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Phenylalanine and Tyrosine Metabolism Analysis in Heterozygotes for Phenylketonuria and in Healthy Individuals

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Abstract

Phenylketonuria (PKU) is an inherited metabolic disorder derived from a deficiency in the enzyme phenylalanine hydroxylase, which converts the amino acid phenylalanine (Phe) into tyrosine (Tyr). Here we aimed to examine the metabolism of Phe and Tyr in heterozygotes for PKU during fasting and after oral overload of Phe (25 mg/kg). Plasma concentration of Phe and Tyr and Phe^2–Tyr ratio were determined under fasting condition or 30, 45, 60, and 90 minutes after Phe overload. The sample consisted of 50 participants: 23 heterozygotes for PKU (10 men and 13 women) and a control group of 27 healthy individuals (13 men and 14 women). The dosage of Phe at 45 and 90 minutes and the micromolar fraction of Phe^2/Tyr after 90 minutes of overload efficiently differentiated PKU heterozygotes. The discriminant function revealed 86% of accuracy. In fact, 94.4% of heterozygotes for PKU were correctly classified.

Keywords

inborn errors of metabolism, heterozygotes, phenylalanine, overload

Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder due to partial or total deficiency of the phenylalanine-4-hydroxylase (PAH), leading to an impaired conversion of amino acid phenylalanine (Phe) into amino acid tyrosine (Tyr).[1]

The PAH gene maps the 12q23.2 region of the chromosome 12 chromosome and contains 13 exons.[2] The PKU is not only characterized by phenotypic heterogeneity, but it also presents molecular heterogeneity. In fact, approximately 600 mutations have been described for the PAH gene.[3] Phenylalanine-4-hydroxylase is mainly localized in the liver, hampering its direct evaluation. In this scenario, biochemical assays for PKU evaluation are indirect and are carried through the measurement of plasma Phe and Tyr.[4]

The accurate classification for heterozygotes is valuable for linkage analysis studies and may assist in estimating the genic frequency as well as in genetic counseling. Studies have demonstrated that a precise discrimination of individuals with tetrahydrobiopterin hyperphenylalaninemia can be achieved by enzymatic assays.[5-7] However, diagnosis of PAH-deficient patients is still a challenge.

Because PAH is particularly activated in liver, enzymatic activity detection do not have practical relevance for heterozygotes screening. However, few investigation using liver tissue have revealed that the activity of this enzyme in heterozygotes is about 30% compared with PHA activity in patients with PKU, which is less than 1%.[8]

In the late 1950s, Hsia and colleagues[9] have demonstrated that oral Phe overload may allow the detection of heterozygotes in the population using the differences in Phe concentration. Indeed, serum levels of Phe were 2-fold higher in heterozygotes after Phe overload (100 mg/kg). These findings were also in line with reduced PAH activity in heterozygotes.[9]

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These investigations primarily initiated other biochemical studies used for PKU detection in heterozygotes. The majority of the employed methods to distinguish PKU heterozygotes from healthy individuals are based on evaluation of blood levels of Phe and Tyr after an oral,[9-11] intravenous[12] Phe overload or deuterium-labeled administration.[13] In this concern notwithstanding the analyses of a single parameter (Phe) may not correctly distinguish both the groups.[14] Therefore, multivariate combinations of different parameters such as levels of Phe and Tyr and the micromolar Phe–Tyr and Phe^2–Tyr ratios have been proposed as discriminant functions with reduced classification error. Additionally, the graphs of micromolar ratios are also recommended for obtaining a higher accuracy in detection of heterozygotes.[10,11,14-18] The use of such methods is simple and low cost and can be justified by the high frequency of heterozygotes in the population (1:50-1:70), as well as by its preventive potential, since it may allow genetic counseling and prenatal diagnosis.[19]

Aim

The present study was tailored to analyze which parameters (Phe, Tyr, Phe/Tyr, and Phe^2/Tyr) possessed the power of distinguishing PKU heterozygotes from healthy individuals. This approach can be used to track PKU heterozygotes in high-risk families.

