Clinical, biochemical, and molecular characterization of an Egyptian group of patients with phenylketonuria and hyperphenylalaninemia: a pilot study

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Background
Phenylketonuria (PKU) is one of the preventable causes of intellectual disability. It is the most prevalent disorder of amino acid metabolism in the white population.

Aim and objective
The following were the aims and objectives of the study: first, assessment and correlation of clinical and biochemical progress of patients; second, assessment of the effect of age at diagnosis on the clinical outcome, third, follow-up of dietary compliance of patients; and third, phenylalanine hydroxylase gene sequencing for selected cases.

Participants and methods
This paper investigated 104 patients with PKU, including 79 early-diagnosed patients, 22 late-diagnosed patients, and three untreated patients, regarding clinical characteristics and Phe metabolic control. Thorough history taking, clinical and neurological assessment, and follow-up of dietary compliance with monthly assessment of blood Phe levels were performed. Four patients were selected for phenylalanine hydroxylase gene sequencing as a pilot study.

Results
Regarding early-diagnosed patients, 3.8% had attention-deficit and hyperactivity disorder. As for the late-diagnosed patients, 68.2% showed abnormal behavior, with an intelligent quotient range of 38.0–90.0. Hyperactivity was detected in 33.3%% of the untreated group, and the intelligent quotient ranged from 45.0 to 67.0. Parental consanguinity was found in 67.3% of cases. On average, 72% of patient had well-controlled blood phenylalanine levels.

Conclusion
There is a wide range of clinical heterogeneity among patients with PKU. Several factors determine the clinical outcome, including age at diagnosis, untreated blood phenylalanine levels, degree of compliance to dietary therapy, interventional therapies, as well as blood–brain barrier selectivity.

Keywords:
attention-deficit hyperactivity disorder, autism, dietary compliance, genotype-phenotype, hyperphenylalaninemia, intelligence quotient, phenylketonuria

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Introduction

Phenylketonuria (PKU) is the most prevalent disorder of amino acid metabolism in the white population (Eisensmith *et al.*, 1995; Scriver and Kaufman,2001). The major cause of PKU is insufficiency of the enzyme phenylalanine hydroxylase (Blau *et al.*, 2010). This enzyme catalyzes the transformation of phenylalanine into tyrosine, which is indispensable intermediate in the biochemical reactions necessary for neurotransmitter production (Wang *et al.*, 2013). Less commonly, PKU can be the result of deficiency of tetrahydrobiopterin (BH4), the cofactor of phenylalanine hydroxylase (PAH) enzyme (Wang *et al.*, 2013). Untreated cases of PKU experience mainly intellectual disability (ID) (Scriver and Kaufman, 2001). Dietary restriction of phenylalanine can prevent such sequela if it was started very early in life (Gregory *et al.*, 2007). Recent introduction of newborn screening for PKU in Egypt has led to the appearance of a growing community of patients with PKU who are in need for attention and scientific research.

Participants and methods

Approval of Alexandria Faculty of Medicine Ethical Committee to carry on the research was obtained. Professional genetic counseling was offered to all patient families. Informed written consent was taken from all parents or guardians for enrollment in the research. It was explicitly mentioned to the patients

and their parents that they have the right to refuse and their refusal would not affect their access to the offered medical care. This study was conducted on 104 patients aged 1–18 years diagnosed with PKU who are following low-Phe diet. Patients with malignant PKU (BH4 cofactor deficiency) were not included. Patients with mild hyperphenylalaninemia (HPA) whose untreated blood Phe levels are consistently less than or equal to 6 mg/dl were not included as they are not on diet-controlled treatment. The patients were referred from four Egyptian governorates. Each individual participating in the study was subjected to detailed history taking, clinical and neurological examination, in addition to follow-up of dietary compliance and monthly blood Phe levels for 3 consecutive months. Four patients were selected for PAH gene sequencing as a pilot study. Two cases were randomly selected from patients with high Phe tolerance, and other two cases were selected from patients with low-Phe tolerance cases. Phe tolerance can be defined as the amount of Phe per kg of body weight or mg per day, which maintains blood Phe concentrations within the target range of 2–6 mg/dl (van Wegberg *et al.*, 2017).

