

Tetrahydrobiopterin responsiveness in patients with phenylketonuria

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Abstract

Objectives: To investigate the BH₄ response in a group of patients with phenylketonuria (PKU) in order to offer this alternative treatment to the responsive patients.

Design and methods: The 24-h-long Phe/BH₄ loading test was performed on 64 PKU patients requiring dietary treatment.

Results: All patients with mild-PKU and 75% of patients with moderate-PKU were BH₄ responsive, while only 11% of classic-PKU patients showed good/partial response ($P < 0.0001$). The percentages of Phe decrease after the BH₄ loading test were significantly different in the three PKU phenotypes (mild PKU: 67.9 ± 18.7 ; moderate PKU: 37.4 ± 16.8 ; and classical PKU: 21.9 ± 13.7 ; ANOVA with Bonferroni correction: $P < 0.0001$). We report four mutations (P147S, D222G, P275S, and P362T) not previously associated with BH₄ responsiveness, all of them combined with mutations with zero predicted residual activity.

Conclusion: Both the percentage of Phe decrease and the Phe value achieved 24 h after BH₄ loading are valuable data in predicting a response. We report four mutations not previously associated with BH₄ responsiveness.

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Keywords: Tetrahydrobiopterin; Phenylketonuria; Hyperphenylalaninemia; BH₄ responsiveness; PAH deficiency; PKU; PAH gene mutations

Introduction

Phenylketonuria (PKU; OMIM 261600) is an autosomal recessive metabolic disease caused by a deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1), a hepatic enzyme that catalyses the conversion of phenylalanine (Phe) to tyrosine, using tetrahydrobiopterin (BH₄) as a coenzyme. PAH deficiency causes an accumulation of Phe and a lack of tyrosine in tissues and biological fluids.

Untreated PKU leads to mental retardation, epilepsy, and other neurological abnormalities, while early detection and treatment with a Phe-restricted diet prevent Phe accumulation and brain damage and results in almost normal neurological development [1].

The BH₄ loading test has traditionally been used for the differential diagnosis of hyperphenylalaninemia caused either by PAH deficiency or by several rare inherited defects in BH₄ metabolism. Patients with mutations in the PAH gene show poor BH₄ response, while patients with primary defects in BH₄ metabolism have good response together with a pterin profile in serum and/or urine, which permits the identification of the defect. However, in 1999 Kure et al. [2] described four BH₄-responsive patients with known mutations of the PAH gene. Several other patients

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have been described since then [3–7], a result which has been attributed to the higher purity [8] and dose of BH₄ used for the loading test (20 mg/kg body weight at present versus 7–10 mg/kg body weight used before) [9]. Moreover, BH₄ treatment of responsive patients has resulted in successful control of blood Phe levels with a progressive relief or withdrawal from the Phe-restricted diet [10–13].

Several mutations of the PAH gene have been reported to be BH₄ responsive [14–18], and basic investigation groups are currently studying the reasons for this responsiveness [19,20]. However, since most PKU patients are double heterozygous for two different mutations, and since there are more than 400 mutations described in the PAH gene (PAH mutation database: <http://www.pahdb.mcgill.ca>), it is difficult to predict BH₄ responsiveness. Moreover, even a combination of known mutations may vary in phenotype, so that for PAH (except for null mutations) no phenotype–genotype correlations can be assumed [7].

Our aim was to investigate the BH₄ response in a group of PKU patients on a Phe-restricted diet monitored in our hospital by means of a combined Phe/BH₄ loading test, so as to be able to offer this alternative treatment to the responsive patients.

Patients and methods

Patients

From a group of 84 patients with phenylketonuria monitored in our hospital (Reference Centre for PKU in Catalonia), 64 patients (age range: 1 month–37 years) were selected to undergo the Phe/BH₄ loading test. Inclusion criteria for patients were as follows: (a) known mutations in the PAH gene or mutations in the course of investigation and pterin profile and dihydropteridine reductase activity excluding a primary defect in BH₄ metabolism, (b) need for dietary treatment (tolerance lower than 600 mg/day), and (c) patients' or their parents' acceptance of the loading test. Twenty patients in all were excluded, for the following reasons: because they did not accept (five patients), because the BH₄ loading test had already been performed in a sibling with the same genotype without response (five patients), because there were social or familial problems (nine patients), and because of pregnancy (one patient).

