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The value of simultaneous determination of blood large neutral amino acids and tetrahydrobiopterin metabolites in the diagnosis of atypical hyperphenylalaninemia

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Abstract

Tetrahydrobiopterin deficiency in newborns with atypical hyperphenylalaninemia requires rapid and accurate diagnosis and the ability to distinguish it from the classical type to prevent early irreversible neurological damage. The study aimed to evaluate neopterin and biopterin (products of tetrahydrobiopterin recycling pathway) and amino acid profiles (used in supplementation therapy) in patients with hyperphenylalaninemia after optimizing ultra-performance liquid chromatography coupled with tandem mass spectrometry to simultaneously measure neopterin, biopterin, and amino acids in dried blood spots. The study enrolled preselected infants with classic ($n = 46$), atypical ($n = 14$) hyperphenylalaninemia, and a control group ($n = 50$).

Result Tandem mass spectrometry detected neo/biopterin in the blood with a sensitivity and specificity of 100%. The mean neo/biopterin levels were significantly lower in the atypical cases (4 ± 1 and 3 ± 1 nmol/L) than the classic (49 ± 13 and 50 ± 12 nmol/L) and control (15.2 and 15.3 nmol/L) groups and correlated with phenylalanine and phenylalanine to tyrosine ratio (*all* $P < 0.05$). The study compared classic and atypical hyperphenylalaninemia cases with the control group. Both classic and atypical cases exhibited decreased levels of arginine, valine, and leucine compared to controls. Classic cases showed increased levels of citrulline, ornithine, and methionine, while atypical cases showed increased citrulline levels only. Comparing atypical versus classic cases, atypical cases exhibited decreased levels of citrulline, ornithine, methionine, arginine, leucine, and valine (all $P < 0.05$). Correlation analysis revealed negative associations between ornithine and biopterin and between arginine and neopterin in classic PKU cases. These findings highlight distinct metabolic differences between classic and atypical PKU.

Conclusion The optimized method detected neo/biopterin in the blood with accuracy and precision. The characteristic pattern of neo/biopterin in the blood makes it possible to differentiate between classic and atypical hyperphenylalaninemia with a sensitivity and specificity of 100%. The amino acid profile could add value when treatment with large neutral amino acids is considered.

Keywords Tandem mass spectrometry, Neopterin, Biopterin, Atypical hyperphenylalaninaemia

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Introduction

Phenylketonuria (PKU) is an inborn error in the metabolism of the amino acid phenylalanine (Phe) due to abnormalities in the phenylalanine hydroxylase (*PAH*) enzyme. PHA is a mixed-function oxidase that catalyzes the hydroxylation of phenylalanine to tyrosine. Partial or complete deficiency of this enzyme leads to an accumulation of phenylalanine, resulting in hyperphenylalaninaemia and abnormalities in the metabolism of many derivatives of the aromatic amino acids [1, 2]. More additional information about PKU epidemiology and global phenotype and genetic distribution is presented in this article [3]. Based on blood Phe level at the diagnosis, PKU is classified into three types: classic PKU with Phe > 1200 µmol/L, moderate PKU with Phe ranging from 600 to 1200 µmol/L, and mild PKU or atypical PKU with Phe ranging from 120 to 600 µmol/L [2]. Tetrahydrobiopterin (BH4) is an essential cofactor for many amino acid hydroxylases, including phenylalanine, tyrosine, and tryptophan hydroxylases. Defects in the enzymatic conversion of phenylalanine to tyrosine, tyrosine to L-Dopa, and tryptophan to 5-hydroxytryptophan cause HPA and reduce dopamine and serotonin levels in the central nervous system. The enzymatic functions of these amino acid hydroxylases are typically dependent on the presence of BH4, oxygen, and iron. The reaction occurs at the active catalytic site of the enzymes involving a non-heme iron molecule. BH4 activates oxygen binding to iron into an oxo-iron complex, which allows the enzymatic hydroxylation of their substrates as Tyr, Phe, or tryptophan, and the oxidized intermediate of BH4 will be converted back to BH4 via the recycling pathway. The degradation products of the reaction pathway include neopterin and biopterin, which are removed by the body as they cannot be further reused; therefore, patients with classic PKU excrete more pterins in urine compared with healthy persons, and the amount of excreted metabolites is directly proportional to blood Phe levels [4, 5]. BH4 deficiencies or failure of its synthesis results in five genetic disorders characterized by a mild increase in the blood phenylalanine level and severe central nervous neurological disease associated with progressive mental and intellectual deterioration despite adequate dietary control of blood Phe. Treatment with synthetic BH4 usually corrects the blood Phe levels without any dietary restriction, and substitutions of dopamine and serotonin precursors prevent further neurological damage. Therefore, it is critical to distinguish BH4 deficiencies from PAH gene deficiencies, and the early measurement of neo/biopterin becomes vital for the proper diagnosis [6–9]. Branched-chain amino acids (BCAAs), valine, leucine, and isoleucine are essential amino acids involved in several disorders, particularly liver cirrhosis, renal failure, sepsis, and cancers [10, 11]. BCAAs are member constituents of the large neutral amino acids, namely phenylalanine, tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, arginine, lysine, and histidine. These LNAAs use the same transporter to cross the blood-brain barrier [12, 13], and dietary supplementation with large doses of phenylalanine-deficient LNAAs decreases the access of phenylalanine into the brain in patients with PKU, thus may be valuable in patients with poor Phe dietary restrictions [12, 13]. The metabolic balance and competition for the protein transporter between the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and BCAAs may influence the synthesis of brain neurotransmitters, particularly dopamine, norepinephrine, and serotonin [14]. In healthy individuals, all of LNAAs but tyrosine are essential amino acids; however, in phenylketonuria and due to deficiency of PHA, tyrosine also becomes an essential amino acid. Different LNAA therapeutic formulas were used as an alternative to Phe dietary restriction, with some combinations containing arginine lysine, ornithine, or citrulline, neither of which is an LNAA. LNAA supplementation in PKU has several therapeutic effects as reducing the blood and brain Phe concentration and increasing cerebral neurotransmitter concentrations via increasing their precursors of cerebral essential amino acid concentrations [11, 15, 16].