Professional genetic counseling was offered to parents before the test with respect to the aim of the study, the nature of the disease, and the benefits of detecting the causative mutations in their children. In addition, post-testing genetic counseling session was offered after the test results were obtained.

Overall, 2-ml venous blood samples for molecular testing were withdrawn from the patients under aseptic technique. Extraction of genomic DNA from 300 μl of each blood sample was carried out. Quality and quantity of extracted DNA were checked by Nanodrop spectrophotometer at 260 and 280 wavelengths. PCR amplification was performed for each of the thirteen exons of PAH gene in each of the four patients. PCR was carried out by GeneAmp PCR system 9700 (Applied Biosystems, Foster City, California, USA). The sequence of the primers used for the analysis and the conditions of the PCR reaction are available upon request. Bidirectional Sanger dideoxy chain termination sequencing reaction was performed on 13 amplicons covering the thirteen exons of PAH gene.

Obtained data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (IBM Corp., Armonk, New York, USA). Qualitative data were described using number and percentage. The Kolmogorov–Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, SD, and median. Significance of the obtained results was judged at the 5% level.

The used tests were as follows:

(1) χ^2-test was used for categorical variables, to compare between different groups
(2) Fisher's exact or Monte Carlo correction was used for correction for χ^2 when more than 20% of the cells have expected count less than 5
(3) Student's *t*-test was used for normally distributed quantitative variables, to compare between two studied groups
(4) *F*-test (analysis of variance) was used for normally distributed quantitative variables, to compare between more than two groups, and post-hoc test (Tukey) for pairwise comparisons
(5) Kruskal–Wallis test was used for abnormally distributed quantitative variables, to compare between more than two studied groups, and post-hoc (Dunn's multiple comparisons test) for pairwise comparisons.

Results

Clinical characterization

Age, sex, and parental consanguinity

The overall age range was 1.0–17.50 years. Approximately 51% were males and 49% were females. The male to female ratio was 1.03:1.

Patients were categorized according to the age at diagnosis into three groups: group 1 included early-diagnosed patients with age at diagnosis less than 3 months. This group included 79 (76%) children. Group 2 represented the late-diagnosed patients, including 22 children (representing 21.2%) with age at diagnosis ranging from 3 months to less than 7 years. Group 3 consisted of three (2.8%) patients, referred to as untreated patients, who were diagnosed at the age of 7 years or more. Parental consanguinity was found in 67.3% of cases.

Anthropometric measurements

Regarding weight, 3.8% of group 1 and 4.5% of group 2 were underweight. Overweight was encountered in 10.1% of group 1 and 13.6% of group 2, whereas obesity was detected in 5.1% of group 1 and 13.6% of group 2. As for height, 12.7% of group 1 and 9.1% of group 2 had short stature. All patients had head circumference within normal range for age (Table 1).

Mental status

Group 1 has normal cognitive function. The intelligent quotient (IQ) in group 2 ranged from 38.0 to 90.0, with mean of 63.92 ± 14.37. Two patients were nontestable

in group 2, whereas five patients represented missed data. Normal intelligence was encountered in one patient of group 2 with mild HPA, having an IQ of 90. Four other patients were found to be slow learners (Table 2).

In group 3, the IQ ranged from 45.0 to 67.0, with mean of 57.0 ± 11.14. The difference in the mean IQ between group 2 and group 3 is not statistically significant.

Seizures

Among the late-diagnosed and untreated patients as well, 16 patients had classic PKU, of whom only two patients (representing 12.5%) had seizures. The seizures were controlled after dietary management in one of them, whereas the other needed additional treatment with antiepileptic drugs.

Motor system examination and cerebellar function

Only one patient of the 25 late-diagnosed and untreated patients experienced persistent fisting, hand tremors (postural and kinetic), and poor cerebellar function, representing 4%.

Behavior

Abnormal behavior was detected in some patients, which ranged from attention-deficit and hyperactivity disorder (ADHD) features (attention-deficit hyperactivity disorder) including hyperactivity, impulsivity, aggressiveness, and inattentiveness to autistic features, including poor eye contact, eye contact on stress, stereotypic behavior, and body rocking (Table 3).

