

## Tetrahydrobiopterin responsiveness: results of the BH<sub>4</sub> loading test in 31 Spanish PKU patients and correlation with their genotype

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### Abstract

Tetrahydrobiopterin (BH<sub>4</sub>) responsiveness in patients with mutations in the phenylalanine hydroxylase (PAH) gene is a recently recognized subtype of hyperphenylalaninemia characterized by a positive BH<sub>4</sub> loading test. According to recent estimates, this phenotype may be quite common, suggesting that a large group of individuals may benefit from BH<sub>4</sub> substitution, eliminating the need of life-long dietary restrictions. This underscores the importance of identifying BH<sub>4</sub>-responsive patients in each population, establishing the association with specific PAH mutations. In this work, we describe the results of a pilot study performed with 31 Spanish PAH-deficient patients subjected to a BH<sub>4</sub> loading test. Overall, 11/31 (37%) showed a positive response with a 30% decrease in blood Phe levels 8 h after the BH<sub>4</sub> challenge, and three additional patients, considered slow responders, showed this decrease only after 12–16 h. We report for the first time a patient homozygous for a splicing mutation with a slow response, suggesting an effect of BH<sub>4</sub> supplementation on PAH gene expression. Most of the responsive patients belong to the mild hyperphenylalaninemia (MHP) or mild phenylketonuria phenotypic groups. In MHP patients we report for the first time the results of parallel single Phe doses confirming the utility of these analyses for a better evaluation of the response. Genotype analysis confirms the involvement in the response of specific mutations (D415N, S87R, R176L, E390G, and A309V) present in hemizygous patients, and provide relevant information for the discussion of the potential mechanisms underlying BH<sub>4</sub> responsiveness.

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### Introduction

(6R)-L-Erythro-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) is the essential cofactor in the hydroxylation of phenylalanine (Phe), catalyzed by hepatic phenylalanine hydroxylase (PAH). A deficiency in any of the components of the PAH system results in hyperphenylalaninemia (HPA), the majority of them caused by mutations in the PAH gene and treated with a Phe-restricted dietary

therapy [1]. On the basis of Phe levels at diagnosis and Phe tolerance, these patients can be phenotypically classified as classic, moderate, mild phenylketonuria (PKU), and mild hyperphenylalaninemia (MHP), the latter not requiring dietary restriction [2]. More infrequent forms of HPA are due to defects in the biosynthesis or regeneration of BH<sub>4</sub>. A standardized BH<sub>4</sub> loading test allows the distinction between PAH and BH<sub>4</sub> defects, although additional analyses (urinary pterins and dihydropteridine reductase activity in erythrocytes) are necessary for an exact diagnosis [3]. A novel subtype of PAH deficiency termed BH<sub>4</sub>-responsive has recently been recog-

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nized after several independent reports of patients showing a decrease in plasma Phe levels in a BH<sub>4</sub> loading test [4–6]. This opens the promising possibility of a novel therapeutic strategy for some PKU patients, overcoming the psychosocial burden of a life-long restricted dietary therapy. However, there are several points still at issue, such as the protocol and interpretation of the BH<sub>4</sub> loading tests, the long-term effectiveness and cost of BH<sub>4</sub> monotherapy, and the exact molecular mechanisms underlying the BH<sub>4</sub>-response.

The proportion of BH<sub>4</sub>-responsive PKU patients is difficult to decide, as some of the reports in the literature offer contradictory results [7,8]. Most of the MHP patients have been found to be BH<sub>4</sub>-responsive [5,6]. However, as these patients are not on dietary therapy, the relevance of such studies is limited. From the other phenotypic classes, most of the BH<sub>4</sub>-responsive patients are mild PKU and a correlation with mutations bearing partial activity has been suggested [6,9]. The need for the elucidation of the molecular mechanism underlying BH<sub>4</sub> responsiveness has been stressed, as this could aid in the classification of potential BH<sub>4</sub>-responsive patients according to their genotype.

