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Genotype-predicted tetrahydrobiopterin (BH_4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency

Iva Karačić [a], David Meili [b], Vladimir Sarnavka [c], Caroline Heintz [b], Beat Thöny [b], Danijela Petković Ramadža [c], Ksenija Fumić [d], Duško Mardešić [a,c], Ivo Barić [a,c,*], Nenad Blau [b,*]

[a] School of Medicine, Zagreb, Croatia
[b] Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Steinwiesstrasse 75, 8032 Zürich, Switzerland
[c] Department of Pediatrics, University Hospital Center, Zagreb, Croatia
[d] Clinical Institute of Laboratory Diagnosis, University Hospital Center, Zagreb, Croatia

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ABSTRACT

Specific mutations in the gene encoding phenylalanine hydroxylase (*PAH*), located on chromosome 12q22-24.1, are linked to tetrahydrobiopterin (BH_4; sapropterin)-responsive phenylketonuria (PKU). Diagnosis is usually done through the newborn screening for PKU, followed by a BH_4 loading test. So far, more than 60 mutant alleles, presenting with a substantial residual PAH activity (average ~47%), were identified in more than 500 patients worldwide. We investigated the predictive value of BH_4-responsive *PAH* mutations in Croatian population. From a group of 127 PKU patients, 62 were selected (based on the genotype) as potentially BH_4-responsive and 39 loaded with BH_4 (20 mg/kg). The overall frequency of BH_4-responsiveness (>30% blood phenylalanine reduction within 24 h) was 36% (14 out of 39 patients with 23 different genotypes), significantly less than expected. The best responders were patients with mild hyperphenylalaninemia (4/4; 100%), followed by mild PKU (8/9; 89%), and classical PKU (2/26; 8%). The most common BH_4-responsive genotypes were p.E390G/p.R408W and p.P281L/p.E390G. These genotypes correspond for approximately >30% residual PAH activity. The p.E390G mutation was 100% associated with BH_4-responsiveness, regardless of the second allele (p.R408W, p.P281L, p.F55Lfs, p.L249P). With regard to the predicted relative PAH activity of recombinantly expressed mutant alleles, there was a significant ($p < 0.002$) difference between BH_4-responders and non-responders.

In a general Croatian PKU population, disease-causing mutations were identified on 226 alleles (99%). There were 35 different mutations: 21 missense, 8 splice site, 3 nonsense, 2 single nucleotide deletions, and 1 in-frame deletion. Four mutations are reported for the first time: p.E76D, p.L333P, p.G346E, and IVS8-2A > G. Five mutations accounted for over two-thirds of investigated alleles: p.L48S, p.R261Q, p.P281L, p.E390G, and p.R408W. Thus, the Croatian PKU population seems to be more homogenous than some other Mediterranean or Central European populations.

This study reveals the importance of a full genotype for the prediction of BH_4-responsiveness. In contrast to previous assumption and with exception of the p.E390G mutation, single allele mutations are not reliable for the selection of potential PKU candidates for pharmacological therapy with BH_4.

Introduction

Phenylketonuria (PKU; OMIM #261600) is an autosomal recessive metabolic disease caused by hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency [1]. Over 500 different mutations, identified on *PAH* gene, are responsible for a large spectrum of clinical phenotypes [2], from mild hyperphenylalaninemia (MHP), a variant that does not require treatment, to classical PKU that leads to severe neurological impairment when untreated. Although phenylalanine restriction has been the mainstay of successful dietary treatment since 1953 when first initiated [3], it imposes a substantial burden on individuals with PKU and the family. This synthetic, highly restrictive diet is associated with a risk of nutritional deficiencies and phenylalanine control, despite good compliance, is sometimes difficult to achieve. However, compliance is often poor, particularly as individuals reach adolescence [4]. Moreover, there is information on poor phenylalanine control before and during pregnancy in women with PKU, which can adversely influence fetal health [5]. Hence there is a need for an alternative treatment of PKU.

BH_4, a catalytic cofactor for PAH, has been shown to activate residual PAH activity and partially restore oxidative Phe

* Corresponding authors.
E-mail addresses: ibaric@kbc-zagreb.hr (I. Barić), nenad.blau@kispi.uzh.ch (N. Blau).