The Phe levels at diagnosis of the patients included in the study ranged from 360 to 2813 $\mu\text{mol/L}$, with an average \pm SD of $1384 \pm 667 \mu\text{mol/L}$. In spite of the continuum phenotypes, patients were arbitrarily classified for diagnostic and therapeutic purposes according to the metabolic phenotype [21,22] into classic PKU (Phe levels at diagnosis or unrestricted nutrition $>1200 \mu\text{mol/L}$ and tolerance: 250–350 mg Phe/day; 38 patients), moderate PKU (Phe levels on unrestricted nutrition between 600 and 1200 $\mu\text{mol/L}$ and tolerance: 350–400 mg Phe/day; 16 patients), and mild PKU

(Phe levels on unrestricted nutrition between 360 and 600 $\mu\text{mol/L}$ and tolerance: 400–600 mg Phe/day; 10 patients). Tolerance is defined as the highest Phe intake tolerated while keeping blood Phe levels within the safe range (120–360 $\mu\text{mol/L}$).

All children or their guardians in this study signed an informed consent agreement in accord with the Helsinki Declaration of 1964, revised in Edinburgh in 2000. Our hospital Ethics Committee approved the study. Compassionate use authorization for the BH₄ loading test and treatment, when necessary, was obtained from the health authorities.

Methods

Combined Phe/BH₄ loading test

The combined Phe/BH₄ loading test was performed according to the protocol recommended by Blau et al. [23] for patients on the diet, with a slight modification. In short, after at least 3 h fasting, 100 mg Phe/kg body weight was given orally. After 3 h, a single dose of 20 mg BH₄/kg body weight was administered orally. Blood samples for amino acid analysis were taken at 3 h before Phe loading and at 0, 3, 7, 11, and 21 h after the BH₄ loading (instead of 27 h stated in ref. 23). Two portions of urine for pterin analysis were collected, during the 12 h before the Phe load and during the 4–8 h after BH₄ administration, and these samples were frozen in light-protected bottles. Food intake with Phe restriction was maintained throughout the study. BH₄ was obtained from Schircks Laboratories (Jona, Switzerland) and Phe (oral use) from Roig Farma, SA (Terrassa, Spain). The BH₄ responsiveness was defined as a decrease in plasma Phe of more than 30% of the value before the BH₄ challenge [23] within a period of 21 h after BH₄ loading.

Biochemical procedures

Plasma phenylalanine and tyrosine were analyzed by ion exchange chromatography with ninhydrin detection (Biochrom 20, Pharmacia Biotech, Cambridge, England) [24]. Urinary biopterin and neopterin were determined by HPLC with fluorescence detection (Perkin Elmer, Serie 200, Norwalk, CT, USA) [25]. Mutations in the PAH gene were analyzed as previously described [26]. Missense mutations were considered as functionally null mutations when the predicted residual enzyme activity (PRA) *in vitro* was reported to be zero [26].

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 11.0). The chi-square test was used to compare the percentage of BH₄-responsive patients

among classical, moderate, and mild PKU patients. The ANOVA test with Bonferroni correction was used to compare the percentages of BH₄ response, plasma Phe, and urine biopterin concentrations in the three groups of PKU patients. The Pearson test was used to determine the correlations between the different variables studied. The Student *t* test for paired data was used to analyze the differences between basal and postload percentages of urine biopterin concentrations. Differences were considered significant when $P < 0.05$.

Results

In our PKU patients, 28 out of 64 (44%) responded with a decrease greater than 30% in plasma Phe concentrations 21 h after a single dose of BH₄, and in 9 of these 28 responder patients the decrease was greater than 65% (Fig. 1). Metabolic phenotype, Phe level at diagnosis, and 21 h after BH₄ challenge, genotype, residual PAH activity (if known), and BH₄ response for the 28 BH₄-responsive patients are summarized in Table 1, while the same variables for the 36 nonresponsive patients are summarized in Table 2.