With the advances in laboratory technology, tandem mass spectrometry emerged as the most significant development in clinical diagnostics tools, especially in the field of inborn errors of metabolism and newborn screening programs. It enables simultaneous analysis of several molecules as the large neutral amino acids and BCAAs in dried blood spots (DBS), and detection of various metabolic disorders, therefore allowing the expansion of screening of metabolic diseases in a single run [17]. The current study aimed to (1) re-evaluate the usefulness of measuring neopterin and biopterin in the DBS in a cohort of patients having either classic or atypical phenylketonuria and to (2) assess the status of blood large neutral amino acids and their relation to neopterin and biopterin. The ultimate goal is to provide an easily applicable method for screening and follow-up of patients with PKU and evaluation of the associated amino acid disorders, particularly those under treatment with various regimens of dietary restriction or BH4 replacement therapy.

Patients

The study investigated a cohort of 60 patients selected for metabolic screening of phenylalanine metabolism-related inborn errors due to clinical symptoms of PKU. The study was conducted at the National Liver Institute and Ain Shams University, involving the departments

of Clinical Biochemistry, Pediatric Hepatology, and Human Genetics, from March 2019 to March 2021.

The majority of the cases diagnosed with typical PKU (98.2%) were beyond the neonatal period, while only 1.2% were identified through neonatal screening. Atypical PKU cases were selected through a special screening program at the Department of Human Genetics, which examined patients with atypical presentations referred from other pediatric clinics for further diagnosis. The average age of presentation for classic PKU and atypical PKU was approximately 56.7 and 50.6 months, respectively. All patients were undergoing treatment primarily involving dietary restrictions and a special amino acid supplementation formula to manage their condition. However, prior to PKU testing, the treatment was temporarily stopped, and patients refrained from any kind of supplementation for 24–48 h to ensure accurate metabolic status assessment without the influence of ongoing treatment. All participants were clinically evaluated for neurological and intellectual mental development and met the diagnostic criteria for PKU [1]. The BH4 loading test was used to discriminate patients with high phenylalanine levels due to PAH deficiency from patients with a mild increase in Phe levels due to BH4 deficiency or enzyme defects in the biosynthesis or regeneration of the cofactor BH4. Of all enrolled patients, 46 patients (28 male and 18 female) met the criteria of typical hyperphenylalanemia and were classified as classical PKU caused by PAH deficiency, and 14 (8 male and 6 female) were diagnosed as atypical PKU with BH4 deficiency. The number of each group does not reflect the incidence of the disease among the population because it is a selective screening for rare cases. It also enrolled 50 healthy participants free from any evidence of metabolic, systemic, or chronic diseases, matching the age and gender as a control.

Blood samples were collected under aspect venipuncture and simultaneous determination of neo/biopterin and an array of amino acids (phenylalanine, tyrosine, citrulline, Ornithine, arginine, leucine, valine) in DBSs by HPLC-MS/MS.