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metabolism in a substantial number of PKU patients. Although this finding was suggested many years ago [6,7], not much attention has been paid to this issue until 1999 when Kure et al. [8] reported patients with PAH deficiency who had responded to oral BH_4 intake by lowering their blood Phe levels. Since then, an increasing number of BH_4-responsive PAH-deficient patients has been reported [9–22]. Continued treatment with BH_4 in responding patients has been shown to increase Phe tolerance, reduce or eliminate the need for Phe-free protein supplements or even to completely replace the diet [23–29]. One of the greatest issues still remains how to identify BH_4-responsive individuals in a large and heterogeneous pool of PAH-deficient patients.

BH_4 loading test result depends on many methodological factors such as preload plasma Phe level, the patient's age at test (newborn vs. older) [12,18,30–32], Phe intake during the test, amount of administered BH_4 and dosage scheme, cut-off levels of Phe reduction and duration of BH_4 test. An optimized 48-h BH_4 loading protocol, with two BH_4 administrations (20 mg/kg/d) on two consecutive days and with four blood samplings (T_0, T_8, T_{16}, and T_{24}) after BH_4 administration, has been proposed for this reason [33]. Nevertheless, one study reported a significant number (>50%) of initially positive BH_4 responders (Phe reduction > 30% in short-term loading test lasting up to 24 h) who did not respond to long-term BH_4 treatment [27]. Also, there are several reports on patients with no significant response to single-dose loading test, but with a marked decrease in plasma Phe after several days of BH_4 administration [12,34]. These inconsistencies stress the need for an additional approach to evaluation of BH_4-responsiveness.

Specific mutations in the *PAH* gene, many of them characterized by substantial residual activity when recombinantly expressed in different cell systems, are repeatedly found to be associated with BH_4-responsiveness [10,35,36]. This is in accordance with data from BH_4 loading tests indicating an incidence of BH_4-responsiveness of >80% in mild variants of PKU patients with an overall incidence of >40% in general PKU population [37]. Up to 10% of classical PKU patients respond in BH_4 loading test (with a usual 30% cut-off in blood Phe reduction) and they are a more difficult target to properly evaluate BH_4-responsiveness. This is because some severe PKU patients had responded to BH_4 by lowering Phe levels for 20%, which was defined as a significant response for this phenotype. Thus, Fiege and Blau [30] propose to modify the cut-off level for BH_4-responsiveness accordingly to the patient's clinical phenotype. However, there is no accurate correlation between genotype and BH_4-responsiveness, still with many reported responding inconsistencies within the same genotype [36]. So far, mutational analysis provides useful information on potential non-responders comprising two null mutations but the prediction of BH_4 responders remains incomplete [37].

The aim of our study was to provide more information on predictive value of genotype for BH_4-responsiveness and to summarize the mutation spectrum of the *PAH* gene in Croatian PKU population. We initially suggested that the presence of a mutation with *in vitro* substantial residual activity, compared with the wild type enzyme, on at least one *PAH* gene copy would be sufficient for BH_4-responsiveness.

Patients and methods

Patients

From a group of 127 patients diagnosed with hyperphenylalaninemia (HPA) (the highest blood phenylalanine 300–3630 μmol/L) from Croatia in whom *PAH* gene mutation analysis had been done, 39 patients were included in BH_4 loading test. Although we selected 62 patients, only 39 individuals accepted to perform the BH_4 loading test. In four families two sibs were included. Selection criteria were only based on genotype. Inclusion criteria were: (a) presence of at least one BH_4-responsive mutation; or (b) presence of at least one mutation termed as unclear in correlation to BH_4-responsiveness; or (c) presence of at least one mutation with so far unknown response to BH_4. A mutation was classified as BH_4-responsive if it was present either in homozygous or functional heterozygous form in BH_4 responders from data in different publications (for further explanation on definition of BH_4-responsive or unclear mutations see Zurflüh et al. [37]). Patients with two null mutations (with no residual activity) were excluded from the study. For mutation classification in relation to BH_4-responsiveness and for additional information on *PAH* gene mutations we used data from BIOPKU database (www.bh4.org/BH4DatabasesBiopku.asp) and a locus-specific knowledgebase PAHdb (www.pahdb.mcgill.ca). There was an almost equal distribution between females (19/39) and males (20/39) (age ranged 1–24 years; mean 11 years) entering BH_4 trial. BH_4 deficiency was excluded in all patients by measuring urinary pterins and dried blood dihydropteridine reductase activity. Patients were assigned to one of the three phenotype categories according to the highest plasma Phe concentration before introducing the diet or after protein loading test (180 mg/kg/d of Phe intake over 5 days): 4/39 patients (10%) were classified as MHP (phenylalanine levels $\leqslant$600 μmol/L), 9/39 patients (23%) were assigned to mild PKU (Phe levels 601–1200 μmol/L) and 26/39 patients (67%) to classical PKU (Phe levels >1200 μmol/L).