All patients with mild PKU and 75% of patients with moderate PKU were BH₄ responsive, while only 11% of classic PKU patients showed good/partial response (chi-square test: $P < 0.0001$). The Phe values during the Phe/BH₄ loading test in the PKU patients with mild, moderate, and classic phenotypes are shown in Fig. 2. The percentages of plasma Phe decrease after the BH₄ loading test were significantly different in the three PKU phenotypes (mild PKU: 67.9 ± 18.7 ; moderate PKU: 37.4 ± 16.8 ; and classical PKU: 21.9 ± 13.7 ; ANOVA with Bonferroni correction: $P < 0.0001$) (Fig. 3). The plasma Phe decreased to safe levels in the mild PKU patients ($298 \pm 196 \mu\text{mol/L}$),

while the final Phe achieved higher levels in the moderate PKU ($726 \pm 270 \mu\text{mol/L}$) and in the classic PKU ($932 \pm 236 \mu\text{mol/L}$) (ANOVA with Bonferroni correction, $P < 0.0001$).

A negative correlation was observed between plasma Phe at diagnosis and the percentage of BH₄ response (Pearson test, $r = -0.581$; $P < 0.001$), while a positive correlation was noted between Phe at diagnosis and the maximum Phe values before the BH₄ challenge ($r = 0.377$; $P = 0.002$).

A positive correlation was observed between the plasma Phe levels 11 and 21 h after the BH₄ challenge (Pearson test, $r = 0.691$; $P < 0.001$). However, the percentages of responsive patients were quite different when considering the 11-h response (25% with good/partial response). Twelve out of 28 patients would have been classified as non-responders using an 11-h BH₄ loading test.

Regarding urine biopterin concentrations, both basal biopterin ($1.21 \pm 0.74 \text{ mmol/mol creat}$) and biopterin/pterin ratio (biopterin $\times 100 / [\text{biopterin} + \text{neopterin}]$) (41.4 ± 11.6) were significantly different from postload levels ($5.15 \pm 2.96 \text{ mmol/mol creat}$ and 74.6 ± 13.1 , respectively) (paired Student *t* test, $P < 0.0001$). No correlation was observed between the percentage of response or the 24-h postload plasma Phe concentrations and the postload total biopterin or biopterin/pterin ratio.

In our study, 17 mutations in the PAH gene appeared to be associated with BH₄ responsiveness, 14 of them combined with null or functionally null mutations. Ten of these have already been associated with BH₄ responsiveness: L48S, I65T, E178G, R241H, R243Q, R261Q, V388M, E390G, Y414C, and D415N (Table 3), while four further mutations are newly reported in BH₄-responsive patients: P147S, D222G, P275S, and P362T. Moreover, we observed three mutations, two of them with known residual activity, combined with other missense responsive mutations (Y168H/V388M, L348V/V388M, and A309V/L48S) in

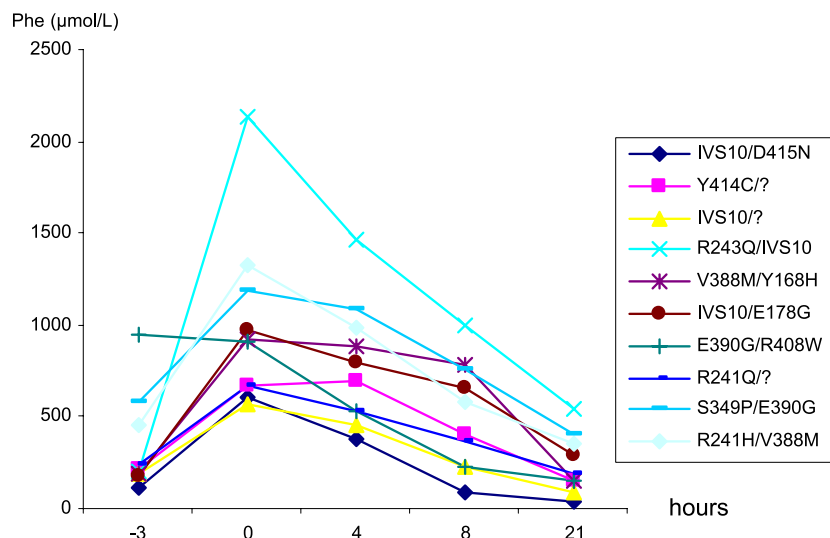


Fig. 1. Plasma Phe concentrations in the BH₄ loading test of the nine good responder patients.