Methods

The sample enrolled 23 obliged heterozygotes from 18 to 45 years of age (10 men and 13 women). The control group was composed by 27 individuals (13 men and 14 women) from 18 to 44 years of age. All participants were in fasting condition for at least 10 hours. Five blood samples were collected. The basal sample was collected during fasting (T0). Afterward, amino acid Phe (25 mg/kg weight) diluted in water was orally administered, and 4 more samples were subsequently collected 30, 45, 60, and 90 minutes after the overload (T30, T45, T60, and T90, respectively).

After the sample collection, plasma was separated and frozen at $-20^{\circ}C$ until the quantitative analyses of the amino acids Phe and Tyr. Women who were pregnant or taking contraceptive pills were not included in the study. Dosage of Tyr and Phe was duplicated in accordance with the fluorometric methods of McCaman and Robins[20] and Philips,[21] respectively.

In accordance with Brazilian Resolution #196/96, the present study was approved by the Ethic Committee of the Institute of Health Sciences of the Federal University of Pará under the protocol #088/08.

Statistics

Both groups were statically compared using 4 parameters, namely, plasma levels of Phe and Tyr and the results of the Phe–Tyr and Phe^2–Tyr ratios. Longitudinal interferences (within-subject) were applied by means of tests for paired samples, such as Student *t* and Wilcoxon tests. Transversal comparisons (between-subject) were made using Student *t* test and Mann-Whitney *U* test. The evaluation of the operational performance of the amino acids was made using receiver–operating characteristic (ROC) curve. The variables with better performance, in accordance with ROC curve, were submitted to discriminant analysis model to validate the efficiency of the model. The level of significance was set at $P < .05$.

Results

The longitudinal analysis of Phe, Tyr, Phe/Tyr, and Phe^2/Tyr (Figure 1) revealed that the maximum peak of level was at 30 and 45 minutes after Phe overload for control and heterozygote groups, respectively. After overload, all of the obtained values were higher than baseline in both the groups ($P < .05$). In the control group, the highest peak of Tyr happened 30 minutes after overload, and all subsequent values were statically different from fasting condition ($P < .05$, baseline). In the heterozygote group, Tyr reached the highest peak 60 minutes after overload and only T45 and T60 reached statistical difference compared to T0 ($P < .05$, Figure 1B). Considering the between-group analysis, Phe was significantly higher in the heterozygote group in T0, T45, T60, and T90. In control group, higher values of Tyr were found in T30, T45, and T90. The Phe–Tyr and Phe^2–Tyr ratios displayed higher values in heterozygote group in all tested intervals ($P < .05$).

Table 1 details the indicators of sensitivity, specificity, and cutoff for the Phe, Tyr, Phe/Tyr, and Phe^2/Tyr parameters. These indicators were used in the ROC curve construction (Figure 2).

Figure 3 depicts the ROC curve used in comparison of the 3 best-obtained results (Phe T45, Phe T90, and Phe^2/Tyr T90). These parameters were chosen because they ablest expressed the differences between heterozygotes and the control groups, thus being selected to the multivariate model (discriminant analysis).

Using the discriminant functions $Y' = -0.0996\,X1 + 0.9928\,X2 + 0.0668\,X3$ and $Y'' = 0.1000\,X1 + 0.9801\,X2 - 0.1717\,X3$, a diagram was plotted to support the classification of each patient from the heterozygote and control groups in accordance with the predictive variables in which X1, X2, and X3 were Phe T45, Phe T90, and Phe^2/Tyr, respectively. Table 2 shows the final classification of both the groups in line with the discriminant functions.

Figure 4 displays 6 individuals from the heterozygote group that, after the Phe overload, whose values were lower than expected for PKU homozygotes (false negative) and 3 individuals from the control groups who presented values similar to heterozygotes (false positive).

Discussion

The absence of satisfactory techniques to detect the enzyme PAH in PKU heterozygotes allied to the difficult molecular analysis of PAH gene due to vast number of mutations has contributed to the implementation of biochemical reliable tests to distinguish between PKU heterozygotes from healthy individuals.