Analytical method

HPLC ACQUITY UPLC H-Class with an analytical column (C18, 1.7 µm, 2.1 × 50 mm) (Waters, MA, USA) was used with a mobile phase prepared from an HPLC-grade water (Millipore, Diamond, USA), an HPLC-grade methanol, acetonitrile, HCL, and formic acid (Thermo Fisher, USA). The extracted samples of DBS (Whatman, NJ, USA) were eluted at a column temperature of 50 °C and a flow rate of 0.3 mL/min for 3 min for a mobile phase A (95%) (0.2 formic acid) and mobile phase B (5% Methanol), followed by a mobile phase B (90%) over 3 min then mobile phase B (10%) for 4 min. Then, the column was washed by mobile phase A (98%) for 5 min to equilibrate the column. The mass spectrometer was set in MRM mode to acquire the peak signal by constant infusion of standard solution at a flow rate of 10 µL/min and a capillary voltage (0.7 kV) at 150 °C. Gas station (Peak Scientific Instruments, Scotland) used nitrogen as dissolvent (900 l/h at 600 °C) gas and argon as collision gas (0.15 mL/min) [18].

The chromatographic separation was accomplished in < 1 min, and the analytical method was validated for linearity, accuracy, precision, lower limit of detection, lower limit of quantitation, and stability. Standards of neo/biopterin (Sigma-Aldrich, Darmstadt, Germany), at concentrations ranging from 0 to 100 nmol/L generated the calibration curves by plotting each peak area detected by the mass spectrometry against its corresponding standard point. Fig. 1 shows the calibration curves derived from the standard points and the slope equation with regression coefficients for the linearity of neo/biopterin.

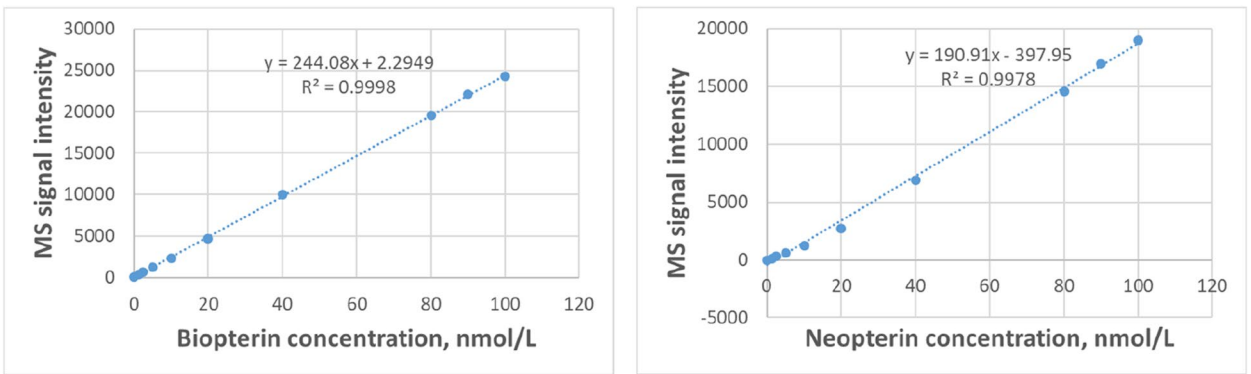

Fig. 1 Calibration curve for biopterin and neopterin concentration nmol/L in DBS with the curve, showing the equation and coefficient of determination (r^2). Slope and *Y*-intercept are reported as mean ± SE

The lower limit of detection of neo/biopterin ranged from 1.5 to 2.5 nmol/L: accuracy = %RE = % (measured − theoretical)/theoretical concentration; precision = % RSD = % standard deviation /mean. The same-day and between-day accuracy and precision ranged from 97 to 105 for both neo/biopterin. DBS spiked with the low (2.5 nmol/L), mid (20 nmol/L), and high (100 nmol/L) standards were stored for either 1week at RT or 12 weeks at −20 °C, and their extracts were used to determine the stability and recovery. The recovery was 105 ± 6% and 106 ± 3% for neo/biopterin at different storage conditions and times. The performance of the optimized tandem mass spectrometry method was sensitive, accurate, and precise for measuring neo/biopterin in the human blood. Determination of phenylalanine, tyrosine, citrulline, ornithine, arginine, leucine, and valine in DBSs by an optimized HPLC-MS/MS method [18] is used for routine laboratory analysis of amino acid in the inborn errors of metabolism unit.

Statistical analysis

The statistical analysis was performed using SPSS 23 (SPSS Inc., CA, USA). The nonparametric Kruskal-Wallis test and the Mann-Whitney were used to perform the multiple comparisons across the groups. Spearman correlation analysis assessed the relationships between neo/biopterin and the amino acid profile. The level of statistical significance was set at $P < 0.05$. Two-by-two table was used to calculate the sensitivity and specificity of neo/biopterin in atypical and classic PKU.