Our group has recently investigated the effect of BH<sub>4</sub> on a number of mutant PAH proteins associated with BH<sub>4</sub> responsiveness in several expression systems and it seems that the basis of the response is multifactorial [10]. Some mutant proteins have a decreased binding affinity for the cofactor, while in others BH<sub>4</sub> exerts a chaperon-like effect protecting them from degradation. There appears to be a general protective effect against inactivation in the *in vitro* system, probably related to preventing the generation of destructive oxygen species [10]. Moreover, it has also been suggested that BH<sub>4</sub> could regulate PAH gene expression or stabilize PAH mRNA [11,12].

Our group has studied in detail the molecular basis of PKU in Spain, revealing a great genetic heterogeneity and a mutational spectrum markedly different from Northern Europe [13,14]. The present work describes the result of a pilot study with 31 patients who were subjected to a BH<sub>4</sub> loading test, with the aim of analyzing BH<sub>4</sub> responsiveness in our population and the correlation of the response with the patients' genotypes.

## Patients and methods

### Patients

During the years 2000–2004 all the PKU patients born in Madrid were routinely subjected to a BH<sub>4</sub> loading test, following the official guidelines. Urinary pterin analysis and DHPR activity measurements excluded a BH<sub>4</sub> deficiency in these cases. In addition, 23 PKU patients under follow-up and selected on the basis of their

phenotype or their genotype (with mutations previously reported to be associated with BH<sub>4</sub> responsiveness) were included in this pilot study. Overall, six MHP patients, 12 mild PKU, five moderate PKU, and eight classical PKU patients were subjected to the BH<sub>4</sub> loading test. The relevant genotype and phenotype data of the patients are detailed in Table 1.

### Genotype analysis

Genotype identification was performed by DGGE analysis and direct sequencing, as previously described [13]. Genomic DNA from patients and family was extracted from whole blood using standard procedures [15]. "Broad-range" DGGE was employed to localize the mutations prior to sequencing [16]. Direct cycle sequencing was performed with BigDye Terminator v.3.1 mix (Applied Biosystems) and analyzed by capillary electrophoresis on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). The Mendelian inheritance was confirmed in all cases by DGGE or sequencing analysis. In four patients with only one allele identified after DGGE analysis the 13 exonic fragments of the PAH gene were sequenced discarding the presence of the second mutation in the studied regions of the PAH gene.

### Amino acids and pterin analysis

Blood phenylalanine (Phe) and tyrosine (Tyr) were determined fluorimetrically after reversed-phase high performance liquid chromatography (HPLC) analysis, based on the method of Allen et al. [17], adapted for whole blood samples spotted onto filter paper. The method was previously validated comparing the Phe and Tyr values obtained in blood filter samples and the corresponding plasma samples from PKU patients and controls. The HPLC system was Model HP1100 from Agilent technologies with a fluorescence detector. Amino acids were eluted from filter blood spots with perchloric acid. After filtration, the samples were injected onto a zorbax eclipse XDB-C18, 250 × 4.6 mm, I.D., 5 μm HPLC column. Phe and Tyr were detected by their natural fluorescence at λ<sub>EX</sub> 215 nm and λ<sub>EM</sub> 283 nm.

Pterin analysis in plasma and urine samples was performed by HPLC analysis, using the column described above, and according to established methods [18]. Pterins were monitored fluorimetrically at λ<sub>EX</sub> 360 nm and λ<sub>EM</sub> 440 nm.

