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Determination of Neopterin and Biopterin in Dried Blood Spot by Tandem Mass Spectrometry in Classic and Atypical Hyperphenylalaninemia

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ABSTRACT

Background: Tetrahydrobiopterin is a coenzyme of phenylalanine hydroxylase and other enzymes essential for the synthesis of tyrosine and other neurotransmitters. Despite proper nutritional regulation of blood phenylalanine levels, tetrahydrobiopterin deficiency results in progressive neurologic illness.

Objective: The study aims to optimize a mass spec-dependent assay for quantitative measurement of biopterin and neopterin as distinctive markers of the atypical phenylketonuria due to tetrahydrobiopterin deficiency.

Patients and methods: The study enrolled 46 patients with typical hyperphenylalaninaemia, 14 atypical cases, and 50 healthy children as a control group. Quantitative measurements of biopterin, neopterin, phenylalanine, and tyrosine were performed by ultra-performance liquid chromatography-mass spectrometry in the dried blood spots of patients and control samples.

Results: Validation of the analytical protocol was performed at a biopterin and neopterin concentration range from 0 to 100 nmol/l. The regression coefficients for the linearity of the calibration curves exceeded 0.98. The lower limit of detection of biopterin and neopterin ranged from 1.5 to 2.5 nmol/l. The intra-day and inter-day precision and accuracy ranged from 96% - 105%, and from 97% - 110%. The mean recoveries were 105 ± 7% for biopterin and 106 ± 9% for neopterin. The short and long-term stability of the stored samples was seven days at room temperature and 12 weeks at -20 °C. Biopterin and neopterin were significantly higher in the classic group compared to the atypical and control group ($p < 0.05$), with their level being in the order classic > control > atypical group. Phenylalanine levels had a significant positive correlation with biopterin and neopterin levels in classic phenylketonuria ($p < 0.05$).

Conclusions: The performance of the developed assay for biopterin and neopterin in the dried blood spot by ultra-performance liquid chromatography-mass spectrometry was accurate and precise thus, provide a legitimate diagnostic tool for cases of atypical phenylketonuria.

Keyword: Tetrahydrobiopterin, Neopterin, Biopterin, Hyperphenylalaninemia, Liquid chromatography-mass spectrometry.

INTRODUCTION

Tyrosine (tyr), a precursor to the neurotransmitters dopamine and norepinephrine in the central nervous system, is in insufficient supply if phenylalanine (Phe) cannot be converted to tyrosine (tyr). Because of a shortage or absence of the essential amino acid phenylalanine (Phe), phenylketonuria (PKU) is an autosomal recessive illness. Tetrahydrobiopterin (BH4), a cofactor for the enzyme phenylalanine hydroxylase (PAH), causes the condition known as atypical PKU [1].

If PKU is not treated, it leads to gradual and permanent brain damage. During the first few years of life, the brain is particularly vulnerable to the neurotoxic effects of increased Phe. Elevated Phe disrupts brain tissue's myelination, synaptic sprouting, and dendritic pruning [2]. Preventing mental retardation and serious neurological consequences such seizures and spasticity is possible with PKU screening, diagnosis, and therapy during the newborn period [3-6].

The most prevalent inborn metabolic mistake, PKU, is most prevalent in Ireland, Eastern Europe, and North America [1]. Based on the plasma Phe level upon diagnosis, PKU is divided into three categories: classic PKU (plasma Phe >1200 mol/l), moderate PKU (plasma Phe 600-1200 mol/l), and mild PKU (plasma Phe 120-600 mol/l). About 50% of instances of PKU are classified as classical, 30% as moderate, and 20% as mild. Only 2% of those with hyperphenylalaninemia had a PAH gene mutation rather than a deficiency in BH4 production or recycling [7].

Five separate hereditary disorders that mirror PKU with increased phenylalanine levels and the potential for neurological damage despite early treatment are caused by the inability to synthesis or renew the PAH cofactor BH4. Without the requirement for a Phe-restricted diet, the administration of synthetic BH4 quickly returns plasma Phe levels to normal [8].

Patients with hyperphenylalaninemia (HPA) experience a depletion of catecholamines and serotonin because BH4 is a necessary cofactor for PAH, tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase in addition to all three isoforms of nitric oxide synthase (NOS), the key enzymes in the biosynthesis of dopamine and serotonin [9–12].

Although sufficient dietary control of blood phenylalanine levels is maintained, BH4 deficiency is a serious condition that causes gradual neurologic deterioration. BH4 is a possible therapeutic target for

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some PKU patients, especially those that are also accompanied by BH4 deficits [13].

Therefore, even in newborns with modestly higher blood Phe levels, the capacity to differentiate between BH4 abnormalities and PAH shortages is essential [14–17].

Tandem mass spectrometry is the method of choice for determining Phe, neopterin, and biopterin concentrations. Enzymatic-colorimetric assays (ECA), bacterial inhibition assays (BIA), and high-performance liquid chromatography (HPLC) are further techniques for detecting Phe [18]. Neonates with PKU might benefit from a useful diagnostic tool by having neopterin and biopterin levels measured by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) in dried blood spots or plasma during PKU screening [19, 20].

AIM OF THE WORK

Optimize and establish a UPLC-MS-dependent assay for measuring biopterin and neopterin in the dried blood spot as a diagnostic tool for the atypical cases of phenylketonuria due to tetrahydrobiopterin (BH4) deficiency.