BH_4 loading test

BH_4 loading was performed at Department of Pediatrics, University Hospital Center Zagreb, after obtaining an informed consent from all participants or their parents including the approval of the institutional ethics committee. Three or four days before BH_4 loading (classical PKU vs. milder forms) and during the entire testing period patients had no dietary restrictions, moreover, they were encouraged to consume Phe-rich food. BH_4 (6R-BH_4 dihydrochloride; Schricks Laboratories, Jona, Switzerland) was administered orally to all patients as a single dose of 20 mg/kg body weight. Blood was collected just before BH_4 administration (T_0), and 8 (T_8), 24 (T_{24}), and 48 h (T_{48}) after the loading. We simplified the criteria suggested by Fiege et al. [17] to define BH_4-responders as follows: "responder", reduction of blood Phe by $\geqslant$30% within 24 h and "slow responder", reduction of blood Phe by <20% at T_8, and $\geqslant$20% but <30% at T_{24}. One patient was classified as "not clear" with the reduction of blood Phe by $\geqslant$30% at T_8, and <20% at T_{24}. No side effects were observed during the BH_4 loading test. Phe and BH_4 were measured from dried blood spots; Phe was analyzed using tandem-mass spectrometry and BH_4 was measured according to the method previously published [38].

Mutational analysis

One hundred and fourteen families with HPA (127 patients), all but four patients detected by Guthrie test within neonatal screening program, were enrolled in a comprehensive analysis of *PAH* gene mutations in Croatia in the last 17 years. According to the previously mentioned criteria, 78% of patients suffer from classical PKU, 14% from mild PKU whilst MHP phenotype is present in only 8%. Analyzed patients comprise 78% of total Croatian PKU population. Patients and/or parents signed informed consent for mutational analysis. Genotyping was performed as follows: DNA was isolated from dried blood spots using the QIAamp DNA Micro Kit (Qiagen). PCR was performed using Hot FirePol DNA Polymerase (Solis Biodyne) and standard thermal cycling, i.e., 15 min denaturation at 95 °C followed by 37 cycles of 30 s at 95 °C, 45 s at 56 °C,

45 s at 72 °C, and final incubation for 10 min at 72 °C (on a GeneAmp PCR System 9700 (Applied Biosystems)). Primers flanking all exons are listed in Table 1. The same primers were used to directly sequence the amplified products with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were purified by gel filtration (using MultiScreen HV 96-well filter plates from Millipore with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing results were compared with the wild-type sequence of the *PAH* gene (Accession numbers NM_000277.1 and NG_008690.1) using the Mutation Surveyor (Demo) Software v3.20 (SoftGenetics). Gene analysis of 39 PKU families (included in this study) was done elsewhere as described in two previous publications on PKU mutations in Croatia [39,40].

The relative PAH activity of mutant alleles was calculated from the data published in the BIOPKUdb (www.biopku.org). It was calculated as a sum of activities for alleles 1 and 2, when expressed recombinantly in different cell systems, divided by two. For alleles expressed in more than one cell system, an average PAH activity was used.

Statistical analysis

Statistical analysis was performed using WinSTAT 2007.1 for Excel (R. Fitch Software, Germany). Wilcoxon test was used to compare the predicted relative PAH activity between BH_4-responders and non-responders.

Results

Mutational spectrum in Croatian PKU population

In our mutation frequency assessment we included 114 PKU families. In one family there were three independent mutant alleles (affected child and uncle) what was the reason for investigating the total of 229 independent alleles. Disease-causing mutation was identified on 226 alleles, corresponding to a diagnostic efficiency of approximately 99%. There were 35 different mutations, including 21 missense mutations, 8 splice site, 3 nonsense, 2 single nucleotide deletions, and one in-frame deletion. The commonest PKU mutation p.R408W accounted for 36% of mutant alleles. Following mutations p.L48S, p.P281L, p.E390G, p.R261Q, and p.R158Q accounted for 10%, 8%, 7%, 6%, and 5.5% of mutant alleles, respectively. Five mutations accounted for over two-thirds of investigated alleles (Table 2). Four mutations have not been previously reported: p.E76D, p.G346E, p.L333P, and IVS8-2A > G. Croatian PKU population is more homogenous than some Mediterranean or Central European populations as Zschocke et al. [40] already described. We found homozygosity in 24% of genotypes (for following mutations: p.R408W, p.P281L, p.E390G, p.L249P, p.L48S, and p.R158Q) and only 10 allelic combinations accounted for over 50% of investigated families in Croatia (Table 3). Thus, the investigation of these allelic combinations in accordance to BH_4 response was of great interest for our population.

