

Incidence of BH₄-responsiveness in phenylalanine-hydroxylase-deficient Italian patients

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Abstract

Background: Hyperphenylalaninemia (HPA) is an inherited metabolic disorder due to deficiency of the enzyme phenylalanine hydroxylase (PAH) or its cofactor tetrahydrobiopterin (BH₄). BH₄-responsiveness in PAH-deficient HPA is a recently described characteristic of most milder phenotypes. BH₄-responsive patients show reduction of plasma phenylalanine (phe) levels after oral administration of BH₄.

Aim: Determination of the incidence of BH₄-responsiveness among a non-selected, cohort population of PAH-deficient hyperphenylalaninemic patients and evaluation of phenotype–genotype correlations.

Patients and methods: All patients born in Lombardy (Italy) between January 2000 and December 2004, and affected by HPA (107 patients) were classified after BH₄ loading test, analysis of urinary pterins, and determination of DHPR activity in blood, and investigated for BH₄-responsiveness. 6R-BH₄ (20 mg/kg) was administered orally as a single dose and plasma samples were obtained at time-points 0, 4, 8, and 24 h after BH₄ administration. In patients with basal plasma phe levels ≤360 mmol/L a combined phe (100 mg phe/kg) and BH₄ (20 mg/kg) loading test was performed. Patients were defined “responsive to BH₄” when plasma phe levels decreased by 30% 8 h after oral BH₄ administration.

Results: BH₄ significantly lowered blood phe levels in 91 (85%) of 107 patients affected by PAH-deficient HPA. Most responsive patients were affected by mild HPA (77%), a smaller percentage by mild (7%) and moderate (7%) phenylketonuria (PKU). One patient with classical PKU was responsive to BH₄. Eighteen mutations were found to be associated to the BH₄-responsive phenotype.

Conclusions: BH₄-responsiveness is shown by a consistent number of PAH-deficient hyperphenylalaninemic patients and seems to be common in milder phenotypes. Genotype is not the only factor determining BH₄-responsiveness.

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Introduction

Hyperphenylalaninemia (HPA) (Mc Kusick 261600) is a common inherited error of aminoacid metabolism due to deficiency of the enzyme phenylalanine-hydroxylase (PAH). A minor number of cases of HPA is due to mutation of one of the enzymes involved in the synthesis

or recycling of its cofactor, tetrahydrobiopterin (BH₄). Differential diagnosis between the two forms of HPA is performed by the BH₄ loading test, analysis of urinary pterins, and determination of DHPR activity in blood. Patients affected by BH₄-deficiency show normalisation of plasma phenylalanine (phe) levels after oral administration of BH₄. Recently also patients affected by PAH-deficiency have been shown to respond to oral administration of BH₄ by lowering plasma phe levels.

Thus, considering responsiveness to the BH₄ loading test, HPA can be classified into three different categories:

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non-responsive PAH-deficiency, responsive PAH-deficiency, and BH₄-deficiency.

Reports of BH₄-responsiveness in PAH-deficient HPA patients are numerous in literature, since description of the very first patients in 1999 [1,2].

Most BH₄-responsive patients are affected by mild HPA (MHP, phe levels 120–360 μmol/L). A smaller number of BH₄-responsive patients shows more severe phenotypes with low phe tolerance, undergoing a diet therapy. In the latter group, BH₄ may represent a valid alternative to the standard dietary treatment. Pharmacological therapy with BH₄ was introduced as mono-therapy or in combination with a less restricted diet in a number of patients and seems to be successful [3–6]. First correlations between BH₄-responsive phenotype and genotype were described [7], but the incidence and prevalence of BH₄-responsiveness in PAH-deficiency and the genotype–phenotype association have to be further investigated.

Aim of the study

The aim of our study was to determine the incidence of BH₄-responsiveness in a non-selected, cohort population of HPA patients, and to verify genotype–phenotype correlation in BH₄-responsiveness.