PATIENTS AND METHODS

The study was carried out in the Departments of Clinical Biochemistry and Molecular Diagnostics and Department of Pediatric Hepatology, National Liver Institute, Menoufia University, and the Department of Human Genetics, Ain Shams University from March 2018 to March 2021.

The study included three groups of patients. The classic PKU (c-PKU) group enrolled 46 patients (28 males and 18 females), with a mean age of 58±35 months, diagnosed by extended metabolic screening and laboratory documentation of HPA> 1200 ng/ml by tandem mass spectrometry. The atypical PKU (a-PKU) group enrolled 14 pediatric patients (8 males and 6 females) with a mean age of 50 months with their blood Phe level < c-PKU level.

The a-PKU diagnosis based on oral tetrahydrobiopterin and phenylalanine loading test. When initial plasma Phe concentrations fall by at least 30% after 8 hours or by 50% after 24 hours, the BH4 loading test is deemed positive [8–11].

The normal healthy control (NHC) group enrolled 50 healthy participants, matching the other groups in terms of age and gender, with no clinical, laboratory, or imaging evidence of PKU. NHC was also free from other metabolic, systemic or chronic liver diseases.

All participants were evaluated clinically and by analysis of samples obtained by sterile venipuncture technique. Hematological and chemical parameters including liver function tests and renal function tests were performed by the Beckman Coulter (AU480) (Beckman Instruments, Fullerton. CA, USA). Complete blood count (CBC), bleeding time, INR by Coulter counter T660 (Coulter electronics, hielaech, fl., USA). Amino acid phenylalanine, tyrosine, biopterin and neopterin by ultra-performance liquid chromatography-mass spectrometry (Waters UPLC-MS/MS, USA).

Reagents and standards

Thermofisher supplied HPLC quality methanol, acetonitrile, hydrocloric acid, and formic acid. Millipore pure water purification equipment (Diamond T, USA) was used to get HPLC grade water. Sigma-Aldrich also sold pterin standards such as biopterin and neopterin (Merck KGaA, Darmstadt, Germany).

Sample preparation and instrument settings

Whole blood was drawn from patients and the control group onto a Guthrie card made of Whatman 903 filter paper purchased from (GE Healthcare in New Jersey, USA). The card was then dried for 4 hours at room temperature on a dry, horizontal, and non-absorbent surface before being stored at 20 °C in vacuum-tight sealed plastic bags. The dried blood spot (DBS) was punched with four 6-mm diameter punches, placed in a vial with 150 µl of 20 mmol/l HCl, and sonicated in an Elma Transsonic T/780 bath (Singen, Germany) for ten minutes. The sample was then fed into the LC-MS/MS apparatus after the extracted solution had been centrifuged at 12000 rpm for 15 min [21].

The triple-quadruple tandem mass spectrometer (ACQUITY UPLC H-Class. Waters Corporation, MA, USA) and positive electrospray ionisation probe made comprised the LC-MS/MS system. The analytical column was a Waters ACQUITY UPLC HSS T3 C18, 1.7 m, 2.1x50 mm column, at a temperature of 50°C. Biopterin and neopterin were eluted using a gradient of 0.3 ml/min flow rate, 95% mobile phase A (0.2 formic acid), and 5% mobile phase B (methanol), followed by a linear gradient of mobile phase B (90%) over 3.5 min and mobile phase B (10) over 4.5 min. For six minutes, 98% mobile phase A was used to equilibrate the column before the next sample was injected. Continuous infusion of pure standards (5 µmol/l) at a flow rate of 10 µl/min was maintained in order to optimise the technique and parameter settings to produce the highest production signals and the [M + H] + signal's maximum signal [20].

The source's capillary voltage was running at 0.7 kV and 150°C at the time. A gas station (Peak Scientific Instruments, Scotland) generated the nitrogen used as desolvation and cone gas, while argon was utilised as collision gas. The collision cell gas flow was set at 0.15 ml/min, while the desolvent gas flow was set at 900 l/h and 600 °C. Two-time window multiple reactions monitoring was used for the MS/MS investigations [21, 22].

Calibration curves and method assessment

Validation of the detection method for the following parameters: linearity, the lower limit of detection (LLOD), and quantitation (LLOQ), precision, accuracy, and stability were established before running the

patient's samples. Linearity was evaluated with a freshly prepared 9 standards points of biopterin and neopterin mixture at a concentration of (0, 1.25, 2.5, 5, 10, 20, 40, 80, 100 nmol/l). Plotting the peak area of biopterin and neopterin vs nominal concentrations was done to create the calibration curves. For standard solutions, the resulting curves were linear across the concentration range of 0 - 100 nmol/l. The calibration curve's lowest point, known as the LLOD, has a signal-to-noise ratio (S/N) > 3.

The LLOQ is the lowest point on the calibration curve whose S/N ratio is > 10, and its measured value is ≤ 20% of the nominal value. The limit of detection for biopterin and neopterin in the assay ranged from 1.5-2.5 nmol/l. The regression coefficients for linearity of the calibration curves exceeded 0.99 **(Figure 1).**

To control for the matrix effect during the assay, five DBS samples were spiked with a mixture of biopterin and neopterin standards to yield final concentration of 0, 5, 10, 20, and 100 nmol/l, then dispensed into filter paper, dried, and stored at -20 °C. Samples were then processed and checked for the same calibration parameter to ensure that the blood matrix do not interfere with the proper quantitative measurement of the samples.