Hyperactivity was encountered in 3.8% of group 1 and 33.3% of group 3. However, 40.9% of group 2 had one or more ADHD features. Meanwhile, autism was diagnosed in 9.1% of group 2, whereas 18.2% of the same group showed one or more autistic features.

Table 1 Anthropometric measurements in the three studied groups

	Age at diagnosis [*n* (%)]		
	Group 1 (*n*=79)	Group 2 (*n*=22)	Group 3 (*n*=3)
Weight centiles			
Underweight (<5)	3 (3.8)	1 (4.5)	0
Normal (5-<85)	64 (81.0)	15 (68.2)	3 (100.0)
Overweight (85-<95)	8 (10.1)	3 (13.6)	0
Obese (≥95)	4 (5.1)	3 (13.6)	0
Height centiles			
Short (<5)	10 (12.7)	2 (9.1)	0
Normal (5-<95)	66 (83.5)	20 (90.9)	3 (100.0)
Tall (≥95)	3 (3.8)	0	0

Biochemical characterization

Classification according to blood Phe levels at diagnosis

According to blood Phe levels at diagnosis, patients were classified into classic PKU, with Phe levels greater than 20 mg/dl; mild PKU, with Phe levels between 10 and 20 mg/dl; and mild HPA, with Phe levels less than 10 mg/dl. As mentioned before, patients with mild HPA whose untreated blood Phe levels were consistently less than or equal to 6 mg/dl were not included in this study (Table 4).

Overall, 46.2% of all the patients were categorized as classic PKU; 34.6% had mild PKU; and 19.2% had mild HPA, with Phe levels at diagnosis greater than 6 mg/dl (i.e., on dietary therapy).

Compliance to low-Phe diet and formula

Compliance to low-Phe diet and formula was followed through diet diary provided by the investigator to the patients, in which parents recorded the daily intake of food and formula for 3 consecutive months. Patients were considered compliant if they were committed to low-Phe diet and formula in at least 95% of the 90 days in which the dietary intake was recorded (Fig. 1).

The overall compliance was 69.2% for low-Phe diet and ~76% for formula. Regarding group 1, 81% were compliant to low-Phe diet and formula compared with 36.4 and 63.6% in group 2, respectively. In group 3, 100% were not compliant to low-Phe diet and 66.7% were not compliant to formula.

Table 2 Intelligence quotient in the three studied groups

Cognitive IQ	Age at diagnosis [*n* (%)]			Test of significance	*P*
	Group 1 (*n*=79)	Group 2 (*n*=22)	Group 3 (*n*=3)		
NAD	79 (100.0)	1 (4.5)	0		
Slow learners	0	4 (18.2)	0		
ID	0	10 (45.5)	3 (100.0)		
Missed	0	5 (22.7)	0		
Nontestable	0	2 (9.09)	0		
Minimum-maximum		38.0-90.0	45.0-67.0	*t*=0.770	0.455
Mean±SD	-	63.92±14.37	57.0±11.14		
Median		61.0	59.0		

ID, intellectual disability; IQ, intelligence quotient; NAD, no abnormality detected.

Table 3 Behavior in the three studied groups

	Age at diagnosis [*n* (%)]			χ^2	^{MC}P
	Group 1 (*n*=79)	Group 2 (*n*=22)	Group 3 (*n*=3)		
Behavior					
NAD	76[a] (96.2)	7[b] (31.8)	2[b] (66.7)	41.670*	<0.001*
Abnormal	3[a] (3.8)	15[b] (68.2)	1[b] (33.3)		
ADHD features	3 (3.8)	9 (40.9)	1 (33.3)		
Autistic features	0	4 (18.2)	0		
Autism	0	2 (9.1)	0		