Responsiveness to BH_4

The outcome of the loading test with BH_4 in 39 PKU patients with 23 different genotypes is summarized in Table 4 (according to biochemical and genetic phenotype and its' mean predicted

Table 1
Primers used for PCR-amplification and sequencing of all 13 exons plus flanking intronic regions of the human *PAH* gene (see also "Patients and methods").

Exon[a]	Sequence (5′ > 3′)	Reference
1F	CGTGCTGTTTGCAAACCTGC	This work
1R	TGGAGGCCCAAATTCCCCTAACTG	[44]
2F	TGATCATTTAATTGCCCTGGA	This work
2R	GCCTGTTCCAGATCCTGTGT	This work
3F	GCCTGCGTTAGTTCCTGTGA	[44][b]
3R	CTTATGTTGCAAAATTCCTC	[44]
4F	GCCATGTTCTGCCAATCTGT	This work
4R	ATCTCATCCTACGGGCCAT	Denmark[c]
5F	TCATGGCTTTAGAGCCCCCA	[44]
5R	AGGCTAGGGGTGTGTTTTTC	[44][b]
6F	CCGACTCCCTCTGCTAACCT	[44][b]
6R	CAATCCTCCCCCAACTTTCT	[44]
7F	TAGCGTCAAAGCCTATGTCC	This work
7R	AAACCTCATTCTTGCAGCAG	This work
8F	TGGCTTAAACCTCCTCCCCT	[44][b]
8R	CTGGGCTCAACTCATTTGAG	[44]
9F	ATGGCCAAGTACTAGGTTGG	[44]
9R	GAGGGCCATAGACTATAGCA	[44][b]
10F	ACACACCCCAAAATAATGCT	This work
10R	GAGTTCCCAGGTTGCATATC	This work
11F	TGAGAGAAGGGGCACAAATG	[44]
11R	CCACCCACAGATGAGTGGCA	This work
12F	TTCTCCAAATGGTGCCCTTC	Denmark[c]
12R	ACTGAGAAACCGAGTGGCCT	Denmark[c]
13F	GACACTTGAAGAGTTTTTGC	[44][b]
13R	TTTTCGGACTTTTTCTGAT	This work

[a] F, forward; R, reverse.
[b] Without GC clamp.
[c] Personal communication Pia Hougaard and Lisbeth Birk Møller, Kennedy Institute, Denmark.

Table 2
The most frequent mutations in Croatian PKU population which account for over two-thirds of the investigated population (229 independent chromosomes).

Mutation	Number of alleles	Frequency (number of independent chromosomes) (%)
p.R408W (c.1222C > T)	82	36
p.L48S (c.143T > C)	23	10
p.P281L (c.842C > T)	18	8
p.E390G (c.1169A > G)	16	7
p.R261Q (c.782G > A)	14	6
Total	153	67

Table 3
The most frequent allelic combinations in Croatia that account for over half of the investigated PKU population (114 families).

Genotype	Number of patients	Frequency in investigated population (%)
p.R408W/p.R408W (c.1222C > T/c.1222C > T)	15	13
p.R408W/p.L48S (c.1222C > T/c.143T > C)	8	7
p.R408W/p.E390G (c.1222C > T/c.1169A > G)	6	5
p.R408W/p.P281L (c.1222C > T/c.842C > T)	5	4.5
p.R408W/p.R261Q (c.1222C > T/c.782G > A)	5	4.5
p.L48S/p.R261Q (c.143T > C/c.782G > A)	4	3.5
p.L48S/p.R158Q (c.143T > C/c.473G > A)	4	3.5
p.R408W/p.R158Q (c.1222C > T/c.473G > A)	4	3.5
p.R408W/IVS12 + 1G > A (c.1222C > T/ c.1315 + 1G > A)	4	3.5
p.P281L/p.E390G (c.842C > T/c.1169A > G)	3	2.5
Total	58	50.5

Table 4
Tetrahydrobiopterin loading test in 39 Croatian PKU patients selected according to genotype data and predictive value of genotype on BH_4-responsiveness according to predicted relative residual PAH activity.