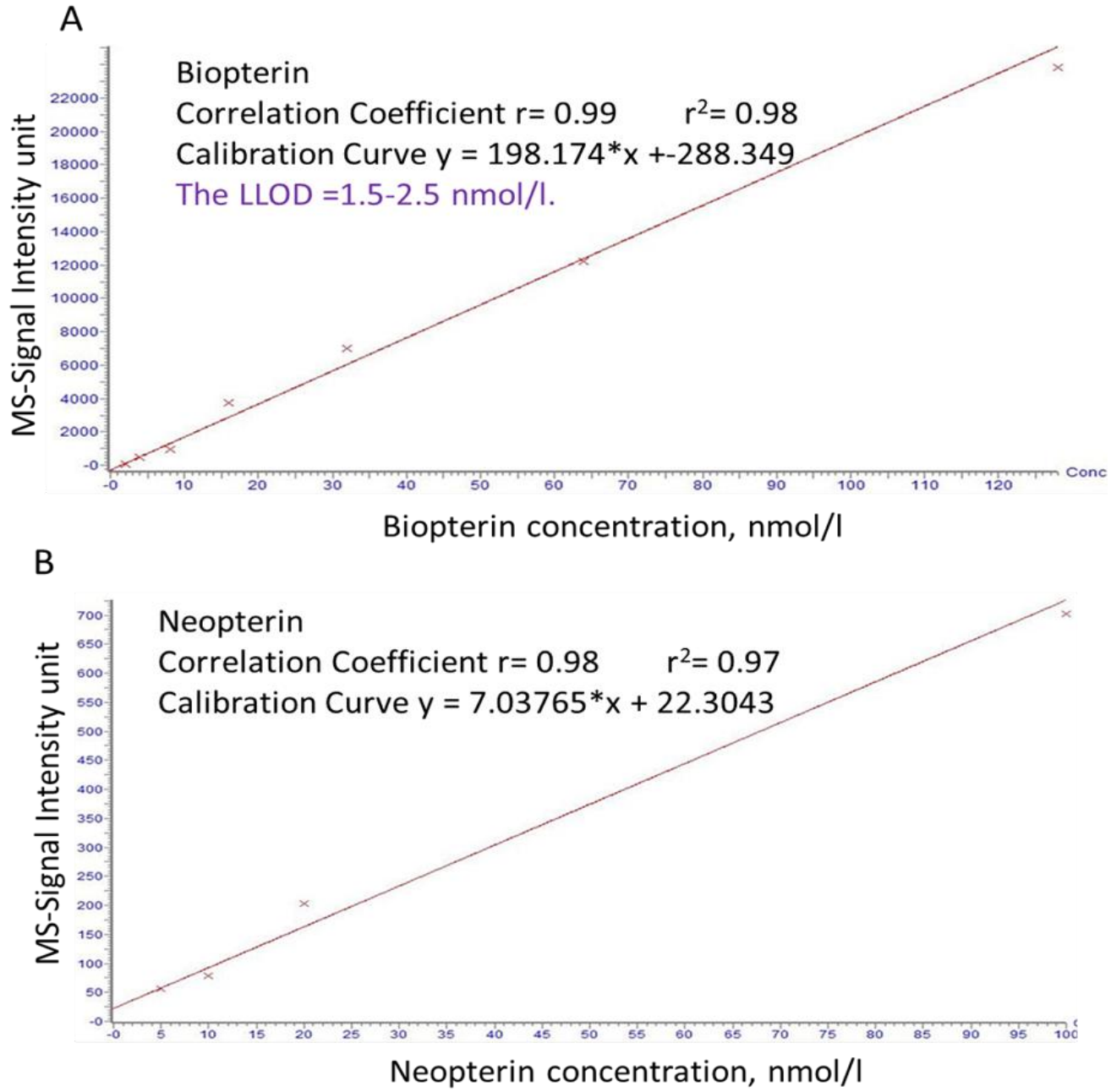

Figure (1): Analysis of linear regression between observed and predicted concentrations: Analysis was assessed by plotting the actual value of the 10-standard containing biopterin and neopterin at the concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80, 100 nmol/l)

The inter-day and intra-day performance of the method.

To determine the intra-day and inter-day' precision and accuracy of the assay method, the lowest 2.5 nmol/l and the highest 100 nmol/l concentrations were assessed in quadruplicates on the same day and on three successive days. The percentage relative error (RE) [% (measured-theoretical)/theoretical concentration] was used to calculate accuracy.

The relative standard deviation (%RSD =% standard deviation /mean) was used to calculate precision. Intra-day and inter-day precision for all matrices evaluated were <15%. Inter-day and intra-day accuracy and precision for all matrices ranged from 96.6% - 105.6%, and from 97.6% to 110.4% **(Table 1).**

The stability was assessed with samples spiked with the low (2.5 nmol/l) and the high (100 nmol/l) standards; 7 days at room temperature (RT), 4 weeks at 4 °C, and 8-12 weeks for long-time storage at -20 °C. The stability test for biopterin and neopterin showed no significant change in the concentration of these analytes at the different storage conditions.