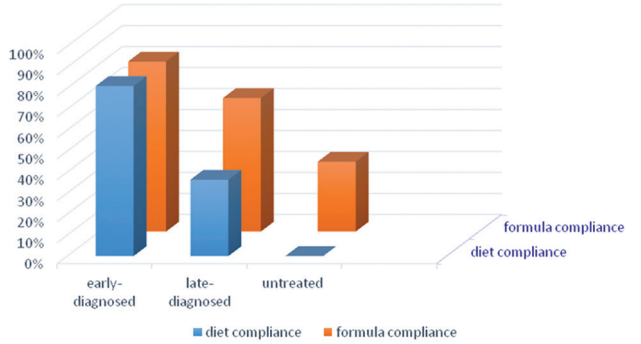
Common letters are not significant (i.e., different letters are significant). ADHD, attention-deficit hyperactivity disorder; MC, Monte Carlo; NAD, no abnormality detected; *P*, *P* value for comparing between the studied groups. *Statistically significant at $P \leq 0.05$.

Table 4 Blood Phe levels at diagnosis

Blood Phe levels	Age at diagnosis [*n* (%)]		
	Group 1 (*n*=79)	Group 2 (*n*=22)	Group 3 (*n*=3)
At diagnosis			
Mild HPA (>6-<10)	18 (22.8)	2 (9.1)	0
Mild PKU (10-20)	29 (36.7)	7 (31.8)	0
Classic PKU (>20)	32 (40.5)	13 (59.1)	3 (100.0)
Minimum-maximum	6.10-43.30	9.10-32.0	23.20-27.0
Mean±SD	17.33±7.86	20.55±6.19	25.23±1.91
Median	17.40	20.60	25.50

HPA, hyperphenylalaninemia; PKU, phenylketonuria.

Figure 1



Compliance to low-Phe diet and formula among the three studied groups.

Blood Phe levels during the study

According to the recommendations of the American College of Medical Genetics and Genomics, the target of dietary management is to keep blood Phe level from 2 to 6 mg/dl. Phe blood level was measured at monthly interval for 3 consecutive months, and the blood Phe levels were compared among the three groups of patients (Table 5).

The mean blood Phe levels in group 1 were within the target range. As for groups 2 and 3, the mean blood Phe levels were all above the upper limit of accepted range but showing gradual improvement.

On average, 72% of patient were well controlled, with group 1 having the highest Phe metabolic control rate (60%), compared with 22.7% for group 2 and 0% for group 3. The difference in blood Phe control among the three groups was statistically significant. A similar control rate was reported in a survey of 10 European centers in which blood Phe levels were within accepted range in 74% for patients aged 1–10 years (Ahring *et al.*, 2011).

Molecular characterization of the selected patients

Four patients were selected for PAH gene sequencing as a pilot study. Table 6 shows the clinical characteristics of the selected patients.

Sequencing results

The transcript ID of the reference sequence was NM_00027. The first patient (P1), whose parents are consanguineous, was surprisingly found to be a compound heterozygote for two pathogenic variants in exon 1 and exon 6 of PAH gene owing to heterozygous deletion of two nucleotides and 22 nucleotides, respectively, creating two frameshift mutations (Figs. 2 and 3). The first variant was annotated c.47_48delCT (p.S16*) and was predicted to create a premature stop codon at position 16 instead of 453 of the amino acid sequence, which is expected to result in the formation of a truncated protein with nonsense-mediated decay. The second variant was annotated c.592_613delTATAAAACCCATGCTTGCTATG (p.Y198Sfs*136). It was predicted to change the amino acid sequence and create a premature stop codon in the other PAH allele at position 333 instead of 453 of the amino acid sequence, which is expected to result in the formation of a truncated protein with nonsense-mediated decay.

The two mutations have been previously known to be disease-causing mutations (Dobrowolski *et al.*, 2009; Doan *et al.*, 2019). The patient had other variants in PAH gene that were predicted to be benign.