Patients and methods

All patients born in Lombardy between January 2000 and December 2004, and affected by HPA (107 cases) were investigated for BH₄-responsiveness. All patients were detected by neonatal screening for HPA (plasma phe >120 μmol/L) and diagnosis was confirmed within the first 25 days of life. Inclusion criteria for the study were: birth at term, uneventful pregnancy, and normal neonatal period. Exclusion criteria were: associated chronic diseases or malformations. No siblings were included in this study. HPA due to BH₄-deficiency was excluded at diagnosis by BH₄ loading test, analysis of urinary pterins, and determination of DHPR activity on blood spot. The BH₄ loading test was performed according to a standardised protocol; 6R-BH₄ was adminis-

tered orally to all patients as a single dose of 20 mg/kg. Plasma samples for the assessment of the aminoacid profile were collected before 4 and 8 h after oral BH₄ administration. In 60 patients, a blood sample for plasma phe was drawn 24 h after BH₄ loading to reveal late-responders [8]. In patients with basal plasma phe levels lower than 360 μmol/L, a combined phe and BH₄ loading test was performed: 100 mg phe/kg body weight was administered orally 3 h before the BH₄ loading test. There was no dietary restriction during the loading test.

BH₄-responsiveness was defined as a 30% decline in plasma phe levels 8 h after oral administration of BH₄.

Patients were assigned to one of the four phenotype categories according to pre-treatment plasma phe concentrations: 83/107 patients (77%) were classified as MHP (plasma phe 120–360 μmol/L), 7/107 patients (7%) were assigned to the mild PKU category (plasma phe 360–600 μmol/L), 7/107 patients (7%) were assigned to the moderate PKU group (plasma phe 600–1200 μmol/L), and 10/107 (9%) to classic PKU (plasma phe >1200 μmol/L).

Mutation analysis was carried out by standard protocols. Restriction enzyme analysis was used to identify some recognised mutations, denaturing gradient gel electrophoresis analysis of the polymerase chain reaction (PCR)-amplified exons and flanking intronic sequences of the PAH gene was carried out directly on PCR-amplified products with the non-guanine–cytidine clamped primers of each exon, using the Applied Biosystem Model 310 DNA Sequencing System and the Rhodamine Terminator Ready Reaction (Perkin Elmer, Italy).

Results

The total incidence of BH₄-responsiveness in our population of HPA patients was 85%, with a significant decline in plasma phe concentration 8 h after oral BH₄ administration in 91 of 107 patients (Table 1). Most HPA patients of the studied population were affected by MHP (83/107, 77%); the majority of these patients were BH₄-responsive (80/83, 96.4%). Nevertheless non-responsiveness to BH₄ was found in three MHP patients, showing percentages of reduction in plasma phe levels of 16.9, 22.6, and 28.6% (Table 2a, Patient 6, 11, and 51).

Table 1
Incidence of BH₄-responsiveness among HPA patients born between January 2000 and December 2004 in Lombardy (Italy)

Year of birth	MHP (responsive)	Mild PKU (responsive)	Moderate PKU (responsive)	Classic PKU (responsive)	Total (responsive)
2000	13 (11)	1 (1)	2 (0)	18 (13)	18 (13)
2001	19 (19)	1 (1)	2 (1)	22 (21)	22 (21)
2002	18 (17)	2 (2)	2 (2)	27 (21)	27 (21)
2003	15 (15)	1 (1)	1 (0)	18 (16)	18 (16)
2004	18 (18)	2 (2)	0	22 (20)	22 (20)
Total	83 (80)	7 (7)	7 (3)	10 (1)	107 (91)

Table 2a
Plasma phenylalanine levels ($\mu\text{mol/L}$) 8 h after BH_4 loading (20 mg/kg) in PAH-deficient patients