Results

The demographic analyses across the different groups

Table 1 displays the demographic criteria of the enrolled patients. Patients in all groups had a matching distribution of age, sex, BMI, and consanguinity, without significant differences across the groups (all $P > 0.05$) except for male predominance and consanguinity that was significantly associated with typical and atypical PKU ($P < 0.05$).

Amino acid profile in the classic and atypical PKU

Table 2 displays the comparison of amino acid profile in the enrolled groups. The blood level of the Phe, Tyr, and Phe/Tyr ratio was significantly higher in the classic PKU than in the atypical PKU and the control groups ($P < 0.05$). The mean blood Phe level was severely elevated (793 μM/L) in classic PKU relative to a mild elevation in atypical PKU (145 μM/L) consistent with the BH4 challenge test. The blood level of Phe, Tyr, and Phe/Tyr ratio was in the order classic PKU > atypical PKU > control.

Compared to the control group, both the classic PKU and the atypical PKU had a significantly decreased level of arginine, valine, and leucine (all $P < 0.05$). The classic PKU had an increased level of citrulline, ornithine, and methionine (all $P < 0.05$); however, in the atypical PKU, only citrulline has such an increased level among other amino acids (all $P < 0.05$). Compared to the atypical PKU, the classic PKU has a significant increase in citrulline, ornithine, methionine, and valine (all $P < 0.01$) and a significant decrease in leucine and arginine (all $P < 0.05$).

Blood neopterin and biopterin in classical and atypical PKU

Table 3 presents the reference values and the statistical comparison of neo/biopterin across the groups. The mean blood level of neop/biopterin was significantly higher in the classic PKU than in the atypical PKU and the control groups (all $P < 0.05$). The blood level of either neopterin or biopterin was the lowest in the atypical PKU than in the classic and the control groups (Fig. 2). Table 4 displayed the

Table 1 Demographic analysis of classic and atypical PKU

	Stat	Classic PKU, $n = 46$	Atypical PKU, $n = 14$	Control, $n = 50$	*P*-value
Age (months)	Min-Max	2–132	0.75–108	0.5–132	
	Mean ± SD	56.7 ± 35.4	50.6 ± 33.8	44.5 ± 39.4	
	Median (IQR)	60 (88%)	48 (58)	36 (84%)	> 0.05
Gender	Male	28 (60%)	8 (57%)	26 (52%)	> 0.05
	Female	18 (40%)	6 (43%)	24 (48%)	
BMI	Median (IQR)	24 (11)	20 (9)	16 (6)	> 0.05
Consanguinity	Yes	36 (87%)	14 (100%)[bc]	14 (31%)	< 0.05
	No	10 (13%)	0 (0%)	36 (69%)	

PKU phenylketonuria, *SD* standard deviation, *Min* minimum, *Max* maximum, *IQR* interquartile range, *K* Kruskal-Wallis test comparison among all groups

*P-value < 0.05 indicates a statistically significant relation

[a] $P < 0.05$ in classic PKU vs. control

[b] $P < 0.05$ atypical PKU vs. control

[c] $P < 0.05$ classical PKU vs. atypical PKU

Table 2 Comparison of amino acids among the groups

AA	Stat	Classic PKU, $n = 46$	Atypical PKU, $n = 14$	Control, $n = 50$	KW *P*-value
Phe (µm/L)	M ± SD	793 ± 415	145 ± 50	75 ± 13	
	Min-Max	300–2211	99.7–250	40–92.5	
	Median (IQR)	748 (465)[a,c]	115 (68)[b,c]	78 (14)	< 0.05
Tyr (µm/L)	M ± SD	48 ± 14	40 ± 13	56 ± 12	
	Min-Max	23–85	23–65	30–75.6	
	Median (IQR)	46 (25)[a,c]	39 (22)[b,c]	59 (18)	< 0.05
P/T ratio	M ± SD	17 ± 8	4 ± 2	1 ± 0	
	Min-Max	4.1–36.2	2.1–10.2	1–1.7	
	Median (IQR)	16 (10)[a,c]	4 (2)[b,c]	1 (0)	< 0.05
Cit	Mean ± SD	69.03 ± 33.39	37.01 ± 3.03	16.04 ± 9.71	
	Min-Max	32–188	32–42	2–34	
	Median (IQR)	62.5 (41)[a,b,c]	37 (5)	13.2 (13)	< 0.05
Orn	Mean ± SD	201 ± 88	101 ± 67	145 ± 81	
	Min-Max	33–348	23–230	29–321	
	Median (IQR)	213 (151)[a,b]	103.5 (100)	138.5 (115)	< 0.05
Met	Mean ± SD	10.03 ± 6.71	2.61 ± 1.93	6.84 ± 5.29	
	Min-Max	1–23	0.2–8	1.7–23	
	Median (IQR)	8.5 (11.87)[a,b,c]	2 (1.3)	4.95 (3.4)	< 0.05
Arg	Mean ± SD	3.15 ± 1.62	5.19 ± 1.53	17.63 ± 10.16	
	Min-Max	0–8.2	2.7–7.3	3.8–34	
	Median (IQR)	2.8 (2.6)[b]	5.2 (2.1)[a,c]	17.45 (20)	< 0.05
Val	Mean ± SD	32.89 ± 16.47	25.91 ± 20.36	76.43 ± 47.82	
	Min-Max	12–65	2–76	3–192	
	Median (IQR)	30 (23)[b,c]	22.2 (25)	66 (59)	< 0.05
Leu	Mean ± SD	19.82 ± 6.29	51.14 ± 6.99	154.82 ± 53.60	
	Min-Max	3–32	39–63	56–249	
	Median (IQR)	21 (8)[b]	52 (10)[a,c]	157.5 (93)	< 0.05

