulation of Phenylalanine in infant's body causing fetal damage (Manta-Vogli & Schulpis, 2017).

Since there is no specific PKU cutoff value of Phenylalanine for the Palestinian population, the CPHL that performs this test for all Palestinian neonates from West Bank uses a cutoff value specified by the PKU neonatal Screening Assay Kit by IBL International, Germany (3 mg/dL). However, due to variations in the PKU cutoff values of Phenylalanine in different countries, the cutoff value used should be examined. Therefore, the purpose of this study was to establish a cutoff value of Phenylalanine for PKU in the West Bank - Palestine and to determine its incidence.

2. Methodology

A retrospective cohort study was conducted using a data for 75,744 specimens obtained from the CPHL (which is the only laboratory in the West Bank that screens for PKU). These specimens were tested for PKU in 2017, the method used for testing was an enzymatic-colorimetric method using (PKU) neonatal Screening Assay kit (RE80015 / RE80019, IBL International, Germany), with no cross-reactivity with the typical substance tested (analytical specificity), 1.55 mg/dL for functional sensitivity (limit of detection). Blood samples were collected between 24 to 72 hours after birth. Regarding linearity, samples showing concentration above the highest standard should be calculated from the linear curve by multiplying the standard used by two. Sample dilution is not recommended because blood presents an endogenous phenylalanine concentration and matrix effects of other substance cannot be excluded according to the kit used.

Blood for testing was obtained from the heel prick of the infant into a cellulose filter paper. A 5-mm spot puncher was used to punch filter paper and get a blood spot, then phenylalanine on the spot paper was eluted with trichloroacetic acid (TCA 3%). Phenylalanine dehydrogenase was added to transform phenylalanine into phenylpyruvate and to reduce NAD+ to NADH, then a substrate -Tetrazolium Salt- (yellow) was added and transformed into violet substrate Formazan by the reduced NADH. The color intensity of Formazan was measured using a spectrophotometer at 570 nm.

The CPHL applies the procedure specified in the IBL kit, a concentration of 2.5-2.9 mg/dL is considered a gray zone and it should be repeated to make sure of the result. If the concentration is above 3 mg/dL after repetition, a new sample should be collected and analyzed again, if it's also above 3 mg/dl it's considered positive. If any sample either a new one or the first one after repetition is below 3 mg/dL, it is considered negative.

3. Ethical approval

The study was a records-based, and there was no direct intervention involvement with patients. However, Data collection forms issued by Palestinian Ministry of Health that include ethical considerations were filled and signed.

4. Statistical Analysis

Data was processed using IBM Statistical Package for Social Sciences (SPSS) version 23 and MedCalc program version 15.8. Receiver Operating Characteristics (ROC) curve analysis was used to calculate the cutoff value. Descriptive statistics were used to assess the percentages while independent sample t – test, paired sample t – test, and Fisher's Exact test were used for inferential statistics. *P*-value <0.05 was considered statistically significant.

5. Results

A total of 75,744 specimens were included in this study from all Palestinian newborns in West Bank region during 2017, from which the results of 1915 specimen were repeated for at least once based on the current methodology adopted by CPHL in Palestine. Among the whole cases, 27 were PKU positive with an incidence rate of (35.6 per 10^5 newborns per year); comprising 59.3% of males and 40.7% of females. This gender difference was statistically insignificant (p=0.108).

The cutoff value obtained by the ROC curve analysis was 3.3 mg/dL with an optimal sensitivity (100%) and specificity (99.^%). Figure 2 shows The Area Under the ROC Curve (AUC) which was found to be 1 (Confidence interval: 1 - 1).

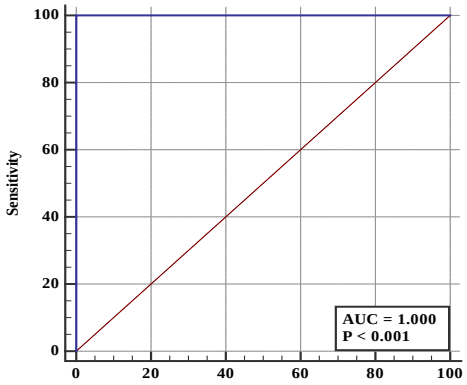

Figure 2. ROC curve analysis to establish the Phenylalanine cutoff value for PKU in the West Bank – Palestine.; AUC: Area under the Curve

Among specimens obtained, 75,576 (99.8%) were found to be true negative (TN), with 1.6 ± 0.467 mg/dL (mean ±SD). A value of 2.6 mg/dL was the most common (mode) among negative results, with a frequency of 397. Figure 3 shows Phenylalanine test results distribution among PKU true negative cases.