The second patient (P2) was found to be homozygote for a pathogenic single base substitution variant in intron 10 of PAH gene (Fig. 4). The variant was annotated g.114621G>A (IVS10-11G>A) (ID: rs5030855). It is predicted to result in splice site change. The patient was

Table 5 Average blood Phe levels during the study

Average Phe follow up	Age at diagnosis [*n* (%)]			Test of significance	Test of significance
	Group 1 (*n*=79)	Group 2 (*n*=22)	Group 3 (*n*=3)		
≤6 (well controlled)	60[a] (75.9)	5[b] (22.7)	0[b]	χ^2=24.876*	^{MC}P<0.001*
>6 (poorly controlled)	19[a] (24.1)	17[b] (77.3)	3[b] (100.0)		
Minimum-maximum	0.89-14.40	2.17-19.10	9.0-22.93	*H*=27.437*	<0.001*
Mean±SD	4.53±2.89	9.37±4.45	16.79±7.11		
Median	3.50	9.32	18.43		
Significance between groups	P_1<0.001*, P_2=0.003*, P_3=0.293				

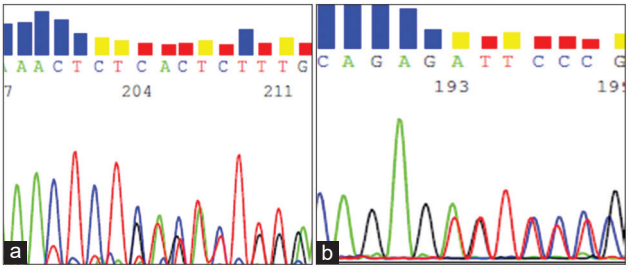
H, *H* for Kruskal-Wallis test, pairwise comparison between each two groups was done using post-hoc test (Dunn's for multiple comparisons test); MC, Monte Carlo; *P*, *P* value for comparing between the studied groups; P_1, *P* value for comparing between <3 months early and 3 months to <7 years late; P_2, *P* value for comparing between <3 months early and greater than or equal to 7 years untreated; P_3, *P* value for comparing between 3 months to <7 years late and greater than or equal to 7 years untreated. *Statistically significant at $P \leq 0.05$.

Table 6 Clinical characteristics of patients selected for genetic testing

Patient ID	Blood Phe levels (mg/dl)			Blood Phe control	Average Phe units/day	Age at Dx	Class	Parental consanguinity	Clinical findings
P1	8	9.1	7.2	Poor	6	Neonatal	Classic PKU	Yes	Normal
P2	12.7	9.3	6	Poor	6	2 years, 6 months	Classic PKU	Yes	GDD, autistic features, IQ=75
P3	1.5	2	2.1	Well	10	Neonatal	Mild HPA	No	Normal
P4	2.1	2	2.4	Well	12	6 years, 10 months	Mild HPA	No	DLD, normal IQ

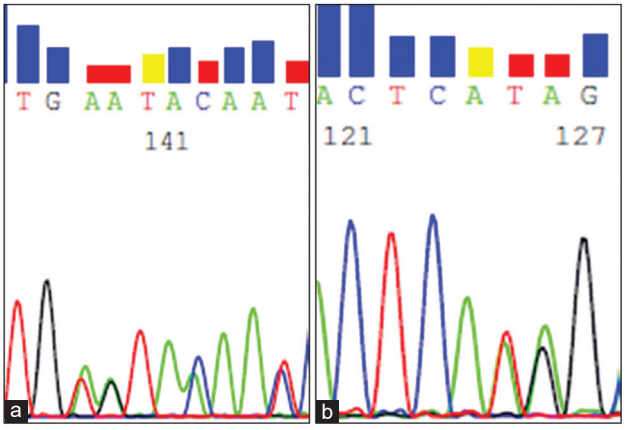
Phe exchange units: 50 mg Phe. DLD, delayed language development; GDD, global developmental delay; HPA, hyperphenylalaninemia; IQ, intelligent quotient; PKU, phenylketonuria.

Figure 2



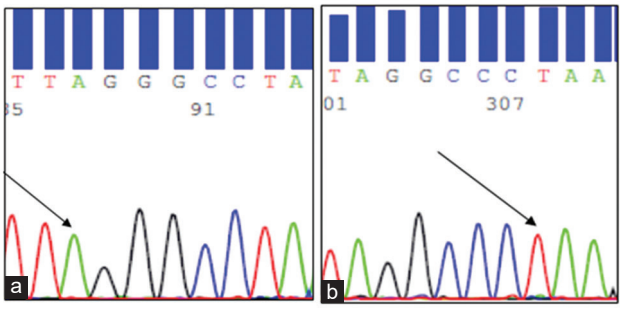
Electropherogram of exon (1) in patient 1 (P1) showing heterozygous deletion of two nucleotides (CT). (a) Forward strand. The change is confirmed in (b): reverse strand.