Non-parametric, Kruskal-Wallis, and Mann-Whitney tests were used to detect the difference across the studied groups, with a *P*-value < 0.05 indicating a statistically significant difference in the median rank presented as median (IQR). Other parametric descriptive statistics such as mean, standard deviation, minimum, maximum, and range are also displayed in the table

Phe phenylalanine, *Tyr* tyrosine, *Phe/Tyr* phenylalanine and tyrosine ratio, *Cit* citrulline, *Orn* ornethine, *Met* methionine, *Arg* arginine, *Val* valine, *Leu* leucine, *IQR* interquartile range, *SD* standard deviation, *Min* minimum, *Max* maximum

[a] $P < 0.05$ in classic PKU vs. control

[b] $P < 0.05$ atypical PKU vs. control

[c] $P < 0.05$ classical PKU vs. atypical PKU

correlation analysis between both neo/biopterin and blood Phe, Tyr, and Phe/Tyr ratio. Both neo/biopterin had a significant positive correlation with Phe levels in classic PKU ($P < 0.001$); however, no similar correlation existed in the atypical PKU or control group ($P > 0.05$). Tyr had a significant correlation with neo/biopterin in atypical PKU only ($P < 0.001$), but neither the classic nor the control group showed such correlations ($P > 0.05$). Neopterin positively correlated with biopterin in all groups (all $P < 0.001$).

The sensitivity and specificity of neopterin and biopterin in diagnosing atypical PKU

The diagnostic status of a patient in this study counts on a gold standard derived from the result of two tests: the Phe level and BH4 loading test. It is assumed that these tests provide confident and unquestionable evidence that the inborn error of metabolism does or does not exist without debating the validity of using these parameters as gold standards. So, the patients were categorized as either having (PKU) or not having (control), then according to the BH4 loading test at a cutoff of 7µg/L as atypical or classic PKU. Table 5 displays the basis for classifying patients with the inborn error of metabolism into classic and atypical PKU, as well as the sensitivity and specificity of neo/biopterin in determining the status of the patients.

Table 3 Blood neopterin and biopterin among the enrolled group

	Statistic	Classic PKU, $n = 46$	Atypical PKU, $n = 14$	Control, $n = 50$	KW *P*-value
Neopterin (nm/L)	M ± SD	49 ± 13	4 ± 1	15 ± 3	
	Min-Max	20–71	3–6	8–22	
	Median (IQR)	48 (19)[a,c]	4 (1)[b,c]	16 (6)	< 0.05
Biopterin (nm/L)	M ± SD	50 ± 12	3 ± 1	15 ± 3	
	Min-Max	28–70	2–5	8–22	
	Median (IQR)	48 (20)[a,c]	3 (1)[b,c]	15 (5)	< 0.05

P-value < 0.05 indicates significance. Mann-Whitney *U* test comparison between the two groups. *P*-value < 0.05 indicates a statistically significant difference between the groups

PKU phenylketonuria, *M* mean, *SD* standard deviation, *Min* minimum, *Max* maximum, *IQR* interquartile range, *K* Kruskal-Wallis test comparison among all groups

[a] A significant difference between the healthy control and the classic PKU groups

[b] A significant difference between the healthy control and the atypical PKU groups

[c] A significant difference between the classical PKU and the atypical PKU groups