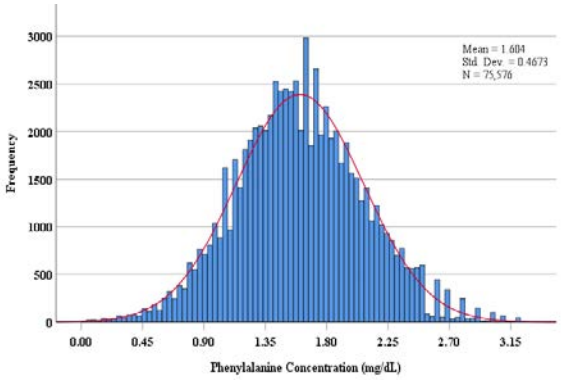

Figure 3. PKU test results distribution of Phenylalanine test results for PKU true negative cases among tested neonates.

However, 141 (0.2%) were found to be false positive (FP) as shown in Table 1. These numbers of TN and FP were obtained according to the calculated cutoff value of 3.3 mg/dL. Regarding true positive results, the highest value obtained was 29 mg/dL while the lowest value was 3.4 mg/dL.

Table 1. Frequency of PKU test result for False Positive and True Positive cases among tested neonates

Phenylalanine Concentration (mg/dL)	No. of FP cases	No. of TP cases
3.3 - 4.9	125	6
5.0 - 6.9	9	5
7.0 - 8.9	4	3
9.0 - 10.9	0	0
11.0 - 12.9	2	0
13.0 - 14.9	0	2
≥ 15.0	1	11
Total	141	27

PKU: phenylketonuria; FP: false positive; TP: True positive

The mean of Phenylalanine values for PKU true positive cases from the first sample without any repetition was 12.25 while the mean after retesting the first sample was 12.30 (p=0.89), while the mean of Phenylalanine value on a second sample that is collected after 10 days from the first one was 15.85 mg/dL. On the other hand, there was a sharp decline of mean Phenylalanine values from 4.05 to 2.52 for False positive results between the results of the first sample and the retesting of it (P <0.001).

Based on the 3.3 mg/dL cutoff value estimated by this study, the cutoff value usually used by CPHL in the West Bank (≥2.5 mg/dL) to distinguish positive from negative cases could be changed to a new one with much less false positive cases (141 case) than the usually estimated number (1888 case) (Figure 4).

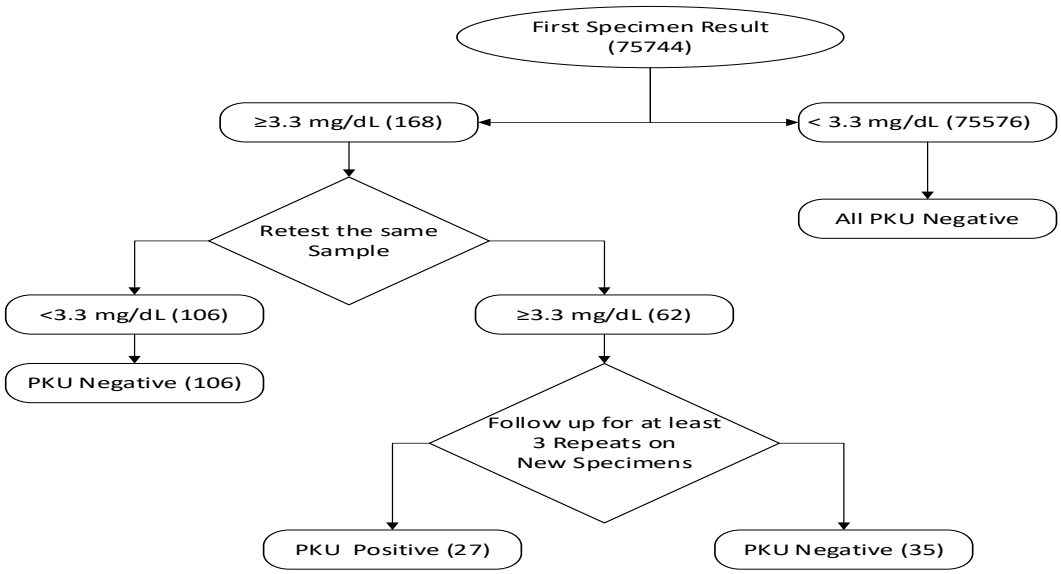

Figure 4. PKU Positive Cases identification based on a cutoff value of ≥ 3.3mg/dL for Phenylalanine