### BH<sub>4</sub> loading test

6R-BH<sub>4</sub> (Schirck's Laboratories, Jona, Switzerland), the biologically active form, was used for all tests. Two days before the loading test and during the entire testing period, patients had no dietary restrictions. Basal urine, plasma, and blood samples are taken and a single

Table 1  
Phenotype and genotype data and results of the BH<sub>4</sub> loading test of the patients included in this study

Reference	Present age	Phe at diagnosis (μM)	Tolerance <sup>a</sup> mg Phe/day	Phenotype	Allele 1	Allele 2	BH <sub>4</sub> response <sup>b</sup>
12710	38 years	420	—	MHP	<b>I65T</b>	<b>A300S</b>	+
18447	1 year	300	—	MHP	<b>A300S</b>	<b>R261Q</b>	+
18801	8 months	420	—	MHP	<b>D415N</b>	R176X	+
18811	7 months	258	—	MHP	<b>S87R</b>	S349P	+
18930	7 months	250	—	MHP	<b>R176L</b>	P281L	+
19548	31 years	440	—	MHP	<b>IVS10-3C&gt;T</b>	<b>IVS10-3C&gt;T</b>	+/-
8438	18 years	850	1000	Mild PKU	I65T	IVS10	—
8439	18 years	950	1000	Mild PKU	I65T	IVS10	—
10806	12 years	1450	850	Mild PKU	R68S	IVS7nt3	—
11332	12 years	890	<2500 <sup>c</sup>	Mild PKU	<b>Y414C</b>	<b>L348V</b>	+
12525	18 years	1030	800	Mild PKU	IVS10	T418N	—
12528	17 years	1000	1000	Mild PKU	<b>Y414C</b>	ND	+
12556	24 years	1000	1000	Mild PKU	P122Q	F39L	—
12572	23 years	583	<2500 <sup>c</sup>	Mild PKU	R408Q	L311P	—
12576	25 years	1060	700	Mild PKU	<b>I65T</b>	<b>P244L</b>	+
12895	8 years	1160	700	Mild PKU	<b>P275R</b>	<b>L348V</b>	+
18236	1 year	1130	700	Mild PKU	<b>I65T</b>	<b>R408Q</b>	+
19068	12 years	500	<2500 <sup>c</sup>	Mild PKU	<b>E390G</b>	IVS12nt1	+
9384	20 years	1660	550	Moderate PKU	I65T	R261Q	—
11353	12 years	2050	450	Moderate PKU	IVS1nt5	L348V	—
12324	9 years	2160	550	Moderate PKU	<b>I65T</b>	<b>V388M</b>	+/-
12562	19 years	1500	350	Moderate PKU	V388M	IVS10	—
12569	9 years	2000	450	Moderate PKU	I65T	S196fs	—
10637	13 years	2300	250	Classical PKU	<b>A309V</b>	IVS1nt5	+/-
11942	10 years	2000	250	Classical PKU	R261Q	R408W	—
14116	5 years	2660	150	Classical PKU	IVS10	ND	—
15618	3 years	2454	150	Classical PKU	IVS10	delF39	—
16550	3 years	2500	150	Classical PKU	R158Q	E280K	—
16714	2 years	2440	150	Classical PKU	R243Q	ND	—
17045	2 years	1900	150	Classical PKU	R261X	R243Q	—
18149	1 year	2660	350	Classical PKU	IVS1nt5	ND	—

Mutations potentially involved in the BH<sub>4</sub> response are depicted in bold.

ND, not determined.

<sup>a</sup> Calculated at 5 years of age, except for patients born after 2000.

<sup>b</sup> +, positive response with ≥30% decrease in Phe levels 8h after BH<sub>4</sub> challenge; —, no response; +/-, slow response with ≥30% decrease in Phe levels 12–16h after BH<sub>4</sub> challenge.

<sup>c</sup> No protein restriction but taking a special Phe-free formula.

dose of BH<sub>4</sub> (20 mg/kg) is administered orally. After the BH<sub>4</sub> challenge, blood filter spots and urine samples are taken up to 24h. In some cases plasma samples are also taken at 0, 4, 8, and 24h for pterin analysis and for validation of the blood filter method for amino acid analysis. Patients with Phe levels at diagnosis <360 μM (MHP patients) were subjected to a combined Phe/BH<sub>4</sub> loading test. A challenge of 100 mg Phe/kg is given 3h before BH<sub>4</sub> loading. Plasma Phe levels were confirmed to peak 3h after the challenge. For better evaluation of the results, MHP patients were also given a single Phe load (100 mg Phe/kg) and monitored for 24h. The single Phe load and combined Phe/BH<sub>4</sub> loading test were performed in 2 consecutive days.