Patient number	Time 0 h	Time 3 h after phe load	Time 8 h after BH_4 load	% (reduction at 8 h)
1	139.00	656.33	23.1	96.5
2	1967.99	—	2009.66	-2.1
3	247.00	819.57	240.81	70.6
4	334.00	791.34	22.57	97.1
5	153.00	595.84	42.82	92.8
6	132.00	246.38	190.49	22.7
7	167.00	803.74	114.04	85.8
8	228.00	780	438	43.8
9	507.00	898.8	550.8	38.7
10	291.00	580.8	66.6	88.5
11	120.60	847.2	604.8	28.6
12	134.89	672.76	127.1	81.1
13	201.48	501.02	36.34	92.7
14	1282.00	—	877.8	31.5
15	1182.00	—	1110	6.1
16	197.63	589.61	145.51	75.3
17	841.21	—	738.42	12.2
18	204.00	430.11	157.55	63.4
19	210.18	380.79	10.32	97.3
20	155.34	341.04	56.24	83.5
21	185.40	399	71.40	82.1
22	291.60	557.4	88.8	84.1
23	1170.30	—	1198.85	-2.4
24	121.00	498.36	43.18	91.3
25	224.54	703.51	183.93	73.9
26	358.00	840.36	204.21	75.7
27	316.80	787	29.97	96.2
28	144.30	630.06	107.57	82.9
29	361.00	—	28.86	92.0
30	224.62	572.37	46.9	91.8
31	814.16	—	287.35	64.7
32	174.28	673.16	122.93	81.7
33	175.95	541.71	314.74	41.9
34	176.95	407.42	126.56	68.9
35	139.79	557.98	100.87	81.9
36	213.66	527	60.1	88.6
37	139.00	768	18	97.7
38	132.00	443	92	79.2
39	267.00	639	123	80.8
40	322.00	774	31	96.0
41	1474.98	—	1440.42	2.3
42	1186.00	—	740	37.6
43	351.45	748.32	96.99	87.0
44	160.14	570.68	149.76	73.8
45	223.25	909.56	147.35	83.8
46	134.00	630.6	110.21	82.5

In patients with plasma phe $<360 \mu\text{mol/L}$ a double phenylalanine (100 mg/kg) and BH_4 (20 mg/kg) loading test has been performed. In bold letters, not responsive patients.

The MHP patient showing a 16.9% reduction of plasma phe at 8 h had a decline of 29.5% at 24 h after BH_4 administration.

Among mild PKU and moderate PKU patients, 100% (7/7) and 42.9% (3/7), respectively, were responsive to oral administration of BH_4 . One classical PKU patient showed a 31.5% decline in phe levels 8 h after BH_4 loading (Table 2a, Patient 14), accordingly he satisfied the criterion for responsiveness to BH_4 .

One single patient was a late-responder: this patient, classified as a classical PKU phenotype showed a 41% decline in plasma phe levels 24 h after BH_4 administration and a non-significant value at 8 h (Table 2b, Patient 49).

The identification of the HPA underlying genotype was available in 74 patients; 66 genotypes were associated to BH_4 -responsiveness at 8 h after BH_4 loading. Mutations on both alleles and genotype classifications are shown in Table 3a. Two new mutations at the locus of PAH gene (969 + 2inst, Patient 35 and T1903I, Patient 86) were identified by genotype investigation.

Genotype analysis revealed 18 different mutations associated with BH_4 -responsiveness (A403V, V245A, I65T, E178G, V230I, T380M, E390G, H201Y, D415N, T92I, A300S, Y414C, P366H, I94S, R261Q, P211T, M276V, IVS4nt5c \rightarrow g) (Table 3a). Most of these responsiveness-associated mutations have already been classified as MHP or mild PKU mutations, two mutations were moderate PKU mutations (I65T and R261Q) [15]. Mutations that occurred most frequently are V245A (19 patients) and A403V (21 patients), being the latter one expressed in homozygosity in four patients. Other three BH_4 -responsive patients expressed homozygosity for I94S, R261Q and IVS4ntc \rightarrow g. I94S is a not yet classified mutation, in our homozygous patient it was expressed as a MHP phenotype (Table 2a, Patient 19). BH_4 -responsiveness in a MHP phenotype was also found in another patient with a not yet classified mutation (P366H) in combination with a null-mutation (R158Q) (Table 3a, Patient 5), corresponding to a condition of functional hemizygosity.