6. Discussion

The incidence rate of PKU in the West Bank of Palestine in 2017 was 35.6 per 10^5 newborns (1 : 2800) less than that in Turkey (1:2600) but Larger than average Arabian countries (1:6000) (Williams *et al.*, 2008). No significant incidence difference was found between males and females; this could be due to PKU being an autosomal recessive inherited disorder and not sex-linked (van Wegberg *et al.*, 2017). The optimum cutoff value of Phenylalanine for PKU for the Palestinian population living in West Bank based on ROC curve analysis was 3.3 mg/dL. Phenylalanine cutoff values for PKU vary from one population to another, for example, according to a study held in Bangkok the established cutoff value was 3.6 mg/dl using ELIZA method, 4 mg/dL using Guthrie method (Wasant *et al.*, 1999), another study in California gave a cutoff value of 4.3 mg/dL (Arnopp *et al.*, 1995). In Brazil, the cut off value was 5 mg/dL (Ramalho *et al.*, 2014). Regarding China, different cities had different cutoff values; 2 mg/dL in both Ankang and Fujian (Zhang & Jiang, 2011; Zhu *et al.*, 2004), 2.3 mg/dL in Shenzhen (Chun *et al.*, 2010), and 2.6 mg/dL in Daqing (Pan *et al.*, 2007).

In the ROC curve analysis, sensitivity refers to rate of true positive cases identified by the test divided by all true positive cases while the specificity refers to the rate of true negative cases identified by the test divided by all true negative cases. The analysis of this data revealed that sensitivity was 100% while specificity was 99.^%; this means that there are no false negative cases and all negative cases are true negative, so the established cutoff value estimated in this study can be used with strong confidence. The 141 (0.2%) false positive cases could be due to prematurity, improper sample collection time, procedural or personal error.

The AUC is a reflection of how good the test is at distinguishing patients with disease and those without the disease. In this study, it was found to be 1.0 for the ROC curve for the first sample, indicating that the test was excellent, and the 3.3 mg/dL value obtained is a definitive cutoff value

Phenylalanine concentrations of PKU cases ranged from >20 mg/dL for classic PKU (4 cases) and between 10 mg/dL to 20 mg/dL for mild PKU (9 cases), and <10 mg/dL for benign PKU (14 case). These different types of PKU according to phenylalanine concentrations could explain the variations in phenylalanine concentrations among the PKU positive cases (Williams *et al.*, 2008).

Repeated measures analysis for PKU for positive cases showed no significant difference between the mean of the first result and the retesting of the first sample, while the same analysis showed a sharp decrease in the mean concentration of phenylalanine in the false negative cases. So, the result of retesting the first sample is an excellent indicator for true positive cases. On the other hand, the mean phenylalanine concentration in the true positive cases increased dramatically between the first sample (12.25 mg/dL) and the second sample (15.85 mg/dL) that is tested after about 10 days, $p = 0.018$. This increase in phenylalanine concentration ascertains the presence of PKU in these neonates. Most false positive cases (115 case) became negative from the retesting of the first sample, while the rest (26 case) became negative on the second sample.

7. Recommendations

Some serious steps should be taken in preventing infants from leaving hospitals without making this important test. Also, parents should collaborate with centers and hospitals and follow the instructions when the test is performed. There is an urgent need to educate parents of PKU patients to follow the restricted diet. Moreover, the right protocol should be followed taking into account the appropriate sample collection time.

To reduce the cost of testing and repeating tests without actual need, we recommend the CPHL to adopt 3.3 mg/dl as a definitive cutoff value of Phenylalanine for PKU from the first test result. If the first test result is more than 3.3 mg/dL, then the sample must be retested, and new samples are necessary to rule out false positive cases.

Acknowledgments

The authors would like to acknowledge and thank the Palestinian Ministry of Health (PMOH) (Central Public Health Laboratory / Public Health Directorate) whose data was used in the creation of this publication. Also, we would like to extend our sincere gratitude to the Director General of Allied Medical Services, PMOH, Mr. Osama Najjar, for his assistance with data collection.

References

Arnopp JJ, Lorey FW, Currier RJ, Eastman JW, Velazquez KB, Morales DR, & Cunningham GC. 1995. Results of screening for phenylketonuria using a lower cutoff value in early collected specimens. *Screening*, **3(4)**, 193-199.

Blau N, Shen N, & Carducci C. 2014. Molecular genetics and diagnosis of phenylketonuria: state of the art. *Expert Rev Mol Diagn*, **14(6)**, 655-671. doi:10.1586/14737159.2014.923760

Borrajo GJ. 2016. Newborn Screening for Phenylketonuria: Latin American Consensus Guidelines. *Journal of Inborn Errors of Metabolism and Screening*, **4**, 1 - 5. doi: 10.1177/2326409816682764

Chun HX, Xin HX, & Qi Z. 2010. Exploration on cut off value of PKU screening in neonates by ROC analysis. *Maternal and Child Health Care of China*, **21**, 025.