Patients were considered to have a positive response in the BH<sub>4</sub> loading test if they showed a decrease in blood Phe values ≥30% 8h after the BH<sub>4</sub> challenge,

considering the starting value the one measured before BH<sub>4</sub> administration, as previously described [19].

## Results and discussion

From the 31 patients included in this study, 11 showed a positive response in the BH<sub>4</sub> loading test (Table 1), five MHP patients and six mild PKU. Three additional patients (one MHP, one moderate, and one classical PKU) may be considered as slow responders, as a >30% reduction was reached only 12–16h post-loading, as discussed below.

Concerning MHP patients, this is the first report of the parallel evaluation of single Phe doses. It has been previously suggested that decreases of Phe values in these patients may be partly due to spontaneous elimi-

nation of plasma Phe [6,19]. This can be readily observed in the results obtained in Phe loading tests performed (Table 2 and Fig. 1). Most MHP patients without a BH<sub>4</sub> challenge already show a decline in Phe levels >30% after 12h, and in one of them (18811), this decrease was already observed after 8h. This could lead to misleading conclusions. However, Phe levels fall more rapidly after the BH<sub>4</sub> challenge, and most of the patients show a 35–60% decrease in Phe values levels already after 4h. These results confirm that ideal testing for MHP patients should include a single Phe load for a better evaluation of the response.

Most of the MHP patients in our series showed a positive response, correlating with other reports in the literature [5,6]. The majority of the missense mutations detected in our responsive MHP patients has been previously reported in earlier BH<sub>4</sub> trials (D415N, A300S, I65T, and R261Q). The exceptions are mutations R176L and S87R which are present in functionally hemizygous patients (i.e., in combination with a functionally null mutation), confirming their direct involvement in BH<sub>4</sub> responsiveness. Mutations conferring the MHP phenotype are associated with high residual activity in expression studies [20–22]. One can speculate that the high residual PAH activity in combination with a stabilizing BH<sub>4</sub> effect or the activation of PAH gene transcription could explain why most MHP patients are responsive.

Of particular interest is patient 19548, who was recently detected in a pilot study aiming to prevent maternal hyperphenylalaninemia, and consisting in the detection of adult undiagnosed MHP women in the Madrid region who were not screened in the newborn period. This patient presented Phe levels at diagnosis of 440 μM and has led an asymptomatic life, finishing higher education. She was classified as MHP and subjected to a BH<sub>4</sub> loading test and genotype analysis. During the week previous to the BH<sub>4</sub> loading test, her Phe levels ranged from 230 to 424 μM with normal dietary intake and she was therefore subjected to a combined Phe/BH<sub>4</sub> load. Genotype analysis surprisingly revealed that

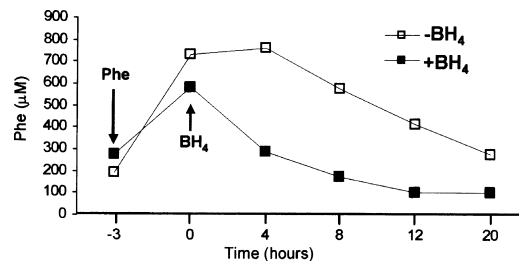


Fig. 1. Combined Phe/BH<sub>4</sub> (+BH<sub>4</sub>) loading test and single Phe loading (-BH<sub>4</sub>) in the MHP patient 18930 (R176L/P281L). Phe was given as a single dose (100mg/kg) and 3h later 20mg/kg BH<sub>4</sub> was administered orally.

she is homozygous for IVS10-3 C>T, a splicing mutation which causes exon 11 skipping [23]. In vitro splicing studies had shown that there is some normal splicing (the splicing efficiency of the mutant transcript was 10–30% of that of the wild-type) [23], which could explain the mild phenotype exhibited by the patient and the highly variable Phe levels under normal diet before, during and after the test. Concerning the BH<sub>4</sub> loading test, the patient has a slow response, with a 30% reduction in Phe levels after 12h. This is the first report of a splicing mutation potentially associated with BH<sub>4</sub> responsiveness. The response can be explained assuming that BH<sub>4</sub> exerts some effect on PAH gene transcription or PAH mRNA stabilization, previously suggested [12,24], which would increase the levels of normally spliced transcript and thus residual PAH protein and activity.