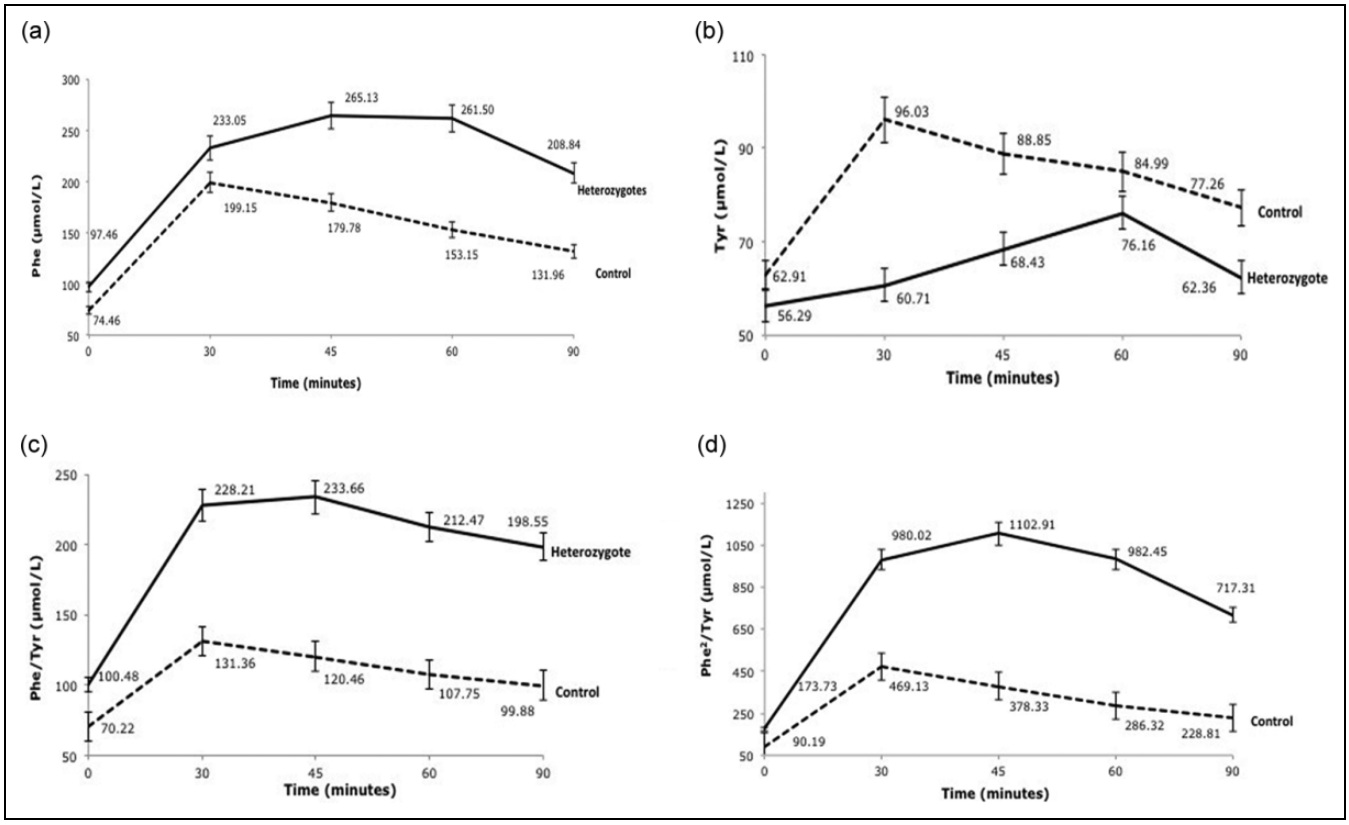

Figure 1. Mean and standard error of the Phe (a), Tyr (b), Phe/Tyr (c), and Phe^2/Tyr (d) parameters in the control group (n = 27) or the heterozygote group (n = 23) in the basal condition or 30, 45, 60, and 90 minutes after the Phe oral overload (T0, T30, T45, T60, and T90, respectively). Phe indicates phenylalanine; Tyr, tyrosine.

Table 1. Sensitivity, Specificity, and Cutoff for the Variables Phe, Tyr, Phe/Tyr, and Phe^2/Tyr.