Genotypes of eight non-responsive patients were detected (Table 3b). In our population homozygosity for the L48S mutation was not associated with BH_4 -responsiveness (Patients 49 and 74), the decline in plasma phe values was 29.7 and 18.8%, respectively. Moreover, in two patients presenting heterozygosity for the same mutation (Patients 23 and 59) plasma phe levels showed almost no variation after BH_4 administration. Non-responsiveness was found in association with a heterozygous genotype with four non-classified mutations (R53H, R297H, L15/516sdelCT, T278N) combined with a classic or a moderate PKU mutation (Patients 6, 51, 2, and 63).

Discussion

This is the first study on BH_4 -responsiveness in an unselected PAH-deficient cohort of HPA patients, born in Lombardy between January 2000 and December 2004, classified and investigated for responsiveness to oral administration of BH_4 at a standardised dose.

Most of our patients are affected by milder phenotypes, based upon the genotype pattern of the Mediter-

Table 2b

Plasma phenylalanine levels ($\mu\text{mol/L}$) 8 and 24h after BH_4 loading (20mg/kg) in PAH-deficient patients

Patient number	Time 0h	Time 3h after phe load	Time 8h after BH_4 load	Time 24h after BH_4 load	% (reduction at 8h)	% (reduction at 24h)
47	186.00	703	29	219	95.9	68.8
48	285.01	669.94	246.52	97.87	63.2	85.4
49	1515.97	—	1089	881.65	28.2	41.8
50	149.44	865.55	33.37	42.15	96.1	95.1
51	260.20	437.99	363.81	308.87	16.9	29.5
52	164.25	674.93	138.73	90.72	79.4	86.6
53	602.61	—	199.34	97.2	66.9	83.9
54	167.40	425.4	43.8	90.6	89.7	78.7
55	165.75	416.73	129.65	82.47	68.9	80.2
56	170.10	438.33	37.4	27.17	91.5	93.8
57	232.35	450.9	169.29	93.7	62.5	79.2
58	458.22	—	212.93	134.06	53.5	70.7
59	1524.08	—	1571.6	1523.18	-3.1	0.1
60	236.01	777.51	88.2	49.26	88.7	93.7
61	449.25	—	97.6	187.07	78.3	58.4
62	1927.64	—	1922.92	1911.13	0.2	0.9
63	1692.68	—	1661.06	1571.43	1.9	7.2
64	143.20	480.26	42.43	58.45	91.2	87.8
65	154.13	730.18	123.79	64.32	83.0	91.2
66	216.36	595.91	211.45	106.75	64.5	82.1
67	181.30	710.9	146.85	72.63	79.3	89.8
68	152.28	939.42	109.96	64.32	88.3	93.2
69	177.98	530.27	82.49	78.83	84.4	85.1
70	1309.40	—	1270.77	1231.5	3.0	5.9
71	147.58	476.23	103.76	50.43	78.2	89.4
72	241.42	530.8	172.8	107.48	67.4	79.8
73	173.21	518.85	127.06	113.51	75.5	78.1
74	705.98	—	572.96	786.28	18.8	-11.4
75	205.42	570.95	49.26	70.32	91.4	87.7
76	339.79	1062.63	169.4	78.6	84.1	92.6
77	124.29	551.18	120.23	80.42	78.2	85.4
78	325.85	538.97	85.42	81.9	84.2	84.8
79	129.00	400	33.36	23.31	91.7	94.2
80	229.45	957.87	61.76	150	93.6	84.3
81	213.65	721.87	45.24	97.17	93.7	86.5
82	486.44	—	14.55	203.74	97.0	58.1
83	151.00	529.00	129.00	92.00	75.6	82.6
84	229.00	451.00	180.00	106.00	60.1	76.5
85	231.00	511.00	35.00	106.00	93.2	79.3
86	125.00	683.00	51.00	44.00	92.5	93.6
87	448.00	—	238.00	173.00	46.9	61.4
88	119.00	360.00	43.00	33.00	88.1	90.8
89	119.00	453.00	162.00	109.00	64.2	75.9
90	246.00	576.00	135.00	88.00	76.6	84.7
91	217.00	724.00	85.00	38.00	88.3	94.8
92	149.05	546.52	39.06	64.47	92.9	88.2
93	132.72	417.17	41.39	34.18	90.1	91.8
94	233.15	536.79	41.56	69.97	92.3	87.0
95	361.53	—	30.54	87.69	91.6	75.7
96	190.06	621.85	158.39	89.4	74.5	85.6
97	200.19	773.68	109.61	74.42	85.8	90.4
98	438.56	—	69.78	125.09	84.1	71.5
99	271.3	647.72	109.02	64.7	83.2	90.0
100	174.13	620.06	49.23	35.1	92.1	94.3
101	159.48	588.37	104.07	65.78	82.3	88.8
102	150.28	399.2	172.36	114.05	56.8	71.4
103	166.99	715.78	195.28	107.23	72.7	85.0
104	335.86	621.24	136.57	119.38	59.3	80.8
105	231.66	469.73	195.82	127.82	58.3	72.8
106	2034.32	—	2152.07	2107.34	-5.8	-3.6
107	1583.18	—	1431.65	1373.3	9.6	13.3