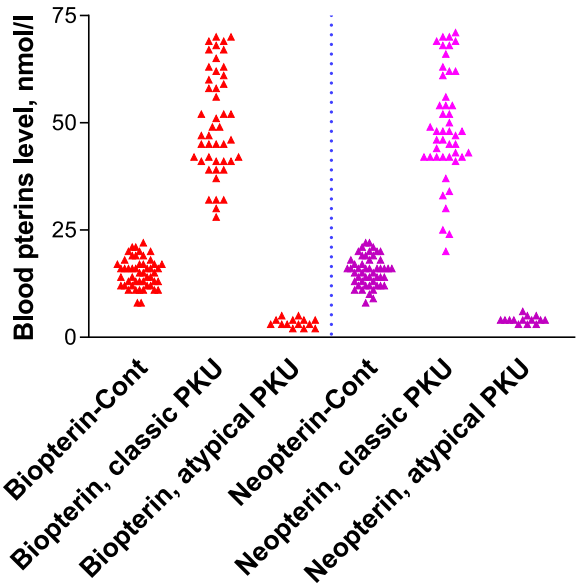

Fig. 2 Column scatter graph of the measured blood biopterin and neopterin levels in different groups

Association of LNAAs, neopterin, and biopterin in hyperphenylalaninemia

Table 6 displays the Spearman correlation analysis between either neopterin or biopterin and a profile of blood amino acids, which reflect the composition of LNAA used in the treatment of PKU. Citrulline, methionine, valine, and leucine did not show any significant correlation with either neopterin or biopterin in all groups (all $P > 0.05$). In the classic PKU, ornithine negatively correlated with biopterin ($r = -0.29$, $P < 0.05$), and arginine negatively correlated with neopterin ($r =$ *0.35, $P <$ 0.05). In atypical PKU, none of the amino acids correlated with either neopterin or biopterin (all $P > 0.05$); however, in the control group, only arginine positively correlated with biopterin ($r = 0.29$, $P< 0.05$).

Discussion

The study emphasized the value of simultaneous determination of neo/biopterin and a panel of amino acid profiles in the DBS and their potential as a non-invasive

Table 4 Correlation between biopterin, neopterin, and Phe in the studied groups

	Neopterin (nm/L)						Biopterin (nm/L)					
	Classic PKU, $n = 46$		Atypical PKU, $n = 14$		Control, $n = 50$		Classic PKU, $n = 46$		Atypical PKU, $n = 14$		Control, $n = 50$	
Cor. = *r* Sig = *p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Phe (μm/L)	0.62**	< 0.01	0.39	0.25	0.28*	0.05	0.64**	< 0.01	0.23	0.421	0.22	0.13
Tyr (μm/L)	0.21	0.15	−0.73**	< 0.01	0.22	0.13	0.18	0.24	−0.71**	< 0.01	0.20	0.16
P/T ratio	0.49**	< 0.01	0.76**	< 0.01	−0.08	0.56	0.56**	< 0.01	0.68**	< 0.01	−0.16	0.28
Biopterin	0.84**	< 0.01	0.88**	< 0.01	0.90**	< 0.01						

PKU phenylketonuria, *Phe* phenylalanine, *Tyr* tyrosine, *PT* phenylalnine to tyrosine ratio, *r* Spearman coefficient

**P*-value < 0.01 indicates a highly significant correlation

Table 5 The sensitivity and specificity of neopterin and biopterin in the classification of PKU into typical and atypical cases

		BH4 loading test		Neopterin		Biopterin	
Classic PKU (Phe > 600 µg/L)	Phe	+	−	Sen (%)	Spe (%)	Sen (%)	Spe (%)
	+	(46)[a]	(0)[b]	100	100	100	100
	−	(0)[c]	(50)[d]				
Atypical PKU (Phe < 120 µg/L)	Phe	+	−	Sen (%)	Spe (%)	Sen (%)	Spe (%)
	+	(14)[a]	(0)[b]	100	100	100	100
	−	(0)[c]	(50)[d]				

PKU phenylketonuria, *Phe* phenylalanine, *BH4* tetrahydrobiopterin, *Sen* sensitivity, *Spe* specificity

Sensitivity (%) = (TP/TP + FN) × 100; specificity (%) = (TN/TN + FP) × 100

[a] TP = true positive

[b] FP = false positive

[c] FN = false negative

[d] TN = true negative

Table 6 Correlation between various amino acids and either neopterin or biopterin in the classic and atypical hyperphenylalanemia

nmol/L	Classic PKU, *n* = 46				Atypical PKU, *n* = 14				Control, *n* = 50			
	Biopterin		Neopterin		Biopterin		Neopterin		Biopterin		Neopterin	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Cit	0.01	0.97	−0.01	0.96	−0.10	0.73	−0.14	0.63	0.09	0.56	0.14	0.33
Orn	−0.29*	0.04	−0.21	0.17	−0.38	0.18	−0.34	0.23	0.05	0.74	−0.08	0.59
Met	0.07	0.63	0.12	0.44	−0.15	0.62	−0.07	0.82	0.11	0.46	0.08	0.56
Arg	−0.21	0.17	−0.35*	0.02	−0.28	0.33	−0.19	0.51	0.29*	0.04	0.20	0.16
Val	0.00	0.98	−0.12	0.44	−0.04	0.90	−0.22	0.46	0.16	0.26	0.10	0.48
Leu	0.17	0.27	0.08	0.61	−0.16	0.59	−0.10	0.74	−0.27	0.06	−0.23	0.11