Patient number	Maximal Phe levels[a] (phenotype)	Allele 1	Allele 2	Phe 0 h	Phe 8 h	Phe 24 h	Phe 48 h[b]	Phe reduction 8 h (%)	Phe reduction 24 h (%)	PAH activity[c]	BH_4 responsiveness
24	1355 (cPKU)	**p.L48S** c.143T > C	**p.L48S** c.143T > C	315	237	140	292	24,8	55,6	39.0	Responder
25	1520 (cPKU)	**p.L48S** c.143T > C	**p.L48S** c.143T > C	655	740	498	582	−13,0	24,0	39.0	Slow-responder
29	1023 (mPKU)	**p.L48S** c.143T > C	**p.R261Q** c.782G > A	1211	nd	759	1060	nd	37,3	39.0	Responder
2	617 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	437	179	239	341	59,0	45,3	37.5	Responder
3	677 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	608	181	115	329	70,2	81,1	37.5	Responder
4[1]	1180 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	411	132	94	340	67.9	77.1	37.5	Responder
5[1]	607 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	519	216	118	261	58,4	77,3	37.5	Responder
6	871 (mPKU)	**p.E390G** c.1169A > G	p.R408W c.1222C > T	389	310	247	363	20,3	36,5	37.5	Responder
7	750 (mPKU)	**p.E390G** c.1169A > G	p.P281L c.842C > T	265	225	146	304	15,1	44,9	37.0	Responder
8	600 (MHP)	**p.E390G** c.1169A > G	p.P281L c.842C > T	332	148	133	412	55,4	59,9	37.0	Responder
9	600 (MHP)	**p.E390G** c.1169A > G	p.F55Lfs c.165delT	285	124	90	239	56,5	68,4	36.5	Responder
10	360 (MHP)	**p.E390G** c.1169A > G	p.L249P c.746T > C	345	138	136	382	60,0	60,6	(36.5)	Responder
27	>1210 (cPKU)	**p.L48S** c.143T > C	**p.R158Q** c.473G > A	1298	1024	908	764	21,1	30,0	24.5	Responder
28	2718 (cPKU)	**p.L48S** c.143T > C	**p.R158Q** c.473G > A	956	951	1069	1016	0,5	−11,8	24.5	Non-responder
17[3]	1658 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	762	733	876	895	3,8	−15,0	20.5	Non-responder
18[3]	2050 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	1281	1298	1218	1370	−1,3	4,9	20.5	Non-responder
19	>1210 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	852	521	763	948	38,8	10,4	20.5	Not clear
20	2153 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	1157	1241	1365	1367	−7,3	−18,0	20.5	Non-responder
21	2118 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	900	859	811	940	4,6	9,9	20.5	Non-responder
22	2039 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	1150	1392	1472	1414	−21,0	−28,0	20.5	Non-responder
23	1920 (cPKU)	**p.L48S** c.143T > C	p.R408W c.1222C > T	898	942	855	1308	−4,9	4,8	20.5	Non-responder
30	2492 (cPKU)	p.R408W c.1222C > T	**p.R261Q** c.782G > A	1096	1361	1160	1256	−24,2	−5,8	20.5	Non-responder
31	3260 (cPKU)	p.R408W c.1222C > T	**p.R261Q** c.782G > A	1084	1080	1150	1088	0,4	− 6,1	20.5	Non-responder
16	>1210 (cPKU)	**p.L48S** c.143T > C	p.P281L c.842C > T	1092	1373	1336	1169	−25,7	−22,3	20.0	Non-responder
35	>1210 (cPKU)	p.R408W c.1222C > T	**p.V245A** + **p.R241C** c.734T > C + c.721C > T	1203	1304	1225	1145	−8,4	−1,8	20.0	Non-responder
26	1466 (cPKU)	**p.L48S** c.143T > C	IVS4 + 5G > T c.441 + 5G > T	879	938	863	944	−6,7	1,8	19.5	Non-responder
32[4]	1573 (cPKU)	p.K363 > Nfs c.1089delG	**p.R261Q** c.782G > A	1152	937	1068	1203	18,7	7,3	19.5	Non-responder
33[4]	1452 (cPKU)	p.K363 > Nfs c.1089delG	**p.R261Q** c.782G > A	1097	954	1006	1025	13,0	8,3	19.5	Non-responder
38	420 (MHP)	**p.I306V** c. 916A > G	p.R408W c.1222C > T	255	83	64	214	67,5	74,9	20.5	Responder
39	720 (mPKU)	**p.I306V** c. 916A > G	p.R111X c.331C > T	335	118	187	283	64,8	44,2	19.5	Responder
34	1211 (cPKU)	p.R408W c.1222C > T	**p.F39del** c.115_117delTTC	1358	1451	1281	1354	−6,8	5,7	11.0	Non-responder
36	2698 (cPKU)	p.R408W c.1222C > T	**p.R158Q** c.473G > A	1377	1164	1158	1118	15,5	15,9	6.0	Non-responder
14	>1210 (cPKU)	p.L249P c.746T > C	**p.R158Q** c.473G > A	632	674	550	741	−6,6	13,0	(5.0)	Non-responder
37	>1210 (cPKU)	**p.R158Q** c.473G > A	?	738	738	784	780	0,0	−6,2	(5.0)	Non-responder
13	1984 (cPKU)	p.L249P c.746T > C	p.R408W c.1222C > T	871	880	792	874	−1,0	9,1	(1.0)	Non-responder
15	2940 (cPKU)	p.L249P c.746T > C	p.P281L c.842C > T	1033	962	1114	1124	6,9	−7,8	(0.5)	Non-responder
1	1082 (mPKU)	**p.I306V** c. 916A > G	p.R261X c.781C > T	295	255	217	263	13,6	26,4	19.5	Slow-responder
11[2]	1306 (cPKU)	p.L249P c.746T > C	p.L249P c.746T > C	1150	1215	1239	1252	−5,7	−7,7	?	Non-responder
12[2]	3055 (cPKU)	p.L249P c.746T > C	p.L249P c.746T > C	1185	1241	1225	1332	−4,7	−3,4	?	Non-responder