In patients with plasma phe <360 $\mu\text{mol/L}$ a double phenylalanine (100 mg/kg) and BH_4 (20 mg/kg) loading test has been performed. In bold letters, not responsive patients at 8 h.

Table 3a

BH₄-responsiveness associated mutations, genotype, reduction in plasma phe (% of basal phe levels) 8 h after BH₄ administration

Number	First allele	Second allele	Genotype	% reduction in plasma phe at 8 h
1	A403V	A403V	MHP	96.5
3	V245A	165delT	MHP	70.6
5	R158Q	P366H	Not classified	92.8
8	I65T	IVS10-11G > A	moderate PKU	43.8
12	E178G	V230I	MHP	81.1
14	R261Q	Y386C	Not classified	31.5
18	T380M	R158Q	MHP	63.4
19	I94S	I94S	Not classified	97.3
21	IVS10-11G > A	V245A	MHP	82.1
22	IVS10del546	V245A	MHP	84.1
25	IVS10-11G > A	A300S	MHP	73.9
26	R158Q	E178G	MHP	75.7
28	V245A	R261Q	MHP	82.9
30	165delT	A403V	MHP	91.8
31	Y414C	R261Q	Mild PKU	64.7
32	P281L	V245A	MHP	81.7
33	P281L	T380M	MHP	41.9
35	V245A	1VS9 + 2insT	MHP	81.9
37	A403V	A403V	MHP	97.7
38	V245A	R261Q	MHP	79.2
39	L48S	E390G	MHP	80.8
40	R261X	A403V	MHP	96.0
42	R261Q	R261Q	moderate PKU	38.7
43	IVS10-11G > A	P211T	Not classified	87.0
44	R158Q	H201Y	MHP	73.8
45	L48S	D415N	MHP	83.8
47	A403V	A403V	MHP	95.9
48	S67P	T92I	MHP	63.2
50	A403V	A403V	MHP	96.1
53	P281L	Y414C	Mild PKU	66.9
55	A300S	L287V	MHP	68.9
56	V245A	A403V	MHP	91.5
57	M276V	A300S	MHP	62.5
58	A300S	I65T	MHP	53.5
60	R158Q	A403V	MHP	88.7
61	S411X	D415N	MHP	78.3
64	V245A	A403V	MHP	91.2
65	R261Q	V245A	MHP	83.0
66	R261Q	A300S	MHP	64.5
67	P281L	V245A	MHP	79.3
69	V245A	Y414C	MHP	84.4
72	IVS10-11G > A	V230I	MHP	67.4
73	V245A	47_48delCT	MHP	75.5
75	V230I	A403V	MHP	91.4
77	V245A	R261Q	MHP	78.2
79	A403V	E178G	MHP	91.7
80	Y414C	A403V	MHP	93.6
81	R408Q	A403V	MHP	93.7
83	IVS7+1G > A	V245A	MHP	75.6
84	IVS2+5G > C	A300S	MHP	60.1
85	A403V	V388M	MHP	93.2
86	A403V	T1903I	MHP	92.5
87	R261Q	M276V	Not classified	46.9
88	A403V	A300S	MHP	88.1
89	IVS4-5C > G	IVS4-5C > G	Mild PKU	64.2
90	V245A	P281L	MHP	76.6
91	IVS10-11G > A	A403V	MHP	88.3
92	V245A	A403V	MHP	92.9
93	V245A	A403V	MHP	90.1
94	IVS10-11G > A	A 403V	MHP	92.3
95	E390G	A403V	MHP	91.6
97	V245A	R158Q	MHP	85.8