Table 1

Phenotype, genotype, predicted residual activity (PRA) in vitro, Phe values at 0 and 21 h of BH₄ loading, and BH₄ response (effect of BH₄ in the decrease of Phe levels) in the 28 patients with >30% response

Patient number	Metabolic PKU phenotype	Allele 1	PRA ^a (%)	Allele 2	PRA ^a (%)	Phe at 0 h of BH ₄ loading test (μmol/L)	Phe at 21 h of BH ₄ loading test (μmol/L)	Percentage of BH ₄ response
1	Mild	IVS10	0	D415N	72	612	37	94.0
2	Mild	R408W	0	E390G	70	950	153	83.9
3	Mild	Y168H ^c	n.d.	V388M	43	925	150	83.8
4	Mild	Y414C	28	n.i.	n.d.	672	148	78.0
5	Classic	IVS10	0	R243Q	<10	2134	545	74.5
6	Mild	R241Q	23	n.i.	n.d.	668	185	72.3
7	Moderate	V388M	43	R241H	23	1330	359	72.0
8	Mild	IVS10	0	E178G	n.d.	972	288	70.4
9	Mild	S349P	0	E390G	70	1186	402	66.1
10	Moderate	V388M	43	P362T ^b	n.d.	1068	511	52.2
11	Mild	n.i.	n.i.	n.i.	n.d.	737	365	50.5
12	Moderate	R408W	0	P275S ^b	n.d.	903	455	49.6
13	Moderate	IVS2nt5G→C	0	P362T ^b	n.d.	1165	603	48.2
14	Mild	R261X	0	R241H	23	892	477	46.5
15	Moderate	V388M	43	P362T ^b	n.d.	930	505	45.7
16	Classic	S349P	0	P147S ^b	n.d.	1590	892	44.0
17	Classic	R158Q	10	R261Q	27	1363	764	43.9
18	Moderate	IVS2nt5G→A	0	R261Q	27	1032	621	40.0
19	Mild	Ivs4nt5G→T	0	Y414C	28	1035	638	38.4
20	Classic	V388M	43	L348V ^c	33	1007	629	37.5
21	Moderate	L48S	39	A309V ^c	70	1336	853	36.2
22	Classic	R261X	0	I65T	26	1130	741	34.4
23	Classic	L48S	39	R261Q	27	1248	849	32.0
24	Classic	I65T	26	n.i.	n.d.	1332	918	31.0
25	Moderate	IVS2nt5G→A	0	R261Q	27	1224	745	30.9
26	Classic	R261Q	27	n.i.	n.d.	1041	720	30.8
27	Moderate	R408W	0	D222G ^b	n.d.	1207	838	30.6
28	Classic	IVS4nt5G→T	0	IVS4nt5G→T	0	1860	1294	30.4

n.i., not yet identified; n.d., not determined.

^a As percentage of wild type (in cell lysates). Data obtained from PAH database: (<http://www.pahdb.mcgill.ca>).

^b Mutations not previously associated with BH₄ responsiveness: mutations found in a heterozygous state in combination with mutations with PRA reported to be zero. Their association with BH₄ responsiveness has yet to be confirmed.

^c Mutations not previously reported in BH₄-responsive patients. They are found in a heterozygous state in combination with another responsive missense mutation.

BH₄-responsive patients. Their association with BH₄ responsiveness remains to be confirmed (Table 3).

Discussion

Since our aim was to investigate the BH₄ responsiveness of our PKU patients in order to be able to identify the candidates for treatment with this cofactor, we considered two groups of response: good/partial and poor responders. Although decreases in Phe values in a 24-h-long combined Phe/BH₄ test may be partly due to spontaneous Phe elimination [14], investigating this point by repeating the Phe loading [23] in the patients would seem a bit much to ask of the parents. From a practical point of view, our results give enough information to start BH₄ daily therapy in the good responder patients, so as to achieve further evidence about the possibility of increasing the tolerance without the PKU formula. Furthermore, long-term treatment would confirm the BH₄ responsiveness of patients.