nd: not done; ?: not known; 1, 2, 3, 4: pairs of siblings.

[a] The highest Phe pretreatment concentration (μmol/L) or the highest Phe in the protein Phe test (180 mg/kg/d) if performed; mild hyperphenylalaninemia (MHP), mild phenylketonuria (mPKU), and classical PKU (cPKU).

[b] 48-h Phe value as test control – if Phe increased 48 h after BH_4 administration as expected regarding BH_4 pharmacokinetics, loading test was considered more reliable.

[c] Predicted relative residual PAH activity (sum of *in vitro* expressed residual activities of alleles 1 + 2 divided by 2). Data from the BIOPKU database (www.biopku.org). Values in brackets show data calculated from only one allele (second allele not known). Mutations in bold are defined as BH_4-responsive [37].

residual PAH activity [41]). The prevalence of BH_4-responsiveness (at least 30% cut-off of phenylalanine reduction within 24 h) was only 36% (14 of 39 patients). Additionally, there were two slow-responders who decreased Phe for 26.4% and 24% within 24 h post loading (patient 1 and 25). The prevalence of BH_4-responders in different phenotype groups was as follows: 100% (4/4 patients) in MHP group, 89% (8/9 patients) in mild PKU group, and 8% (2/26 patients) in classical PKU group. All MHP patients and 5/8 responding mild PKU patients (patients 2, 3, 4, 5, and 39) showed significant Phe reduction (>50%) already 8 h post loading. In severe phenotypes that responded (two patients plus one slow responder) phenylalanine reduction was significantly (over 20%) expressed 24 h post loading. Interestingly, patient 6 with the same genotype (p.E390G/p.R408W) (and disease severity, accordingly) as patients 2, 3, 4, and 5 showed significantly slower and less effective response to BH_4. We could not explain this by differences in test performance or BH_4 pharmacokinetics, and to our knowledge such poor response of this genotype has never been described before. It is, however, possible that in this patient Phe intake during the test was lower than in other patients with the same genotype. In all patients blood BH_4 levels increased from initial 1.25 ± 1.11 nmol/g Hb (mean ± SD) to 6.06 ± 4.79 nmol/g Hb 8 h after BH_4 administration and decreased to 2.07 ± 1.16 nmol/g Hb after 24 h and 1.64 ± 1.47 nmol/g Hb after 48 h.