(continued on next page)

Table 3a (continued)

Number	First allele	Second allele	Genotype	% reduction in plasma phe at 8 h
97	V245A	R158Q	MHP	85.8
98	G272X	P211T	Not classified	84.1
99	IVS10-11G > A	D415N	MHP	83.2
100	A300S	A403V	MHP	92.1
103	T380M	V388M	MHP	72.7

Table 3b

Genotypes of non-responsive HPA patients (plasma phe reduction \leq 30% 8 h after BH₄ administration).

Patient number	First allele	Second allele	Genotype	% reduction in plasma phe at 8 h
2	IVS10-11G > A	47_48delCT	Not classified	-2.1
6	R261Q	R53H	Not classified	22.7
23	R261X	L48S	Mild PKU	-2.4
49	L48S	L48S	Mild PKU	28.2
51	P281L	R297H	Not classified	16.9
59	R408W	L48S	Mild PKU	-3.1
63	P281L	T278N	Not classified	1.9
74	L48S	L48S	Mild PKU	18.8

reanean population we studied. This specific genotype pattern, presenting a prevalence of MHP mutations, is a major determinant of BH₄-responsiveness among HPA patients, as BH₄-responsiveness is known to be a characteristic of milder forms of HPA, e.g., forms with different degrees of PAH residual enzyme activity [9].

Accordingly, in our population the BH₄ loading test with a standardised dosage (20 mg/kg body weight in one single, oral dose) led to a significant decrease in phe levels in 91/107 patients, being 80/91 MHP patients, classified on the basis of basal plasma phe levels.

However genotypes associated with consistent phenotypes are no guaranty for BH₄-responsiveness: three patients affected by MHP did not show significant reduction of plasma phe after BH₄ administration. The genotypes of non-responsive MHP phenotypes were available in two out of three patients (Table 3b, Patients 6 and 51).

In all mild PKU and three out of seven moderate PKU patients BH₄ administration lowered plasma phe levels significantly. Thus a considerable percentage of patients affected by moderate and mild PKU, undergoing diet therapy according to our therapeutic protocol, showed responsiveness to BH₄ and among these patients BH₄ might represent either a valid therapeutic alternative or a lower dietetic therapy regimen.

Our data showed that classical PKU patients may be BH₄-responsive, according to previous reports [10]. Patient 14 (Table 2a) was classified according to basal phe levels as a classical PKU phenotype and showed responsiveness to BH₄ at 8 h, however he presented a discordant, non-classifiable genotype: R261Q/Y386C (Table 3a). Extended phe determination up to 24 h revealed another classical PKU patient (Table 2b, Patient 49) to be a late-responder, but mutation analysis revealed a mild PKU genotype. The same genotype in

another patient, who was phenotypically classified as a moderate PKU, did not determine responsiveness either at 8 h or at 24 h after BH₄ administration (Table 3b, Patient 74), underlining the inconsistency between both genotype and phenotype at diagnosis, and BH₄-responsiveness and underlying genotypes.