Figure 3



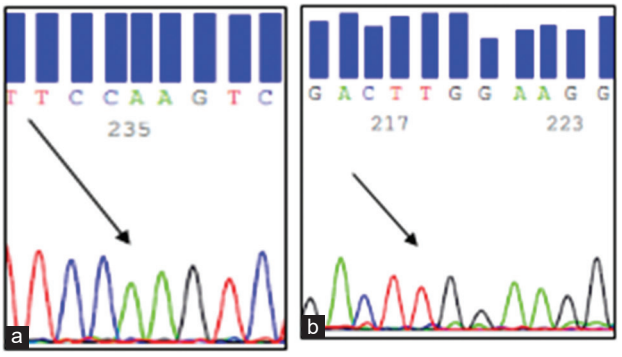
Electropherogram of exon (6) in patient 1 (P1) showing heterozygous deletion of twenty two nucleotides (GTATAAAACCCATGCTTGCTAT). (a) Forward strand and (b) reverse strand.

Figure 4



Electropherogram of patient 2 (P2) showing homozygous single base substitution variant in intron 10 g.114621G>A. (a) Forward strand. The change is confirmed in (b) reverse strand.

Figure 5



Electropherogram of patient 3 (P3) showing homozygous single base substitution variant in exon 7 c.782G>A p.R261Q. (a) Forward strand. The change is confirmed in (b) reverse strand.

found to harbor other variants that were predicted to be benign.

The third patient (P3) was found to be homozygote for a pathogenic single base substitution variant in exon 7 of PAH gene (Fig. 5). The variant detected, c.782G>A

p.R261Q (ID: rs5030849), occurred in a CpG mutation-hotspot on exon 7, leading to the conversion of Arg to Gln at codon 261. The patient's PAH gene is harboring other benign variants. Table 7 shows the pathogenic variants detected in patients 1, 2, and 3.

All variants found in the fourth patient (P4) were predicted to be benign. The patient is a descendent of consanguineous parents. She was diagnosed at the age of six years and ten months with a Phe level of 9.3 mg/dl after seeking medical advice for delayed language development. The patient was otherwise normal with normal cognitive function (IQ = 90).

Discussion

The male to female ratio is consistent with the disease pattern of inheritance being autosomal recessive, as is the case in most inborn errors of metabolism (Eisensmith *et al.*, 1995).

Patient categorization in the current study was based on the observation that IQ improvement rarely occurs if PKU management was initiated after the age of 7, and they usually experience severe mental affection and behavioral problems (Koch *et al.*, 1999; Murphy *et al.*, 2008).

Approximately one-third of cases were born to nonconsanguineous parents. This observation can be explained by the nature of the disease being autosomal recessive and the relatively high incidence in Egypt (1 in 7500), which reflects a relatively high carrier rate (Egyptian Ministry of Health, 2018). Sadek *et al.* (2018) reported a consanguinity rate of 85% among parents of patients with PKU in Upper Egypt. The difference may be attributed to the higher rates of consanguinity in this area of Egypt.

Some patients were found to be underweight in accordance to the study conducted by Verkerk and colleagues in 1994, which declared that patients with PKU might experience growth retardation particularly in the first 3 years. On the contrary, some patients were overweight or even obese. There is a growing number of reports of increased incidence of overweight and obesity in patients with PKU, particularly females (Burrage *et al.*, 2012). It is unclear whether obesity among patients with PKU is a result of the underlying condition, a treatment consequence, or an outcome of inadequate metabolic control (Rocha *et al*. 2013).