Our results suggest that about 44% of PKU patients respond with a decrease greater than 30% in plasma Phe concentrations 21 h after a single dose of BH₄. Considering the metabolic phenotype of the responsive subjects, all patients with mild PKU and 75% of patients with moderate PKU were BH₄ responsive, while only 11% of classic PKU patients were BH₄ responsive. The negative correlation observed between plasma Phe at diagnosis and the percentage of response in our series supports the statement that mild and moderate phenotypes (Phe at diagnosis between 360 and 1000 μmol/L) respond better to BH₄ challenge [7,14,23]. However, some patients (9/28) with classical PKU showed a considerable response, which even reached 74.5% in one patient (number 5, Table 1). Conversely, three patients with moderate PKU showed a percentage of response lower than 30% (patients 43, 46, and 55 in Table 2).

From our results, we deduced that not only were the percentages of decrease in Phe levels important in deciding the candidate patients for future BH₄ therapy, but also that the 21 h Phe values achieved after a single BH₄ dose could

Table 2

Phenotype, genotype, predicted residual activity (PRA) in vitro, Phe values at 0 and 21 h post-BH₄ loading, and BH₄ response (effect of BH₄ in the decrease of Phe levels) in the 28 patients with <30% response

Patient	Metabolic PKU phenotype	Allele 1	PRA ^a (%)	Allele 2	PRA ^a (%)	Phe at 0 h of BH ₄ loading test (μmol/L)	Phe at 21 h of BH ₄ loading test (μmol/L)	Percentage of BH ₄ response
29	Classic	R243X	0	G352fsdelG	0	1520	1077	29.1
30	Classic	R261Q	27	S349P	0	1221	868	28.9
31	Classic	IVS8nt7A→G	0	n.i.	0	866	619	28.5
32	Classic	R243X	0	S349P	0	617	446	27.7
33	Classic	delF39(ex 2)	0	R261X	0	867	638	26.4
34	Classic	IVS4nt5G→T	0	G148D	n.d.	798	594	25.6
35	Classic	R243Q	<10	I65T	26	1445	1164	24.5
36	Classic	I65T	26	n.i.	n.i.	1395	1055	24.4
37	Classic	L48S	39	S349P	0	1104	836	24.3
38	Classic	R111X	0	R243X	0	1541	1166	24.3
39	Classic	IVS4nt5G→T	0	R158Q	10	1355	1030	24.0
40	Classic	R408W	0	IVS7nt1G-A	0	1350	1029	23.7
41	Classic	n.i.	n.i.	n.i.	n.i.	1397	1090	22.0
42	Classic	I65T	23	n.i.	n.i.	1240	980	21.0
43	Moderate	R243X	0	R261Q	27	1011	798	21.0
44	Classic	P122Q	n.d.	IVS10	0	1031	819	20.6
45	Classic	S349P	0	V388M	43	1741	1385	20.4
46	Moderate	IVS1nt5g→t	0	R158Q	10	1300	1035	20.4
47	Classic	G272X	n.d.	n.i.	n.i.	907	754	16.8
48	Classic	I65T	23	I65T	23	1331	1126	15.4
49	Classic	IVS4nt5G→T	0	R243Q	<10	1103	934	15.3
50	Classic	L197fsDEL22bp	0	L197fsDEL22bp	0	938	795	15.2
51	Classic	n.i.	n.i.	n.i.	n.i.	1276	1093	14.3
52	Classic	P122Q	n.d.	IVS10	0	1134	943	12.4
53	Classic	IVS10	0	IVS10	0	1685	1489	11.6
54	Classic	P281S	n.d.	P281S	n.d.	1145	1017	11.1
55	Moderate	V388M	43	n.i.	n.i.	1087	971	10.7
56	Classic	G352fsdelG	0	S349P	0	1344	1204	10.4
57	Classic	IVS8nt1G→A	0	I65T	26	1038	942	9.2
58	Classic	IVS10	0	n.i.	n.i.	1311	1197	8.7
59	Classic	IVS10	0	IVS10	0	1160	1072	7.6
60	Classic	IVS8nt-7a→g	0	IVS8nt+1g→a	0	1035	963	6.9
61	Classic	IVS4nt5G→T	0	IVS10	0	914	872	4.6
62	Classic	1162–1163TGdel	0	N61/N62/T63fsdel stop	0	818	786	4.0
63	Classic	P279fsdelC	0	V388M	43	877	875	2.3
64	Classic	n.i.	n.i.	n.i.	n.i.	1368	1340	2.0

n.i., not yet identified; n.d., not determined.