Folling I. 1994. The discovery of phenylketonuria. *Acta Paediatr Suppl*, **407**, 4-10.

Guest JF, Bai JJ, Taylor RR, Sladkevicius E, Lee PJ, & Lachmann RH. 2013. Costs and outcomes over 36 years of patients with phenylketonuria who do and do not remain on a phenylalanine-restricted diet. *J Intellect Disabil Res*, **57(6)**, 567-579. doi:10.1111/j.1365-2788.2012.01568.x

Guthrie R, & Susi A. 1963. A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants. *Pediatrics*, **32(3)**, 338-343.

Hanley WB. 2004. Adult phenylketonuria. *Am J Med*, **117(8)**, 590-595. doi:10.1016/j.amjmed.2004.03.042

Kose E, Aksoy B, Kuyum P, Tuncer N, Arslan N, & Ozturk Y. 2018. The Effects of Breastfeeding in Infants With Phenylketonuria. *J Pediatr Nurs*, **38**, 27-32. doi:10.1016/j.pedn.2017.10.009

Manta-Vogli PD, & Schulpis KH. 2017. Phenylketonuria Dietary Management and an Emerging Development. *J Acad Nutr Diet*. doi:10.1016/j.jand.2017.05.020

Merrick J, Aspler S, & Schwarz G. 2003. Phenylalanine-restricted diet should be life long. A case report on long-term follow-up of an adolescent with untreated phenylketonuria. *Int J Adolesc Med Health*, **15(2)**, 165-168.

NIH. (2019). What are common treatments for phenylketonuria (PKU)? https://www.nichd.nih.gov/health/topics/pku/conditioninfo/treatments (15.5.2019)

Pan JL, Zhao YQ, Chen YM, & Zhang FX. 2007. Study on Phe cut-off point of neonatal phenylketonuria screening in Daqing. *Chinese Journal of Birth Health & Heredity*, **2**, 65 - 71.

Ramalho AR, Ramalho RJ, Oliveira CR, Magalhaes MM, Santos EG, Sarmento PM, Matos DO, Oliveira MC, Oliveira AL, & Aguiar-Oliveira MH. 2014. Evaluation of effectiveness and outcome of PKU screening and management in the State of Sergipe, Brazil. *Arq Bras Endocrinol Metabol*, **58(1)**, 62-67.

Schlegel G, Scholz R, Ullrich K, Santer R, & Rune GM. 2016. Phenylketonuria: Direct and indirect effects of phenylalanine. *Exp Neurol*, **281**, 28-36. doi:10.1016/j.expneurol.2016.04.013

Schuck PF, Malgarin F, Cararo JH, Cardoso F, Streck EL, & Ferreira GC. 2015. Phenylketonuria Pathophysiology: on the Role of Metabolic Alterations. *Aging Dis*, **6(5)**, 390-399. doi:10.14336/AD.2015.0827

van Wegberg AMJ, MacDonald A, Ahring K, Belanger-Quintana A, Blau N, Bosch AM, Burlina A, Campistol J, Feillet F, Gizewska M, Huijbregts SC, Kearney S, Leuzzi V, Maillot F, Muntau AC, van Rijn M, Trefz F, Walter JH, & van Spronsen FJ. 2017. The complete European guidelines on phenylketonuria: diagnosis and treatment. *Orphanet J Rare Dis*, **12(1)**, 162. doi:10.1186/s13023-017-0685-2

Wasant P, Liammongkolkul S, & Srisawat C. 1999. Neonatal screening for congenital hypothyroidism and phenylketonuria at Siriraj Hospital, Mahidol University, Bangkok, Thailand--a pilot study. *The Southeast Asian journal of tropical medicine and public health*, **30**, 33-37.

Williams RA, Mamotte CD, & Burnett JR. 2008. Phenylketonuria: an inborn error of phenylalanine metabolism. *Clin Biochem Rev*, **29(1)**, 31-41.

Zhang L, & Jiang JY. 2011. Study on the cut-off value of screening test for phenylketonuria of neonate in Ankang area of Shanxi province. *Laboratory Medicine and Clinic*, **16**, 027.

Zhu WB, Chen HQ, & Wang J. 2004. Study on Phe cut-off point of neonatal phenylketonuria screening in Fujian province. *Chinese Journal of Birth Health & Heredity*, **4**, 94 -96.