Spearman correlation between neopterin/biopterin and LNAAs

r Spearman coefficient, *PKU* phenylketonuria, *Tyr* tyrosine, *PT* phenylalanine to tyrosine ratio, *Cit* citrulline, *Orn* ornethine, *Met* methionine, *Age* arginine, *Val* valine, *Leu* leucine

**P*-value < 0.01 indicates a highly significant correlation

diagnostic tool for atypical cases of HPA due to BH4 deficiency. The pattern of increased Phe and reduced Tyr concentrations with elevated phenylalanine/tyrosine ratio characterizes all forms of HPA during newborn screening programs. Depending on the enzyme defect, genotype, and severity of the disease, different forms of PKU with different clinical phenotypes and classifications have been described [19]. Approximately 50% of cases are classical severe PKU, 30% are moderate PKU, and 20% are mild. Only 2% of the HPA have a deficiency in BH4 synthesis without mutation in the PAH gene [7, 20]. The study enrolled patients with the clinical phenotypes of either classic PKU (based on the detection of high blood Phe levels) or patients with atypical PKU with a milder elevation of Phe with evidence of BH4 deficiency by BH4 loading test. Although the BH4 loading test is a relatively old and subjective test, yet due to its simplicity, it is still used in screening and diagnosing atypical cases of PKU in all age groups and pregnant PKU women [21]. Several factors may compromise the interpretation of the test such as BH4 dose, age, sex, diet, and genotype of the patients. Generally, the diagnosis of BH4 deficiencies relies on the finding of an elevated blood Phe concentration with low pterins in the urine or blood and decreases in the enzymatic activity involved in the synthesis and regeneration of BH4 [9].

It is recommended that all infants undergo PKU screening within the first few days of life to enable timely dietary intervention and protect them from potential neurological damage. Ideally, the blood sample for screening is obtained between days 2 and 5, although it can be performed up to 7 days of age [22]. In this study, the majority of enrolled cases were diagnosed beyond the neonatal period, with only 1.8% identified through neonatal screening for PKU. The average age of presentation was 56.7 months in the classic PKU and 50.6 months in atypical PKU, very similar to previous studies involving Egyptian children [23, 24]. Despite PKU being an

autosomal recessive disorder, male predominance was observed in both the typical and atypical PKU groups, consistent with other studies conducted on Egyptian populations [23, 24]. However, such findings of male predominance may not fully represent the characteristics of the entire population, as the participants in this study were not randomly selected, and the sample size for the atypical PKU group was relatively small.

Most developed countries screen for PKU; however, slight information is available regarding the prevalence of PKU in the Middle East compared to other world regions. In the Arabic world, nine countries including Egypt, Bahrain, Kuwait, Oman, Qatar, the State of Palestine, Saudi Arabia, and the Emirates have extensive national neonatal blood screening programs. Other non-Arabic Middle Eastern countries include Turkey, Iran, and the Occupying Jewish State. It is apparent that PKU occurs more often in the Middle East than in other Western countries due to several factors such as the typical large family size and consanguinity [25].

Several studies conducted in different regions have reported a high prevalence of consanguinity among families with PKU. For example, a study in Jordan found parental consanguinity in 137 out of 151 families [26], while a study in the UAE reported 81.5% consanguinity among all detected metabolic disorders, including PKU [27]. Similarly, in Egypt, a study showed that 88% of patients with PKU were born to consanguineous parents [28]. Egypt has established a national newborn screening program for PKU, although limited publications are available estimating the prevalence of PKU in Egypt compared to countries like Saudi Arabia and the United Arab Emirates [29]. The lack of publications on the prevalence of PKU in Egypt can be attributed to various factors, including limited research focus, the absence of a national registry, underdiagnosis and underreporting, limited screening programs, data accessibility, and publication bias, as well as resource constraints.