Thirty-five patients from this study (see Table 4) had at least one mutation with known *in vitro* residual activity of 10% or more (compared with the wild-type enzyme), and we assumed that each patient from this group would respond to BH_4. However, only 14/35 (40%) of these patients responded and one patient was labeled "not clear" (patient 19) as he responded just at T_8, and then elevated Phe significantly at T_{24}. This was not estimated as reliable response to BH_4. Additionally, we loaded with BH_4 four patients with p.L249P, mutation with unknown response to BH_4 (and with no *in vitro* studies on residual enzyme activity). To our knowledge, this mutation was only described in Croatian PKU population. p.L249P was first described by Zschocke et al. [40]. It accounts for 3.5% of mutant alleles and, according to the results of BH_4 loading test, it does not have any substantial residual enzyme activity. Namely, this mutation was associated with BH_4-responsiveness only when in allelic combination with p.E390G (so far 100% responsive mutation), but did not show any response in homozygous or functional heterozygous condition.

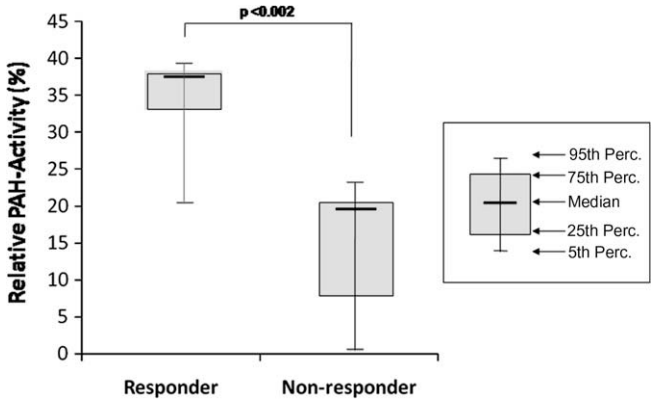

Fig. 1. Comparison of the predicted relative PAH activity in patients with the BH_4-responsive PKU ($n = 14$) and in non-responders ($n = 20$). For definition of the predicted relative PAH activity, see "Patients and methods" and Table 4. Three patients (two slow-responder and one not clear) were not included in the comparison. In four non-responder patients the relative PAH activity was calculated from only one allele, as well as for one responder. In two patients no data were available for the relative PAH activity.

Analysis of predicted relative PAH activities revealed a significant difference ($p < 0.002$) between BH_4-responders and non-responders (Fig. 1). The median relative PAH activity was 37.2% in BH_4-responder (5th–95th percentile: 20.0–39.0%) and 20.0% in BH_4 non-responder group (5th–95th percentile: 0.5–24.3%).

Discussion

This is the first study on BH_4-responsiveness in a cohort of HPA patients where selection criteria for BH_4 loading test were only based on genotype information. We predicted BH_4-responsiveness in individuals with at least one mutation expressing *in vitro* substantial residual activity (>10%). This prediction was based on a current knowledge that specific mutation with some residual activity would be considered as the major determinant of BH_4-responding phenotype. Mutation-predicted prevalence of BH_4-responsiveness was, however, almost threefold higher than prevalence data obtained after our BH_4 loading test. This finding reveals the importance of the complete PAH genotype instead of just a single responsive mutation in predicting the BH_4-responsiveness.

Croatia has relatively homogenous PKU population with around 75% of families comprising classical PKU. Mutational background confirms this finding as the most common mutation p.R408W accounts for 36% of mutant alleles, with p.R408W homozygosity rate of 13%. Total homozygosity rate (24%) is higher than in majority of other European populations (according to data summarized in Guldberg et al. [42]). However, we believed there could be a significant number of BH_4-responsive patients in this population, not just among 22% of milder forms, but also in severe phenotypes. This prediction was based on the fact that "milder" mutations such as p.L48S, p.E390G, p.R261Q, and p.R158Q were following p.R408W with allelic frequency of 10%, 7%, 6%, and 5.5%, respectively, in Croatian PKU population.