^a As percentage of wild type (in cell lysates). Data obtained from PAH database: (<http://www.pahdb.mcgill.ca>).

be an indicator of successful treatment (Table 1). In our experience, preliminary data from 8-month follow-up of the nine patients with good response indicate successful results in eight of them (data not shown). In fact, these eight patients were treated with 5 mg BH₄/kg/day, without PKU formula (patients 1–4 and 6–9 in Table 1) and with considerable liberalization of the protein-restricted diet, and they achieved plasma Phe levels in a range from 160 to 396 μmol/L. However, the classic PKU patient (number 5, Table 1) with good response but high final Phe levels after the loading test needed a higher BH₄ dose (10 mg/kg/day), combined with PKU formula, in order to be able to increase Phe tolerance. These results suggest that treatment with only BH₄ in the other eight classic PKU-responsive patients (Table 1) who achieved 21 h Phe values from 629 to 1294 μmol/L would not be enough to control Phe levels without the PKU formula and a Phe-restricted diet.

Our results also demonstrate the importance of performing the 24-h-long Phe/BH₄ loading test [23]. In our experience from a previous study [27,14], Phe loading (100 mg/kg body weight) before the BH₄ challenge is a better practice than a 3-day-free diet, which may not be accomplished by PKU patients who are not used to eating protein-rich meals. In the present study, a good correlation was observed between Phe levels at diagnosis and those before BH₄ challenge. These maximum Phe values allowed for calculation of the BH₄ response with reliability. Furthermore, the 24-h-long test permitted the identification of slow responders in our series. The number of responsive patients would have been different when considering the 11-h response because 12 out of 28 patients with good/partial 21-h response showed a less than 30% decrease in Phe levels at 11 h following BH₄ challenge. Among these 12 patients, there were classic,