From the remaining patients, six mild PKU patients fulfilled the criteria of a positive BH<sub>4</sub> response (Table 3) and two (one moderate and one classical PKU) reached a 30% decrease in Phe levels only after 16h (slow responders). Increases in Tyr levels were not significant. The maximum decrease in Phe ranged from 45 to 72% at 12–16h post-loading (Table 3). Phe levels rise again at 24h. The estimated elimination half-life of orally administered BH<sub>4</sub> is approximately 4–5h [7,25] which accounts for these results. Although interindividual variations in intestinal absorption of BH<sub>4</sub> have

Table 2

Reduction in blood Phe values during the single Phe and combined Phe/BH<sub>4</sub> loading test in BH<sub>4</sub>-responsive MHP patients

Reference	Genotype	Phe (μM) +BH <sub>4</sub> /-BH <sub>4</sub> <sup>a</sup>		% Reduction in blood Phe levels +BH <sub>4</sub> /-BH <sub>4</sub> <sup>a</sup>			
		-3h	0h	4h	8h	12h	20h
12710	A300S/I65T	649/410	1742/1420	35/11	61/17	75/35	87/54
18801	D415N/R176X	354/295	1074/1098	36/0	87/7	92/36	n.d.
18447	A300S/R261Q	267/n.d.	1868/n.d.	23/n.d.	39/n.d.	59/n.d.	90/n.d.
18811	S87R/S349P	46/188	646/437	59/15	78/33	65/55	67/n.d.
18930	R176L/P281L	277/194	585/735	50/0	70/22	82/43	83/62
Slow responder							
19548	IVS10-3A>C/IVS10-3A>C	1154/698	2202/1468	10/0	16/0	31/0	66/n.d.

n.d. not determined.

<sup>a</sup> In the combined Phe/BH<sub>4</sub> loading test (+BH<sub>4</sub>), the BH<sub>4</sub> challenge (marked as time 0) was given 3h after the Phe load and blood Phe values were monitored up to 20–21h. Single Phe loading (-BH<sub>4</sub>) was monitored for the same period of time.

Table 3  
Reduction in blood Phe values after BH<sub>4</sub> challenge in BH<sub>4</sub>-responsive PKU patients

Reference	Genotype	Phenotype	Basal Phe (μM)	Phe values, μM (% reduction)				
				4h	8h	12h	16h	24h
11332	Y414C/L348V	Mild PKU	441	242 (45)	145 (67)	226 (49)	330 (25)	485 (0)
12528	Y414C/nd	Mild PKU	1094	856 (22)	620 (43)	352 (68)	464 (58)	564 (48)
12576	I65T/P244L	Mild PKU	1391	1301 (6)	941 (32)	1099 (21)	1074 (23)	931 (33)
12895	P275R/L348V	Mild PKU	816	592 (27)	419 (49)	445 (45)	614 (25)	528 (35)
18236	R408Q/I65T	Mild PKU	1097	884 (19)	793 (29)	662 (40)	621 (43)	707 (36)
19068	E390G/IVS12nt1	Mild PKU	732	445 (39)	281 (62)	206 (72)	198 (73)	265 (64)
Slow responders								
10637	A309V/IVS1nt5	Classical PKU	1165	1109 (5)	921 (21)	889 (24)	707 (40)	1091 (5)
12324	V388M/I65T	Moderate PKU	1717	1456 (15)	1674 (5)	1274 (26)	1153 (33)	1694 (3)

been suggested to account for variable responses, in all our patients pterin analysis showed a similar pattern of BH<sub>4</sub> absorbance. The maximum biopterin concentration was always reached after 4h, representing  $85.7 \pm 5\%$  of total pterins (neopterin plus biopterin).