		Sensitivity	Specificity	Distance	Cutoff
Phe	T45	0.869	0.926	0.150	3.960
	T60	0.782	0.926	0.230	3.240
	T90	0.869	0.852	0.197	2.710
Tyr	T30	0.815	0.783	0.286	1.290
	T45	0.778	0.689	0.377	1.220
	T90	0.825	0.652	0.378	1.150
Phe/Tyr	T0	0.738	0.778	0.343	1.340
	T30	0.739	0.815	0.320	2.650
	T45	0.826	0.815	0.254	2.480
	T60	0.957	0.630	0.373	1.910
	T90	0.913	0.778	0.239	2.030
Phe^2/Tyr	T0	0.783	0.815	0.286	1.810
	T30	0.565	0.926	0.441	1.,080
	T45	0.913	0.778	0.239	7.300
	T60	0.739	0.963	0.263	9.500
	T90	0.826	0.889	0.206	6.220

Abbreviations: Phe, phenylalanine; Tyr, tyrosine; T, interval (minutes) from Phe oral overload to dosage.

The parameters here employed were chosen taking into account that the expected hydroxylation rate of Phe for Tyr is smaller in heterozygotes than in healthy individuals. Thus, healthy individuals should present higher plasma levels of Tyr. Considering that heterozygotes may display increased levels of Phe and reduced levels of Tyr, one may propose Phe–Tyr and Phe^2–Tyr ratios usage to augment the distinction between PKU heterozygotes and healthy individuals, although some studies had already described important overlapping zones when these parameters are applied.[4,21]

In the control group, all of the obtained values of Phe after overload were significantly higher than T0. These findings are in agreement with previous studies.[23] The levels of Phe progressively increased and reached the highest peak at 30 minutes after overload administration, suggesting that Phe is being rapidly metabolized into Tyr. Statistical differences for Tyr levels were detected after T30, corroborating the metabolization of Phe into Tyr. What concerns Phe/Tyr and Phe^2/Tyr ratios, the highest peak occurred in T30. These findings are in line with previous studies.[18,24]

In the heterozygote group, the mean value of Phe, Phe/Tyr, and Phe^2/Tyr reached the peak at T45, suggesting that the metabolization of Phe into Tyr was slower for this group compared to the control group. In accordance, Tyr levels were higher at T60. These results were also found by Silva and colleagues.[4]

When both groups were compared during fasting condition and after Phe overload, Phe serum concentration was increased in the heterozygote group, corroborating previous findings.[4,25]