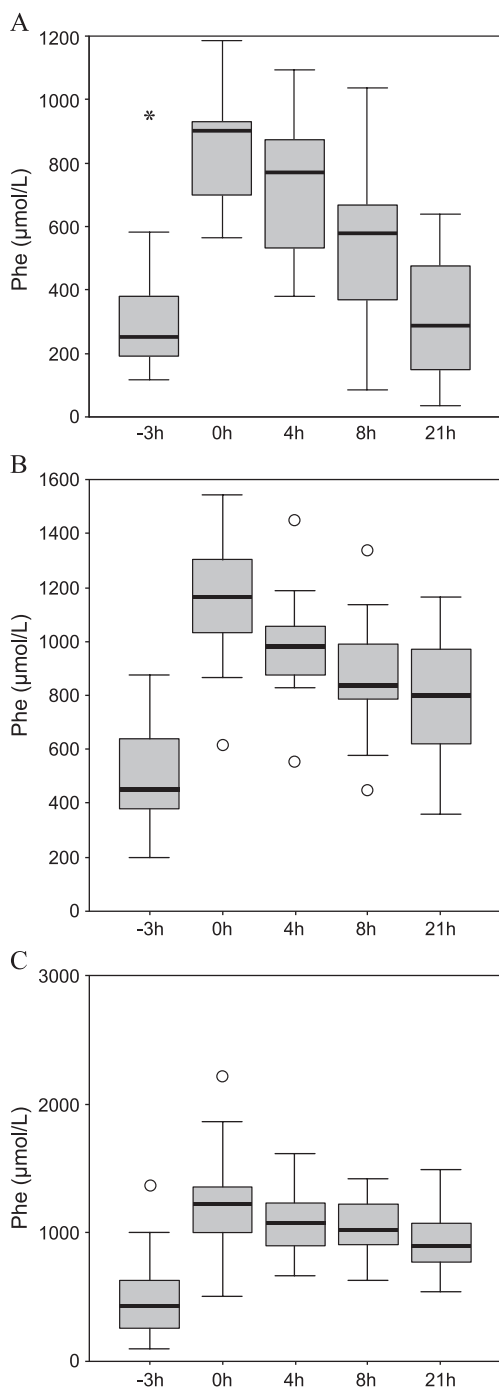


Fig. 2. Phe values during the Phe/BH₄ loading test in the three groups of PKU patients: mild (A), moderate (B), and classic (C) phenotype. The length of the boxes indicates the interquartile space (P₂₅–P₇₅); the horizontal line into the box represents the median (P₅₀), and the error bars indicate the adjacent values, that is, the maximum and minimum values of the distribution, which may not be considered abnormal. The circles indicate the outliers and the star represents an extreme value.

moderate, and mild PKU phenotypes, so that we cannot predict the group of patients which will need 24 h to respond, at least under our conditions. Therefore, a 24-h-long test seems advisable. We slightly modified the protocol of Blau et al. [23] by finishing the test at 21 h

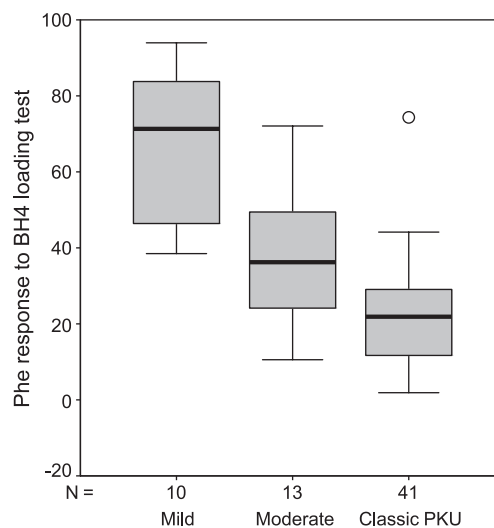


Fig. 3. Response to BH₄ loading test in 64 PKU patients. The circles indicate the outliers.

after the BH₄ loading so as to allow most patients to attend school or work the second day and thereby make it easier for them to participate in the study.

The determination of biopterin and neopterin concentrations in urine, before and 4–8 h after the BH₄ loading,

Table 3
BH₄-responsive mutations in the PAH gene in our series

Mutation	Exon	PRA ^a (%)	PAH domain	Reference
L48S	2	39	Regulatory	[5,7,15]
I65T	3	26	Regulatory	[6,15]
P147S ^b	4	n.d.	Catalytic	
Y168H ^c /V388M	5/11	n.d./43	Catalytic/ Catalytic	
E178G	6	n.d.	Catalytic	[18]
D222G ^b	6	n.d.	Catalytic	
R241H	7	23	Catalytic	[7]
R243Q	7	<10	Catalytic	[7,28]
R261Q	7	27	Catalytic	[6,7,14,15]
P275S ^b	7	n.d.	Catalytic	
A309V ^c /L48S	9/2	70/39	Catalytic/ Regulatory	
L348V ^c /V388M	10/11	33/43	Catalytic/ Catalytic	
P362T ^b	11	n.d.	Catalytic	
V388M	11	43	Catalytic	[14,30]
E390G	11	70	Catalytic	[10,14–16]
Y414C	12	28	Tetramerization	[4,28]
D415N	12	72	Tetramerization	[7,15,28]

n.d., not determined.

^a As percentage of wild type (in cell lysates). Data obtained from PAH database: (<http://www.pahdb.mcgill.ca>) and BIOPKU database (<http://www.bh4.org/>).

^b Mutations not previously associated with BH₄ responsiveness: mutations found in a heterozygous state in combination with missense mutations with PRA reported to be zero. Their association with BH₄ responsiveness has yet to be confirmed.

^c Mutations not previously reported in BH₄-responsive patients. They are found in a heterozygous state in combination with another responsive missense mutation.

allowed for verification that the BH₄ ingestion and absorption were correct. Moreover, the lack of correlation observed between the response percentage or the 24-h postload Phe and the bipterin values confirms that the response was not related to the BH₄ absorption.