In most of the BH<sub>4</sub>-responsive patients (exceptions are 12576, 18236, and the slow responders), Phe levels dropped below 500 μM, within the therapeutic range for their age, suggesting BH<sub>4</sub> monotherapy could be suitable and effective for them. Three patients (12528, 12895, and 19068) are currently enrolled for a long-term treatment trial with BH<sub>4</sub> under no dietary restriction. For patients 12576 and 18236 the results suggest that a combined BH<sub>4</sub> and dietary therapy would be most effective to keep Phe levels within the therapeutic range. In addition, some authors have reported age-dependency of the results of the BH<sub>4</sub> loading test, so patient 18236, at present 1 year old, could be re-evaluated in the following years. For the patients classified as slow responders, the possible contribution of the diurnal plasma Phe variation to the slow response observed must be taken into account. Optimal BH<sub>4</sub> dosage should be adjusted to confirm a positive response and the suitability for a BH<sub>4</sub>-based therapy. At present, patient 10637 is also selected for a long-term trial with BH<sub>4</sub> monitoring the potential increase in Phe tolerance.

In our series, 6/11 (54%) mild PKU patients are clearly BH<sub>4</sub> responsive. Other reports have shown larger proportions [7,26], but differences in the BH<sub>4</sub> loading tests and in the definition of a positive response could account for this. In this regard, we believe it is important that patients have no dietary restrictions during the testing period, to determine precisely the effect of BH<sub>4</sub> supplementation, avoiding the spontaneous elimination of Phe due to PAH mutations with partial activity, and, in short, to offer a more reliable prediction of the result of a potential BH<sub>4</sub> monotherapy in those patients.

All the PKU patients included in this study are compound heterozygous. The presence of mutations D415N and E390G in combination with functionally null muta-

tions confirm their direct involvement in BH<sub>4</sub> responsiveness. In patients 12895 (P275R/L348V), 18236 (I65T/R408Q), 12710 (I65T/A300S), 18447 (R261Q/A300S), 12576 (I65T/P244L), and the slow responder 12324 (V388M/I65T) the contribution of each mutation to the responsive phenotype is unclear, requiring further expression studies. The I65T mutation alone (with a null mutation, see Table 1, or in homozygous patients) [6] does not result in BH<sub>4</sub> responsiveness. A modest decrease in the binding affinity for BH<sub>4</sub> in the I65T mutant protein was found [10], but it appears that the in vivo response depends critically on the second mutant allele also with residual activity (in our patients, R408Q, P244L and V388M, but not R261Q) which determines the molecular composition of the PAH mutant subunits present in the tetrameric form of the enzyme in vivo. Mutation R261Q has been positively associated to the BH<sub>4</sub>-responsive phenotype in one study [6] but it has also been found inconsistently associated with responsiveness [5], which could at first hand be explained by discrepancies between the BH<sub>4</sub> loading tests performed. However, this residue is implicated in the interaction between neighboring subunits and in this case, the second PKU allelic variant in the patient could have a high significance in determining the BH<sub>4</sub> responsiveness.

Mutation A309V, present in an hemizygous patient considered a slow responder (10637), has been demonstrated to be stabilized by BH<sub>4</sub> in an in vitro system [10]. This mutation is located near the dimerization motif and the binding of the cofactor may favor the proper dimer/tetramer formation resulting in reduced protein instability and degradation.

The great genetic heterogeneity in PKU patients justifies the study of BH<sub>4</sub> responsiveness in each population, to determine the prevalence and to identify additional associated mutations, which along with detailed expression studies may help unravel the exact mechanisms underlying this phenomenon which offers a promising possibility to treat a subgroup of PKU patients.

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