Table 7 Pathogenic variants detected in patients 1, 2, and 3

Patient	Alteration region	Variant
P1	Exon 1	Heterozygous deletion c.47_48delCT
P1	Exon 6	Heterozygous deletion c.592_613delTATAAAACCCATGCTTGCTATG
P2	Intron 10	Homozygous single base substitution g.114621G>A (IVS10-11G>A) (ID: rs5030855)
P3	Exon 7	Homozygous single base substitution c.782G>A (p.R261Q) (ID: rs5030849)

The reduced height encountered in some of the early-treated and late-diagnosed patients in the current study might be related to relative unavailability of special low-protein foods as concluded by Thiele *et al.* (2017), who also reported reduced height from birth onward in early-treated patients with PKU.

Several studies described microcephaly among late-diagnosed and untreated patients (Behbehani *et al.*, 1981; Steiner, Acosta *et al.* 2007; Mazur *et al.*, 2010). However, this was not observed in this study.

Not all the late-diagnosed patients had ID. A similar observation was reported by Vliet *et al.* (2018) who described in a systematic review that 59 reported cases of untreated patients with PKU did not have ID, as defined by an IQ greater than or equal to 80.

The mean IQ in groups 2 and 3 was close to the mean IQ for late-diagnosed PKU reported by Gonzalez *et al.* (2011) among Spanish late-diagnosed patients (62) and slightly higher than the mean IQ reported by Abdel-Salam *et al.* (2005) in another group of Egyptian patients (47.7 ± 2.62).

Multiple studies showed a wide variation in the spectrum of mental affection among patients with PKU (Hsia *et al.*, 1968; Rajabi *et al.*, 2016). This can be explained by the brain Phe levels being responsible for the severity of neurological insult in patients with PKU (Weglage *et al.*, 2001). The concentration of Phe in the brain is determined by the amount of Phe transported across the blood–brain barrier through L-type amino acid transporter (LAT1) at which Phe competes with other large neutral amino acids for transport.(Weglage *et al.*, 2001; de Groot *et al.*, 2010) This may denote that the blood level is not the only determining factor and LAT1 can play a role in determining the severity of the phenotype.

The reported seizure rate among the late-diagnosed classic patients with PKU in this study is close to that published by (Veneselli *et al.*, 1998) (12.8%). However, Abdel-Salam *et al.* (2005) reported a higher seizure rate of 35%. The difference can be attributed to the multifactorial nature of epilepsy.

The percentage of patients with tremors in this study is far less than that reported by Gonzalez *et al.* (2011) (93.1%).

ADHD has been linked to PKU in a series of studies conducted by Antshel and Waisbren (2003a,), in which the prevalence of ADHD was found to be 2.5 times more than the prevalence among general population. It is thought that this link is owing to the fact that both patients with PKU and patients with ADHD have low levels of dopamine in prefrontal cortex and striatum, creating a hypodopaminergic state with subsequent noradrenergic state (Arnsten, 2006; Landvogt *et al.*, 2008). Neto *et al.* (2018), also reported attention problems in early-treated PKU children.

In one study, the prevalence of autism among late-diagnosed children with PKU was 5.7% (Baieli *et al.*, 2003). Reiss *et al.* (1986) reported in a review of autism in patients with genetic disorders that the prevalence of autism in patients with PKU is 20%. Several studies reported different rates of autism among patients with PKU (Fombonne, 1997; Khemir *et al.*, 2016). The difference in the reported frequencies of autism may be related to different genetic and environmental background of the studied PKU cohorts.

It is noticeable that compliance to low-Phe diet is inversely related to age at diagnosis. It is expected to be more difficult for children who were on regular diet to be committed to low-Phe diet and stop eating normal diet after getting used to it. In relation to formula compliance, the noncompliant children were having difficulty tolerating the smell of the formula. The early-diagnosed children who started formula intake during the neonatal period had better acceptance and better compliance than the late-diagnosed patients, and the difference in compliance among the three groups of patients was found to be statistically significant. Several studies reported decline in formula compliance with age (Prince *et al.*, 1997; Owada *et al.*, 2000; MacDonald *et al.*, 2006).