The current study optimized a tandem mass spectrometry method to measure neo/biopterin and amino acids in DBS and validated it for linearity, the limit of detection, recovery, quantitation, accuracy, and precision. The tandem mass spectrometry detected a mildly increased level of Phe in all atypical PKU cases, associated with a decrease in the level of neo/biopterin. None of these atypical PKU cases had high neo/biopterin levels, which means the sensitivity of the method was 100% (true-positive atypical PKU are patients with mildly increased Phe with low neo/biopterin) and ensures that all patients will be identified if the test is used in the diagnostic workup or during screening for atypical PKU. The false-positive rate was 0 because all patients were preselected to have the disease, and the probability of having an individual with a normal or mildly elevated Phe level associated with very low neo/biopterin does not exist. Similarly, all cases of classic PKU showed moderate to severe increases in Phe levels associated with high levels of neo/biopterin. None of these hyperphenylalanemia patients had a decreased level of neo/biopterin, and thus, all patients fulfilled the criteria to be categorized as true-positive cases of classical PKU (true-positive classic PKU are patients with mildly to severely increased Phe with a high level of neo/biopterin) and a sensitivity of 100% is ensured. False-positive cases cannot be assumed because all patients were preselected to have the disease, and the probability of having a healthy individual with a very high Phe level does not exist. The control group represents the true-negative cases with normal neo/biopterin and Phe levels. The assay method did not classify any control as having an increased neo/biopterin, so all patients were true-negative, and the specificity of 100% is considered. The false-negative rate is 0 (individuals with normal to moderate increase in Phe and low pterins) which ensures that no incorrectly false-negative case will be rolled in if the assay is used in a screening program. The high sensitivity and specificity of the tandem mass spectrometry method make it an appropriate screening method for PKU and workup for diagnosing atypical PKU as it is fast, reliable, precise, and accurate.

The findings of this study align with existing research in both experimental and clinical settings [6]. In clinical practice, the measurement of neopterin and biopterin levels in the blood by mass spectrometry can aid in diagnosing atypical PKU caused by BH4 deficiency. However, there may be some overlap between pathologically low values in BH4-deficient patients and lower normal range values in classic PKU patients. In such cases, genetic evaluation and assessment of PAH enzymatic activity in liver and kidney tissues may be necessary [30]. Early screening for BH4 deficiency by measuring neopterin and biopterin levels in newborns allows for the detection of BH4 deficiency before irreversible cognitive damage occurs. Therefore, any newborn with phenylalanine levels exceeding 120 μmol/L should undergo BH4 screening. The correlation between neopterin and biopterin suggests that these biomarkers can serve as indicators of disease severity and can be used to monitor treatment response in both classic and atypical PKU [31].

The present study found that both the classic and atypical PKU had a significantly decreased level of methionine, arginine, valine, and leucine; nevertheless, the classic PKU showed a significant increase of citrulline, ornithine, methionine, arginine, leucine, and valine than atypical PKU. Correlation analysis between neo/biopterin and blood amino acids showed that in the classic PKU, ornithine negatively correlated with biopterin, and arginine

negatively correlated with neopterin. Due to the dynamic turnover of the amino acid metabolic pool in the presence of an abnormally high phenylalanine level, a specific pattern or relationship between these amino acids and biopterin/neopterin is not evident. Nevertheless, in such a context, the amino acids profile could be viewed as an adding tool when therapy with large neutral amino acid supplementation is considered [14, 19, 32, 33].

The primary objective was to demonstrate the sensitivity and specificity of neo/biopterin in a diagnostic laboratory workup for atypical PKU and its applicability to the day-to-day routine setting. The discriminative role of the other amino acids that are not directly causing PKU requires further analysis and validation in a larger population with extensive studies to outline the normative ranges of these amino acids in different groups.

Limitations of the study include the relatively small sample size of the atypical cases of PKU due to the low incidence of rare conditions all over the country. The study did not control for dietary intake on the diagnosis. Also, lack of liver biopsy, enzymatic assays, and genetic profiles to confirm the diagnosis and differentiate between different classes of atypical PKU.

Conclusions

The use of tandem mass spectrometry for quantitative measurement of neo/biopterin in DBS represents an accurate, sensitive, time and cost-effective means for early discovery of BH4 deficiencies in neonates and infants presented with atypical PKU. The amino acid profile could provide an additional value when treatment with the large neutral amino acids supplementation is considered a therapeutic modality in atypical PKU patients.

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Authors' contributions
NAS and GAG designed the experiments, collected the sample and patients' data, and optimized and revised the UPLC-MS method. MAO contributed to the protocol development. RG and OZ contributed to the clinical diagnosis and patient evaluation. AK is the corresponding author and is responsible for writing and editing the manuscript. All authors reviewed and approved the manuscript.

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Declarations

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The research follows the ethical standards of the Helsinki Declaration. The IRB of the NLI permitted the protocol (IRB 00134/2018 INTM), with oral or written consents obtained from all contributors.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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