By contrasting spiked and unspiked blood spots made from the same entire blood pool, recovery was assessed. The average recoveries for biopterin were 105±7% and for neopterin they were 106±9%. The assay performance of the developed UPLC-MS/MS protocol was accurate and precise for measuring biopterin and neopterin in the human blood **(Table 1).**

Table (1): Performance of the optimized protocol for quantitative detection of biopterin and neopterin

Analyte	Actual (nmol/l)	Measured (nmol/l) M±SD	Inter-days (n=10)		Intra-day (n=10)		Recovery (%) (nmol/l) M±SD
			Precision CV%	Accuracy	Precision CV%	Accuracy	
Biopterin	0	0.9±0.05	1.68	102.6	2.04	102.5	
	2.5	2.6±0.2	10.8	105.64	9.86	110.2	105±5.6
	20	24.3±3.8	6.82	98.7	7.23	101.3	108±9
	100	96.9±4.9	5.16	96.95	6.23	97.95	96±3
Neopterin	0	1.19±0.3	2.95	112.3	3.16	103.2	
	2.5	2.76±0.15	5.51	110.4	6.21	107.3	105.9±5.9
	20	18.7±5.3	6.3	105.6	5.82	99.6	112±7
	100	97.4±1.62	6.38	97.88	5.72	101.4	97.46±2.5

M: mean, SD: standard deviation, CV coefficient of variation

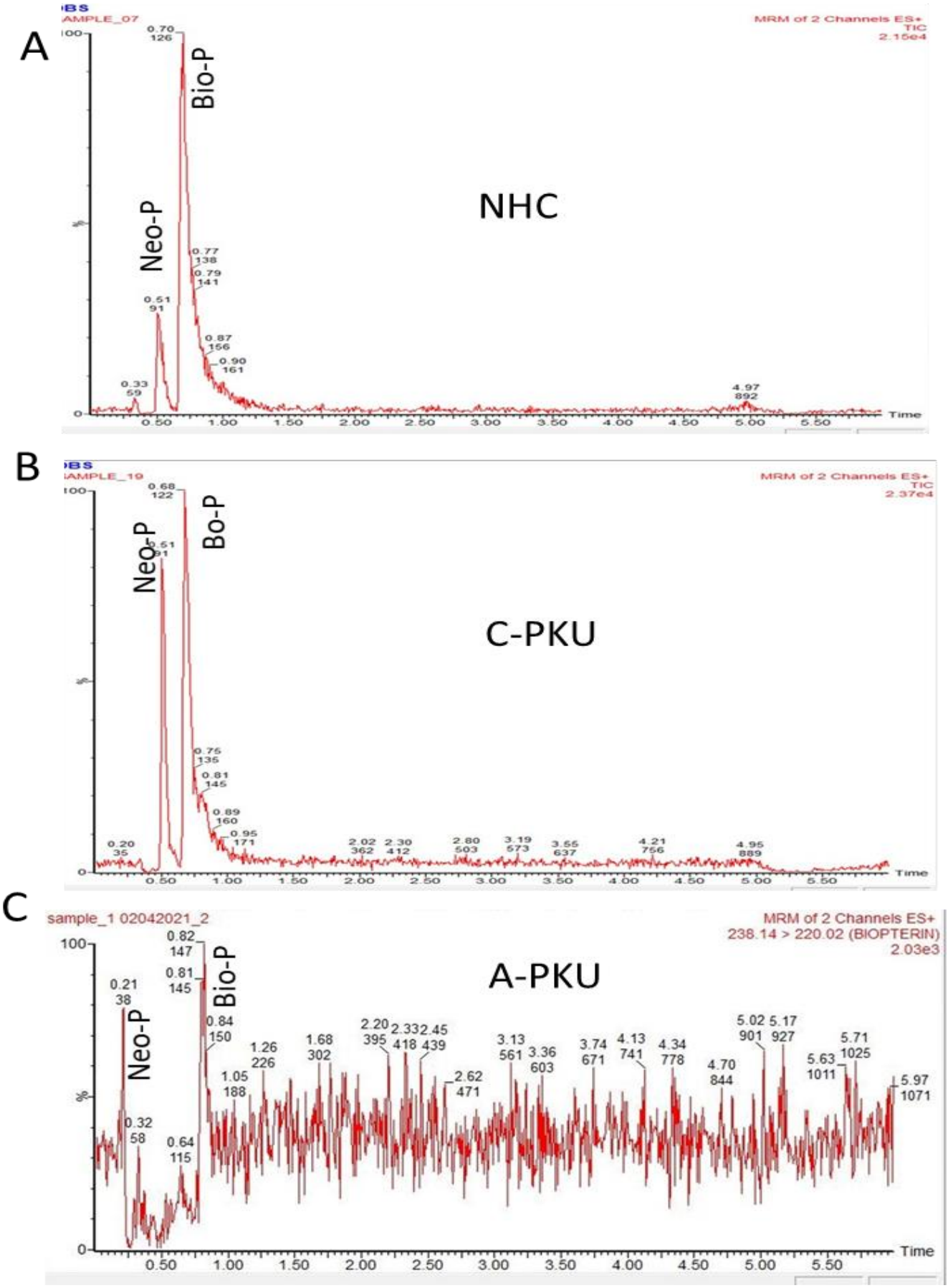

Figure (2): Chromatogram showing the detected peaks of neopterin and biopterin in **(A)** NHC, **(B)** C-PKU, and **(C)** A-PKU. Neop: neopterin, Biop: biopterin, NHC: normal healthy control, C-PKU: classic phenylketonuria, A-PKU: atypical phenylketonuria.

Ethical consent:

The Ethical Institutional Review Board at Menoufia University approved the study. Informed written consent was obtained from parents of all children participants before recruitment in the study, after explaining the objectives of the work. This study was conducted in compliance with the code of ethics of the world medical association (Declaration of Helsinki) for human subjects.