L48S mutation has previously been reported as a BH₄-responsive mutation [11]. In our patients this mutation is related to responsiveness only when associated with an MHP mutation (Table 3a, Patients 39 and 45). Neither homozygosity (Table 3b, Patients 49 and 74) nor functional hemizygoty (Table 3b, Patients 23 and 59) determined significant decrease in plasma phe levels at 8 h. Even other contrasting results from different studies concerning genotype-phenotype correlation in BH₄-responsiveness indicate that PAH gene mutations may not be the only determinants of BH₄-responsiveness. Nevertheless, BH₄ loading tests performed in the same patient but in different days led to different data (Blau, unpublished data) showing that other factors (maybe related to the quality and amount of concomitant food intake or to resting energy expenditure, REE) may interfere with the loading test, influencing the final results. That is why the dietetic intake of phe during the BH₄ loading test, determining individual plasma phe levels, should be standardised.

A 5 days loading test [12] or the 1 week loading test experienced by some authors [8] may overcome these daily result fluctuation, showing more realistic data concerning the responsiveness of a patient to BH₄ and revealing late-responsive patients.

Different responses to the BH₄ loading test in patients with identical genotype have already been described [13]. Different dosages and different BH₄ products used for the BH₄ loading test are most probable factors interfering with responsiveness. In patients undergoing a double loading test (phe and BH₄ administration) the spontane-

ous elimination of phe after a single phe loading test should be assessed, as well. Reductions in phe levels after oral phe administration are reported even without BH₄ supplementation, but in case of BH₄ supplementation a more significant and earlier decline was reported [14].

The pharmacological role of BH₄ is growing, but almost nothing is known about its pharmacokinetic profile. Different absorption, metabolism, first pass-effect and pharmacokinetic parameters, especially half-life time, are important determinants for individual BH₄ plasma levels, e.g., responsiveness [15]. In the rat model absorption of BH₄ after oral administration showed important variations depending on age [16], another important factor in BH₄-responsiveness.

Multiple administration and extended sampling times in the application of a BH₄ loading test might be necessary to detect slow-responders or low-absorbers [8].

Additional, unknown factors might be responsible for BH₄-responsiveness and different models have been hypothesized to explain mechanisms of BH₄-responsiveness. Based on the location and proximity of particular mutations to the BH₄ binding site in the three-dimensional structure of PAH, the hypothesis of Km variants, which might be activated by BH₄, suggesting mechanisms such as low cofactor affinity, was proposed [17]. Other mutations are located in the regulatory domain of PAH gene expression and BH₄ may increase PAH mRNA enzyme activity and protein levels, as shown in the *hph-1* mouse model [18]. Nevertheless, Thony et al. [19] showed in their in vitro protein expression system that an activation of transcription or an mRNA stabilization seem to be excluded and that BH₄ cofactor appears to directly affect the conformational stability and consequently the activity of PAH, rather than by influencing PAH gene expression. BH₄ may have a chaperon-like activity, which stabilizes the PAH enzyme and protects it from proteolytic degradation [20]. Another hypothesis for BH₄-responsiveness might be stabilisation of the tertiary structure or introduction of 3D structural changes in the PAH protein, but final underlying mechanisms determining BH₄-responsiveness still have to be elucidated.

BH₄-responsiveness was shown to play an important role in the population we investigated. Genotype–phenotype correlations in HPA are known to be often inconsistent and even in the prediction of BH₄-responsiveness genetic analysis is not the rate-limiting factor. Possible interactions between different mutations codifying for different regions of the PAH enzyme structure and influence of non-genetic, but proteomic or metabolomic factors have to be considered and investigated.

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