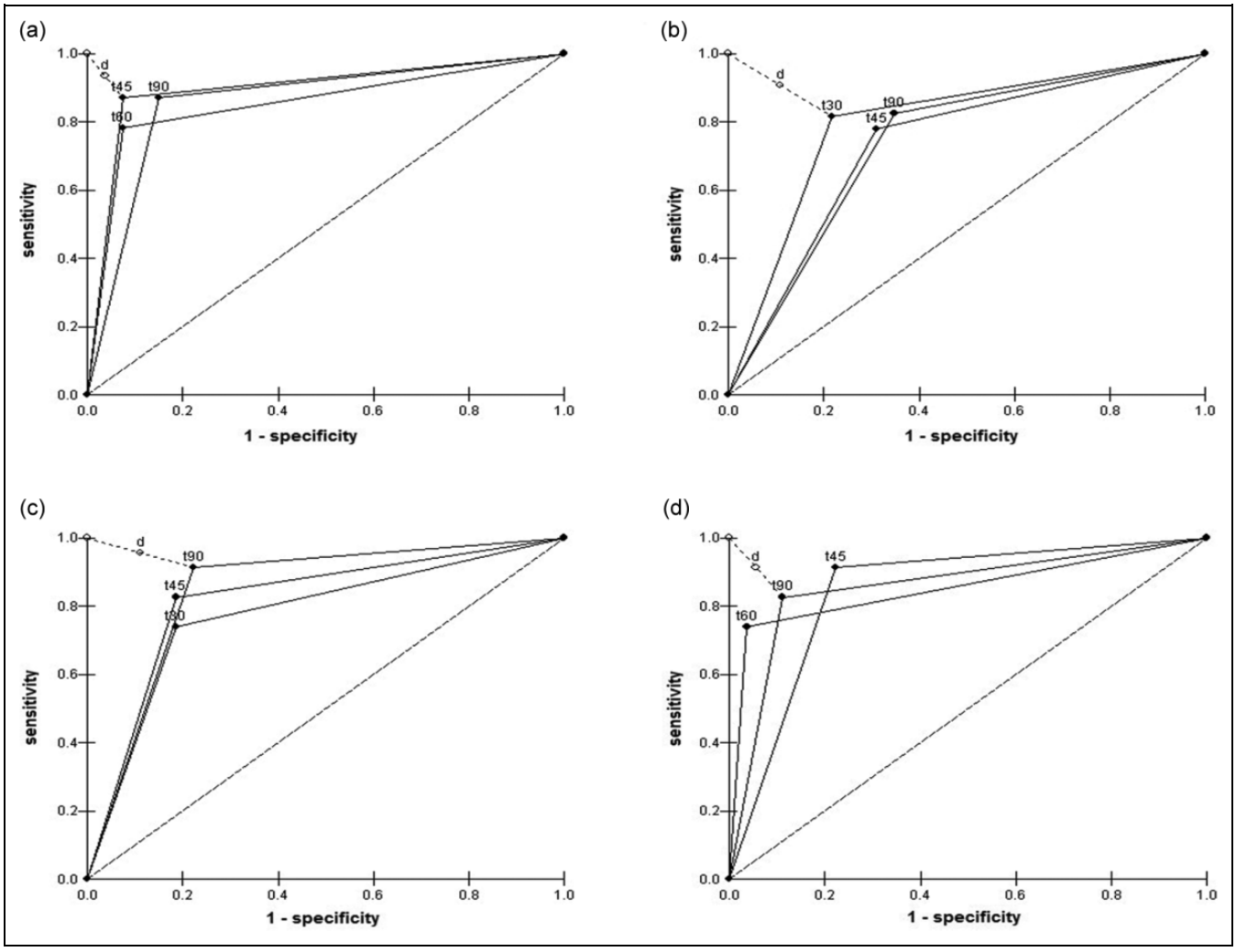

Figure 2. ROC curve of the Phe (a), Tyr (b), Phe/Tyr (c), Phe/Tyr, and Phe^2/Tyr (d) parameters. Phe indicates phenylalanine; Tyr, tyrosine.

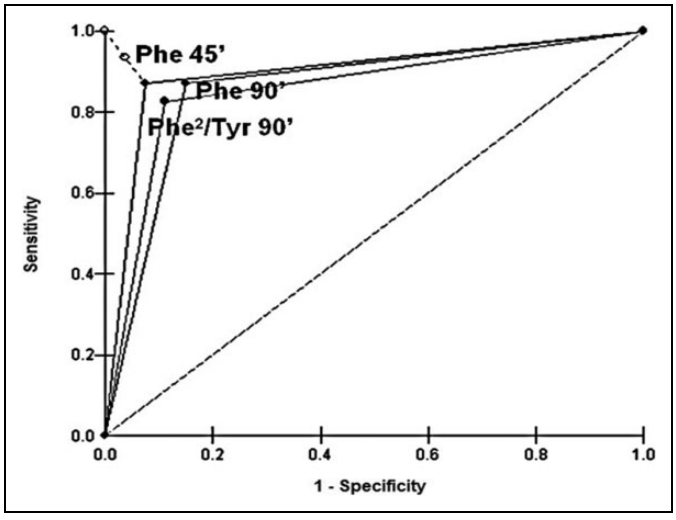

Figure 3. ROC curve of the best parameters (Phe after 45 minutes of the oral Phe overload and Phe and $Phe^2/TyrT90$ after 90 minutes of oral overload). Phe indicates phenylalanine; Tyr, tyrosine; ROC, receiver operating characteristic.

Table 2. Classification of Participants From Both Groups Tested for Discriminant Equation.

Sensitivity	73.91%
Specificity	88.89%
False positive (error type I)	11.11%
False negative (error type II)	26.09%
Prevalence	0.460 or 46%
Test positive predictive value	94.4%
Test negative predictive value	81.25%
Accuracy	0.86 or 86%
Positive likelihood ratio	19.96
Negative likelihood ratio	0.27

The largest difference between the 2 groups occurred at T60. Garcia and coworkers[26] have verified that the levels of Tyr in the heterozygote group were 2-fold increased compared to control and that the major difference also occurred 60 minutes after the overload.