Statistical analysis

SPSS 23 was used to analyse the data (SPSS Inc., CA, USA). Quantitative information was presented as mean±SD, range, median, and interquartile range (IQR). To determine the significance of multiple comparisons, the non-parametric statistical Kruskal Wallis test and the Mann-Whitney test were utilised. In order to evaluate the correlations between the various factors, Spearman correlation analysis was utilised. Frequencies and relative percentages were used to depict qualitative data. To determine differences between two or more sets of qualitative variables, chi square test (X^2) was used. P value less than 0.05 was regarded as significant.

RESULTS

The demographic and clinical characteristics of the different groups:

The demographic and the neurological clinical manifestation of the C-PKU and A-PKU groups are presented in **Table 2.** The enrolled groups were homogenous regarding weight, height, body mass index, and paternal consanguinity, without significant differences across the groups (all p >0.05). Neurological manifestations such as delayed growth (DG), delayed mental development (DMD), delayed learning, speech and hearing (DLSPH), lack of concentration (LOC), seizure, and mental retardation (MR) were the common clinical finding in C-PKU. In A-PKU milder form of these findings were observed. The biochemical and hematological parameters of the enrolled groups are displayed in **Table 3.** There was no significant difference between all studied groups regarding chemical and hematological parameters (all *P*>0.05).

Table (2): Demographic and clinical characteristics of the different groups

	C- PKU N=46	A-PKU N=14	NHC, N=50	**P-value**
WT (kg) Min. – Max. Mean ± SD. Median (IQR)	 3-36 22.5±10.77 25(31-15.625)	 6-35 21.87±8.8 24(30-15)	 3-35 16.08±9 13.5(24- 8)	> 0.05
HG(cm) Min. – Max. Mean ± SD. Median (IQR)	60-115 96.21±16.4 98.5(106-93)	65-130 100.5±13.8 100(110-93.75)	60-120 93.9±19.47 100(110-78.75)	> 0.05
BMI Min. – Max. Mean ± SD. Median (IQR)	8-32 22.2±7.42 24.8(28-17)	12-32 20.69±5.75 19.8(25-16.4)	16.6±4.45 7-28 16.3(19.4-13)	> 0.05
Age (months) Min. – Max. Mean ± SD. Median (IQR)	 2.0-132 57.9±35.7 60(126-38)	 1.5-108 50.7±32.8 48(108-46)	 1.0-132.0 43±40.2 36(132-48)	> 0.05
Gender	N (%)	N (%)	N (%)	
Male Female	28(60) 18(40)	8(57) 6(43)	26(52) 24(48)	> 0.05
Consanguinity Positive Negative	 36(87) 10(13)	 14(100) 0(0)	 14(31) 36(69)	<0.05
Neurological manifestations	N (%)	N (%)	N (%)	
DG	2(4.3)	3(21.4)	0	
DMD	13(28.3)	4(28.6)	0	
DSPH	10(21.7)	1(7.1)	0	
LOC	18(39.1)	6(42.9)	0	

NHC: Normal healthy control, C-PKU: Classic phenylketonuria, A-PKU: Atypical phenylketonuria, DG: Delayed growth, DSPH: Delayed speech, DMD: Delayed motor development, LOC: Lack of concentration, HG: Height, WT: Weight, BMI: Body mass index, Min: Minimum, Max: Maximum, SD: Standard deviation, IQR: Interquartile range.

Table (3): Chemical and hematological parameters across the different groups

Studied variables	NHC, N=50	C-PKU, N=46	A-PKU, N=14	P-value
AST(u/l) Mean ± SD	22.58±5.09	19.6±4.99	19.5±4.16	> 0.05
ALT(u/l) Mean ± SD	22.32±5.41	19.8±3.23	20.4±3.54	> 0.05
TB(mg/dl) Mean ± SD	0.93±0.18	0.87±0.12	0.9±0.14	> 0.05
DB(mg/dl) Mean ± SD	0.27±0.04	0.22±0.01	0.23±0.08	> 0.05
CR(mg/dl) Mean ± SD	0.3±0.03	0.37±0.02	0.36±0.01	> 0.05
Urea(mg/dl) Mean ± SD	13.26±2.87	14.2±3.6	12.7±2.05	> 0.05
HB(mg/dl) Mean ± SD	11.07±1.06	10.9±1.13	10.65±0.99	> 0.05
RBCs Mean ± SD	5.08±0.5	5±0.44	4.9±0.5	> 0.05
Platelets Mean ± SD	312.7±61.7	336.8±61.9	339.7±59.4	> 0.05
BT (min) Mean ± SD	6.49±1.41	5.8±1.31	6.47±1.44	> 0.05
INR Mean ± SD	1.008±0.15	1.04±0.17	1.21±0.19	> 0.05

AST: Aspartate transaminase, ALT: Alanine transaminase, TB: Total bilirubin, DB: Direct bilirubin, CR: Creatinine, HB: Hemoglobin, RBCs: Red blood cells, BT: Bleeding time, INR: International normalized ratio, SD: Standard deviation

Blood biopterin, neopterin, Phe, Tyr, and Phe/tyr ratio in the enrolled groups:

Figure 3 and table 4 present the blood biopterin, neopterin, Phe, tyr, and Phe/tyr ratio in different groups. Biopterin and neopterin were significantly higher in the C-PKU than A-PKU and NHC groups, with their level being in the order C-PKU> NHC> A-PKU. Phenylalanine levels had a significant positive correlation with biopterin and neopterin levels in classic PKU **(Table 5).**