The mechanism of action is still unknown for many mutations associated with BH₄ responsiveness. Some authors have hypothesized that mutations located in the catalytic domain result in mutant enzymes that are Km variants of PAH with an altered binding affinity for BH₄ [20]. These mutations might be located either in the cofactor binding regions or in regions that interact with secondary structures in the protein involved in cofactor binding. However, this hypothesis seems to be unlikely since other authors have not found a correlation between genotype and BH₄ responsiveness [7,28]. Other hypotheses are three-dimensional structural changes and induction of PAH gene expression by BH₄ [5,23]. An additional interesting hypothesis is that interindividual differences in the cellular folding apparatus may determine the tertiary structure and the amount of mutant PAH dimers, by preventing their degradation or by increasing the residual enzymatic activity [28].

Most of the BH₄-responsive mutations found in our series are in the PAH catalytic domain, but not within the BH₄ binding sites (Table 3). Some of them have already been reported (Table 3), but several mutations are newly reported here as being associated with BH₄ responsiveness. Four mutations (P147S, D222G, P275S, and P362T) were found in a heterozygous state with functionally null mutations in our series (Table 1), two of them being proximal to, but not located in, the cofactor binding regions [20].

Three further mutations (Y168H, L348V, and A309V), two of them with known residual activity, were observed in a heterozygous state with other missense responsive mutations in BH₄-responsive patients (numbers 3, 20, and 21, Table 1). The A309V mutation with a 70% residual activity has been studied by Pey et al. [29], who suggest that its location proximal to the cofactor binding regions plays a role for this residue in the stabilization or structural maintenance of these domains.

Regarding the previously reported mutations associated with BH₄ responsiveness (Table 3), in our series the V388M mutation was present in four BH₄-responsive patients in combination with other missense mutations not previously reported as responsive (Y168H, P362T, and L348V) (Table 1), except for one (R241H) [7]. However, when the V388M mutation was associated with two mutations with zero PRA (P279fsdelC and S349P) (Table 2), no response was observed. Moreover, the I65T mutation described as a responsive mutation [6,15] was found in our series in two partially responsive patients (numbers 22 and 24), in one of whom it was associated with a functionally null mutation (R261X), but it was also present in five patients with no response, including patient 48 (homozygous). This mutation was also associated with nonresponse

in other series [14]. A potential explanation for these inconsistencies is that the I65T mutation is located in the regulatory domain of the PAH gene, so that the activity of the mutated enzyme may be subject to regulation by substrate concentrations. Therefore, the Phe tolerance of patients carrying this mutation and also the BH₄ response may partially depend on the target plasma Phe levels [23]. Furthermore, the L48S mutation, also located in the regulatory domain, was described as responsive [5] in homozygosis, but in our series was associated with some response in two patients, in one case in combination with another responsive mutation (number 23, Table 1). However, no response was observed when it was combined with a mutation with zero PRA (S349P) (number 37, Table 2). The R261Q mutation was associated with responsiveness both in combination with null mutations and not (patients 17, 23, 25, and 26, Table 1) and also in other series [6,7,14,15]. However, it was found not to be responsive in another study [17]. Finally, we should note that the mutation R243Q, with some residual enzymatic activity (<10%), was associated with BH₄ responsiveness [28] in patient 5 in combination with a null mutation (IVS10), while patient 35 with very low response also carries it in combination with a known responsive mutation (I65T), so that its real response is not clear. These observations confirm the lack of correlation between genotype and BH₄ responsiveness [7].

In conclusion, all patients with mild PKU and 75% of patients with moderate PKU were BH₄ responsive, while only 11% of classic PKU patients showed good/partial response. Both the percentage of Phe decrease and the final Phe value achieved 24 h after the loading are valuable data in predicting the BH₄ response. We found four mutations not previously associated with BH₄ responsiveness and confirmed the BH₄ response of most of the reported mutations. However, further studies in PAH protein structure are necessary to understand the controversial results, which might be explained by the regulative properties of the cofactor BH₄ on the mutated PAH protein, complicated by the great number of different mutations and combinations in the PAH gene. Considering the difficulties in predicting a good response on the basis of patient genotype, a combined Phe/BH₄ loading test should be recommended for all PKU patients except those carrying null mutations in both alleles.

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