Still concerning Tyr parameter, the levels of this amino acid were decreased in the heterozygotes at T30, T45, and T90. In line with data found by Guldberg et al[22] and Spada et al,[27] the

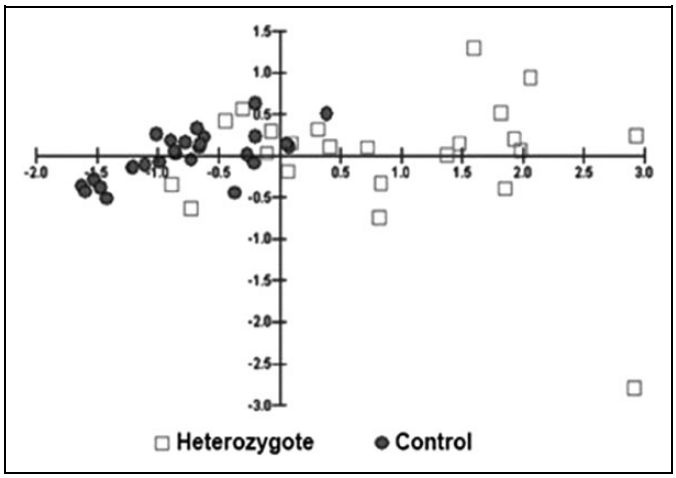

Figure 4. Classification heterozygote and control group participants.

greatest difference between experimental and control groups occurred at T30.

The variables Phe/Tyr and Phe^2/Tyr were increased in the heterozygotes throughout the measured period. This finding is in agreement with those from Silva et al,[19] Song et al,[28] and Abdalla et al.[29]

The analysis of the ROC curve revealed that the best parameters to differentiate PKU heterozygotes from healthy individuals were, in the order, Phe after 45 and 90 minutes of overload and Phe^2/Tyr after 90 minutes. Of note, Phe^2/Tyr at T90 had presented the best performance in the previous study from Abdalla et al.[29]

We intended to increase the differentiation degree between both groups. Therefore, discriminant functions were elaborated using the 3 best parameters obtained here. Thus, we found sensitivity of 73.91% and specificity of 88.89%. The rates of false positive and false negative were 11.11% and 26.09%, respectively. The positive predictive value of the test was 94.4%, and the negative predictive value was 81.25%. In the study of Silva and colleageues,[19] the discriminant function that considered fasting condition was found to be more efficient than the one that considered the variables after aspartame overload. In that work, the discriminant function correctly classified 98% of individuals with 97% of accuracy.

Here, when the discriminant function was applied, 6 false-negative cases were detected in the heterozygote group (26.09%, 4 male and 2 female). In the control group, 3 false-positive cases were detected (11.11%, 2 male and 1 female). In the work from Silva et al,[19] which used aspartame overload instead (100 mg/kg, containing 56 mg/kg of Phe), 3 participants (all women) from the control group presented with Phe values above the expected for healthy individuals and 1 PKU heterozygote displayed lower probable values for this condition. It should be highlighted that the exclusion criteria were previously determined in the present study. Specifically, use of contraceptives pills, menstrual period, and pregnancy were among them because they can potentially interfere with Phe metabolism and mislead to classification of an individual as PKU heterozygote. Among this cited factors, contraceptive pills have the greatest probability of inducing such an error. Additionally, iron deficiency anemia should also be considered. A small possibility of having heterozygotes in the control group can be discarded since the frequency in the population is 1:50 to 1:70.[4,8,30]

Conclusion

The present study is original regarding the dosage of Phe after overload (25 mg/kg) and the distinct time points employed to evaluate the metabolism of amino acid Phe and Tyr. The ROC curve had demonstrated that the Phe at T45 and T90 and Phe^2/Tyr at T90 were the best parameters to differentiate PKU heterozygotes from healthy individuals. Considering our results, a large range of differentiation was obtained after 45 minutes of Phe overload. The discriminant analysis revealed an accuracy rate of 86%. In addition, 94.4% of the PKU heterozygotes were correctly classified.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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