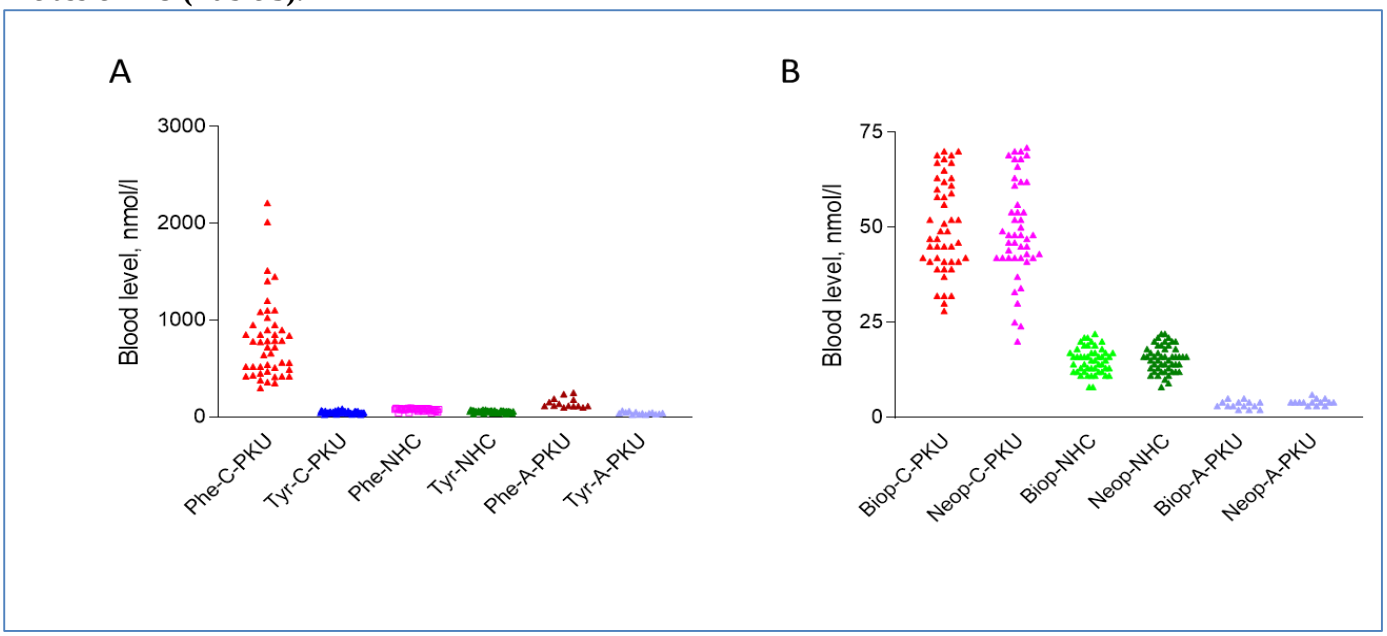

Figure (3): Phe, Tyr, biopterin and neopterin levels in different groups: Column scattered graph of the measured blood (A) Phe and Tyr. (B) Biopterin and neopterin

NHC: Normal healthy control, C-PKU: Classic phenylketonuria, A-PKU: Atypical phenylketonuria, Biop: Biopterin, Neop: Neopterin.

Table (4): Blood biopterin, neopterin, Phe, Tyr, and Phe/Tyr ratio in the enrolled group

Studied variables	NHC, N=50	C- PKU N=46	A-PKU N=14	K P-value	MW U test
Biopterin (nm/L) Mean ± SD	14.96±3.29	50.1±12.18	3.16±0.61	P < 0.05	P1 < 0.01 P2 <0.01 P3 < 0.01
Neopterin(nm/L) Mean ± SD	15.4±3.34	49.35±12.9	3.99±0.84	P < 0.05	P1 < 0.01 P2 < 0.01 P3 < 0.01
Phe (µm/L) Mean ± SD	74.56±13	793.4±90.6	144.6±32.9	P < 0.05	P1 < 0.01 P2 < 0.01 P3 < 0.01
Tyr (µm/L) Mean ± SD	55.6±11.86	47.9±10.9	39.92±8.2	P < 0.05	P1 < 0.01 P2 < 0.01 P3 < 0.01
P/T ratio Mean ± SD	1.36±0.17	17.12±4.01	4.05±0.91	P < 0.05	P1 < 0.01 P2 < 0.01 P3 < 0.01

NHC: Normal healthy control, C-PKU: Classic phenylketonuria, A-PKU: Atypical phenylketonuria, Phe: Phenylalanine, tyr: Tyrosine, PT: Phenylalnine to tyrosine ratio, Min: Minimum, Max: Maximum, SD: Standard deviation, IQR: Interquartile range, K: Kruskal Wallis test comparison among all groups. Mann-Whitney U test comparison between two groups, P1: comparing between NHC and C-PKU group. P2: comparing between NHC and A-PKU group. P3: comparing between C-PKI and A-PKU group.

Table (5): Correlation between biopterin and neopterin, Phe, tyr, levels and P/T ratio across the studied groups

Group		Cor/sig	phe(µm/)	tyr(µm/)	P/T ratio	neopt(nm/)
C- PKU, n=46	biopt(nm/L)	r	0.637	0.176	0.557	0.840
		p	<0.001	0.242	<0.001	<0.001
A- PKU, n=14	biopt(nm/L)	r	0.234	-0.710	0.684	0.881
		p	0.421	0.004	0.007	<0.001
NHC	biopt(nm/L)	r	0.216	0.204	-0.156	0.902
		p	0.133	0.156	0.279	<0.001

NHC: Normal healthy control, C-PKU: Classic phenylketonuria, A-PKU: Atypical phenylketonuria, Phe: phenylalanine, tyr: tyrosine, PT: Phenylalanine to tyrosine ratio, r: Spearman coefficient.