Concerning the sequencing results, patient 1 was compound heterozygote despite having consanguineous parents. It should be denoted that these results were confirmed by repeating the sequencing process, starting from blood sampling, by different laboratory personnel. Patient 1 was diagnosed during the neonatal period after diagnosis of other two elder siblings who were diagnosed after the age of 7 years. She was compliant to the dietary regimen but she had limited Phe tolerance. She had normal developmental history and normal cognitive function (IQ = 89). On the contrary, her elder sister and brother both had ID (IQ of 45 and 59, respectively). The elder sister had poor cerebellar function, persistent fisting, and hand tremors (postural and kinetic). Both of them were noncompliant to low-Phe diet and formula, as they could not tolerate the smell of the formula. This highlights the importance of early age of intake of special PKU formula to facilitate patient getting used to the taste and hence proper compliance.

The pathogenic variant detected in patient 2 has been reported earlier as the most common mutation in European Mediterranean countries, and it is known to be associated with severe phenotype as was also noticed in this patient as having classical PKU and limited Phe tolerance (Pey *et al.*, 2003; Zschocke, 2003). The patient was born to a consanguineous couple and was diagnosed at the age of 2 years and 6 months. The patient had history of global developmental delay with autistic features (body rocking, stereotypic behavior, and eye contact on stress), with an IQ score of 75. The patient showed improved language skills and improved behavior on follow-up visits.

The mutation discovered in patient 2 is reported to result in a residual enzyme activity of 30%, leading to a mild phenotype as observed in our patient (mild HPA with high Phe tolerance). Mild phenotype was reported by Berkovich and colleagues, in a patient having the same variant (Berkovich *et al.*, 2008). The patient was diagnosed neonatally with normal development and cognitive function. The patient's parents are nonconsanguineous, suggesting a possibility of a high carrier rate for this variant among Egyptians. The genotype of this patient also was double-checked by different laboratory personnel to exclude the possibility of laboratory technical errors.

The failure of identification of pathogenic variants in patient 4 may be owing to failure of mutation detection by the sequencing method used in the present study. According to Daniele *et al.* (2006), a small percentage of PAH mutations escape detection in all populations, as the causative mutation may be present in the nonsequenced regulatory sequences, for example, the promoter or nonsequenced parts of 5'UTR and 3'UTR (Ramsden *et al.*, 2016).

Pagani *et al.* (2003), demonstrated benign sequence variants in CFTR gene, which were previously considered as neutral polymorphism, resulted in impaired mRNA splicing and thus acting as pathogenic. The study reported that these variants interfered with a newly discovered regulatory element called composite exonic regulatory element of splicing, indicating that any benign polymorphism in an exon should not be disregarded as it might influence the mRNA splicing process (Pagani *et al.*, 2003). Functional splicing assays are needed to test the effect of the variants on the mRNA splicing process (Pagani *et al.*, 2003).

Furthermore, mild HPA may occur owing to nongenetic causes as reported by Scholl-Burgi *et al.* (2011), who

stated that chronic immune stimulation may cause permanent moderate impairment of the hepatic enzyme phenylalanine hydroxylase, resulting in elevation of blood Phe levels in absence of genetic mutations in PAH gene. The cause of such impairment is still obscure. It has been speculated that reactive oxygen species may be responsible for this inactivation mechanism through modification of the tertiary structure of the enzyme or through oxidation of the enzyme cofactor (BH4) (Fuchs *et al.*, 2011; Scholl-Burgi *et al.*, 2011).

Conclusion

There is a wide range of clinical heterogeneity among patients with PKU. Several factors determine the clinical outcome including age at diagnosis, untreated blood phenylalanine levels, degree of compliance to dietary therapy, interventional therapies, as well as blood-brain barrier selectivity.

Commitment to PKU nutritional regimen is of utmost importance; however, it might be challenging to some patients.

Regular blood Phe monitoring is crucial to guide the nutritional therapeutic plan of the patient.

The Egyptian population may have a relatively high carrier rate for PKU.

In the present pilot study, detection of causative mutations in some patients provided an explanation for the variation in biochemical phenotype of the patients reflected in their untreated blood Phe levels and Phe intake tolerance.

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Conflicts of interest

There are no conflicts of interest.

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