DISCUSSION

The present study aimed to establish a UPLC-MS/MS-dependent analytical protocol for measuring biopterin and neopterin in DBS as a simple laboratory test to differentiate the c-PKU from a-PKU patients that usually miss early detection and diagnosis. The analytical process examined both intra-day and inter-days linearity, precision, recovery, the limit of detection and quantitation by examining the linear connection between the obtained concentration of solutions containing biopterin and neopterin and the known concentration of labelled external standards.

Over a concentration range of 0-100 nmol/l, the standard point curves that were produced were linear. The LLOD for biopterin and neopterin in this assay ranged from 1.5-2.5 nm/l, which indicated a sensitive method to detect a-PKU. The higher sensitivity enables precise determination of biopterin and neopterin even at minute concentration, thus opening a door for a population-based reference range that help in screening larger size population and differentiating c-PKU from a-PKU [21].

The stability of the biopterin and neopterin in the stored DBS samples during screening for and identification of inborn error of metabolism is an important factor. Laboratories specializing in inborn errors of metabolism pay attention to short-term and long-term storage conditions. The results of the optimized protocol showed that the concentration of biopterin and neopterin remained mostly unchanged until 12 days at RT or for long-time storage at -20 °C. These data agree with previously published protocols in the literature that showed in the DBS, BH4 is fully oxidized to biopterin and neopterin, which are stable for up to 16 days at RT, a sufficient time to send samples to the laboratory [23, 24].

As a delayed diagnosis for BH4 disorders results in irreversible mental retardation, biopterin and neopterin analysis in DBS obtained in the first days of life enables an earlier diagnosis of 6-pyruvoyl tetrahydropterin synthase (PTPS) deficiency and

guanosine triphosphate cyclohydrolase-1 (GTP-CH) disorders [25-28]. Therefore, it is important to screen for a BH4 metabolic deficiency in every infant with HPA (phenylalanine levels greater than 120 μmol/l) during newborn screening [21].

Since the introduction of LC-MS/MS into laboratories involved in newborn screening for PKU, the advantage of simultaneous analysis of Phe and biopterin in the same sample collected DBS shortens the period for HPA diagnosis caused by BH4 deficiency [20]. In patients with PKU, the effects of supplying the protein substitute on the plasma concentrations of tyr and other amino acids are important nutritional conditions that require special follow-up. In those who don't have PKU, the ratio of Phe to Tyr is around 1:1, whereas in the PKU it typically ranges from 2.5:1 to 10:1 [20, 21].

During supplementation treatment for PKU with BH4, the majority of mild and moderate PKU cases show a satisfactory clinical response, nevertheless, only 10% of the c-PKU show such a response [29, 30]. These references explore the contentious questions of how, when, and who to test for BH4 response [22, 31].

The most popular laboratory test for detecting, diagnosing, and monitoring inborn mistakes in BH4 metabolism has evolved to be the detection of neopterin and biopterin in various bodily fluids [8-11, 23, 25, 32].

CONCLUSIONS

The application of UPLC-MS/MS for quantitative measurement of biopterin and neopterin in DBS of a screened newborn with hyperphenylalaninaemia is an accurate tool for early detection of tetrahydrobiopterin deficiencies in a-PKU for early diagnosis and treatment to prevent mental and neuro-motor mal-development.

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REFERENCES

1. **Waisbren S, Noel K, Fahrbach K *et al.* (2007):** Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis**.** Mol Genet Metab., (92)**:**63-70.
2. **Feldmann R, Denecke J, Grenzebach M *et al.* (2005):** Frontal lobe-dependent functions in treated phenylketonuria: blood phenylalanine concentrations and long-term deficits in adolescents and young adults**.** J Inherit Metab Dis., 28**:**445-455.
3. **Weglage J, Ullrich K, Pietsch M *et al.* (1997):** Intellectual, neurologic, and neuropsychologic outcome in untreated subjects with nonphenylketonuria hyperphenylalaninemia. German Collaborative Study on Phenylketonuria**.** Pediatr Res., 42: 378-384.
4. **Kure S, Shintaku H (2019):** Tetrahydrobipterin-responsive phenylalanine hydroxylase deficiency. J Hum Genet., 64:67-71.
5. **Subspecialty Group of Newborn Screening, Society of Birth Defects Prevention and Control, Chinese Preventive Medicine Association (2019):** Consensus statement on dietary treatment and nutritional management for phenylalanine hydroxylase deficiency. Zhonghua Er Ke Za Zhi., 57: 405-409.
6. **Yuskiv N, Potter B, Stockler S *et al.* (2019):** Nutritional management of phenylalanine hydroxylase (PAH) deficiency in pediatric patients in Canada: a survey of dietitians' current practices. Orphanet J Rare Dis., 14: 1-13.
7. **Williams R, Mamotte C, Burnett J (2008):** Phenylketonuria: an inborn error of phenylalanine metabolism. Clin Biochem Rev., 29: 31-41.
8. **Longo N (2009):** Disorders of biopterin metabolism. J Inherit Metab Dis., 32: 333-342.
9. **Eichinger A, Danecka M, Moglich T *et al.* (2018):** Secondary BH4 deficiency links protein homeostasis to regulation of phenylalanine metabolism. Hum Mol Genet., 27: 1732-1742.
10. **Vela-Amieva M, Abreu-Gonzalez M, Gonzalez-del Angel A *et al.* (2015):** Phenylalanine hydroxylase deficiency in Mexico: genotype-phenotype correlations, BH4 responsiveness and evidence of a founder effect. Clin Genet., 88: 62-67.
11. **Zhu T, Ye J, Han L *et al.* (2017):** The predictive value of genetic analyses in the diagnosis of tetrahydrobiopterin (BH4)-responsiveness in Chinese phenylalanine hydroxylase deficiency patients. Sci Rep., 7: 1-13.
12. **Ashe K, Kelso W, Farrand S *et al.* (2019):** Psychiatric and cognitive aspects of phenylketonuria: The limitations of diet and promise of new treatments. Front Psychiatry, 10: 561. doi: 10.3389/fpsyt.2019.00561
13. **Boneh A, Francis D, Humphrey M *et al.* (2006):** Three-year audit of the hyperphenylalaninaemia/phenylketonuria spectrum in Victoria. J Paediatr Child Health, 42: 496-498.
14. **Alibakhshi R, Moradi K, Mohebbi Z *et al.* (2014):** Mutation analysis of PAH gene in patients with PKU in western Iran and its association with polymorphisms: identification of four novel mutations. Metab Brain Dis., 29: 131-138.
15. **Rastegar Moghadam M, Shojaei A, Babaei V *et al.* (2018):** Mutation analysis of phenylalanine hydroxylase gene in Iranian patients with Phenylketonuria. Med J Islam Repub Iran, 32: 21. doi: 10.14196/mjiri.32.21
16. **Vieira Neto E, Laranjeira F, Quelhas D *et al.* (2018):** Mutation analysis of the PAH gene in phenylketonuria patients from Rio de Janeiro, Southeast Brazil. Mol Genet Genomic Med., 6(4): 575–591.
17. **Yan Y, Zhang C, Jin X *et al.* (2019):** Mutation spectrum of PAH gene in phenylketonuria patients in Northwest China: identification of twenty novel variants. Metab Brain Dis., 34: 733-745.
18. **Groselj U, Murko S, Zerjav Tansek M *et al.* (2015):** Comparison of tandem mass spectrometry and amino acid analyzer for phenylalanine and tyrosine monitoring--implications for clinical management of patients with hyperphenylalaninemia. Clin Biochem., 48:14-18.
19. **Jung-Klawitter S, Kuseyri Hubschmann O (2019):** Analysis of catecholamines and pterins in inborn errors of monoamine neurotransmitter metabolism-from past to future. Cells, 8(8):867. doi: 10.3390/cells8080867.

20. **Santagata S, Di Carlo E, Carducci C *et al.* (2017):** Development of a new UPLC-ESI-MS/MS method for the determination of biopterin and neopterin in dried blood spot. Clin Chim Acta., 466: 145-151.
21. **Zurfluh M, Giovannini M, Fiori L *et al.* (2005):** Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper. Mol Genet Metab., 86: 96-103.
22. **Blau N, Belanger-Quintana A, Demirkol M *et al.* (2010):** Management of phenylketonuria in Europe: survey results from 19 countries. Mol Genet Metab., (99):109-115.
23. **Blau N (2016):** Genetics of Phenylketonuria: Then and Now. Hum Mutat., 37: 508-515.
24. **Blau N, Longo N (2015):** Alternative therapies to address the unmet medical needs of patients with phenylketonuria. Expert Opin Pharmacother., 16: 791-800.
25. **Blau N, Hennermann J, Langenbeck U *et al.* (2011):** Diagnosis, classification, and genetics of phenylketonuria and tetrahydrobiopterin (BH4) deficiencies. Mol Genet Metab., 104: 2-9.
26. **Opladen T, Lopez-Laso E, Cortes-Saladelafont E *et al.* (2020)::** Correction to: Consensus guideline for the diagnosis and treatment of tetrahydrobiopterin (BH4) deficiencies. Orphanet J Rare Dis, 15: 1-5.
27. **Opladen T, Lopez-Laso E, Cortes-Saladelafont E *et al.* (2020):** Consensus guideline for the diagnosis and treatment of tetrahydrobiopterin (BH4) deficiencies. Orphanet J Rare Dis., 15: 126. doi: 10.1186/s13023-020-01379-8.
28. **Dayasiri K, Suraweera N, Nawarathne D *et al.* (2019):** GTP-cyclohydrolase I deficiency presenting as malignant hyperphenylalaninemia, recurrent hyperthermia and progressive neurological dysfunction in a South Asian child - a case report. BMC Pediatr., 19: 199. doi: 10.1186/s12887-019-1580-x.
29. **Mitchell J, Trakadis Y, Scriver C (2011):** Phenylalanine hydroxylase deficiency. Genet Med., 13: 697-707.
30. **Moat S, Schulenburg-Brand D, Lemonde H *et al.* (2020):** Performance of laboratory tests used to measure blood phenylalanine for the monitoring of patients with phenylketonuria. J Inherit Metab Dis., 43: 179-188.
31. **Blau N, Belanger-Quintana A, Demirkol M *et al.* (2009):** Optimizing the use of sapropterin (BH(4)) in the management of phenylketonuria. Mol Genet Metab., 96: 158-163.
32. **Burton C, Shi H, Ma Y (2016):** Development of a high-performance liquid chromatography - Tandem mass spectrometry urinary pterinomics workflow**.** Anal Chim Acta., 927:72-81.