Our results reveal, however, negative impact of the most frequent mutation, p.R408W, on the second allele with BH_4-responsive mutation (p.L48S, p.R261Q, or p.R158Q). These three mutations have been repeatedly reported as inconsistent in BH_4-responsiveness [10,13,16,18,36], and they cannot be regarded as dominant in compound heterozygous patients as their behavior highly depends on the other mutation. Moreover, we observed to some extent consistent pattern in BH_4-responsiveness for specific combinations of "inconsistent" mutations p.L48S and p.R261Q. They are non-responders (except patient 19 regarded as "not clear") in functional hemizygous form, but found as BH_4-responders in homozygous form (including one slow responder), in compound heterozygosity with MHP mutation as well as in *in trans* combination of this two mutations (p.L48S/p.R261Q). Although similar results were reported in many publications, there are several exceptions such as a report of BH_4-responsive p.R261Q/p.R243X genotype by Spaapen and Estela Rubio-Gozalbo [43] as well as reported non-responder with p.L48S homozygosity by Fiori et al. [16]. Both p.L48S homozygotes (patient 24 and 25) belong to milder form of classical PKU as they increase Phe much slower than typical classical PKU patients when not on diet, but however they can eventually reach high Phe levels (see their pretreatment Phe concentrations from Table 4). As reported by Leuzzi et al. [18] and confirmed in our study, p.L48S homozygotes sometimes show slow response to BH_4 (at T_{24} Phe reduction of 24%). Thus, in a case of this genotype a 30% cut-off in Phe reduction would not be always reliable to estimate BH_4-responsiveness. Trefz et al. [36] found p.R158Q mutation as the most inconsistent of all mutations (500 alleles investigated) in responding to BH_4. Our results confirm this observation, especially in case of compound heterozygosity with p.L48S (patient 27 and 28). Functional hemizygous patients for p.R158Q result in BH_4 non-responsiveness.

Our study confirms the definition of p.E390G mutation as 100% responsive allele. To our knowledge, this mutation has never so far been described as BH_4 non-responsive, regardless of the second allele. The similar was observed for much less frequent p.I306V mutation (full responsiveness in two patients and "slow" responsiveness in the third loaded patient), although the response of the latter needs to be evaluated on more patients.

Our findings reveal following features: one mutation is sufficient to estimate response to BH_4 just in case of MHP mutations (e.g., p.E390G). In all other cases mutational combination (i.e., complete genotype) should be used to indicate and adjust, still not standardized, BH_4 loading test. On the basis of allelic mutational combination we were able to predict BH_4-response for the majority of patients. The exceptions were genotypes with mutations with unknown response to BH_4 and so far unmeasured *in vitro* residual activity (p.L249P), with *in cis* mutations (p.V245A + p.R241C), and highly inconsistent genotype (p.L48S/p.R158Q). Genotype information is also useful for the selection of a target population among patients with classical PKU (i.e., by excluding patients with two null mutations), as well as to point to patients who are probable slow-responders not to be missed by too rigorous loading protocol (e.g., some p.L48S homozygotes).

The predicted relative PAH activities (Table 4 and Fig. 1) were significantly higher in BH_4-responders, compared with the non-responder group. It seems that a substantial residual PAH activity resulting from a combination of both alleles is needed to predict 100% responsiveness in PKU patients.

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References

[1] C.R. Scriver, S. Kaufman, Hyperphenylalaninemia: phenylalanine hydroxylase deficiency, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, B. Vogelstein (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 1667–1724.
[2] C.R. Scriver, The *PAH* gene, phenylketonuria, and a paradigm shift, Hum. Mutat. 28 (2007) 831–845.
[3] H. Bickel, J.W. Gerrard, E.M. Hickmans, Influence of phenylalanine intake on phenylketonuria, Lancet 2 (1953) 812–819.
[4] J.H. Walter, F.J. White, S.K. Hall, A. MacDonald, G. Rylance, A. Boneh, D.E. Francis, G.J. Shortland, M. Schmidt, A. Vail, How practical are recommendations for dietary control in phenylketonuria?, Lancet 360 (2002) 55–57.
[5] A.S. Brown, P.M. Fernhoff, S.E. Waisbren, D.M. Frazier, R. Singh, F. Rohr, J.M. Morris, A. Kenneson, P. MacDonald, M. Gwinn, M. Honein, S.A. Rasmussen, Barriers to successful dietary control among pregnant women with phenylketonuria, Genet. Med. 4 (2002) 84–89.
[6] S. Milstien, S. Kaufman, Studies on the phenylalanine hydroxylase system in liver slices, J. Biol. Chem. 250 (1975) 4777–4781.
[7] A. Niederwieser, H.C. Curtius, Tetrahydrobiopterin deficiencies in hyperphenylalaninemia, in: H. Bickel, U. Wachtel (Eds.), Inherited Diseases of Amino Acid Metabolism, Georg Thieme, Stuttgart, 1985, pp. 104–121.
[8] S. Kure, D.C. Hou, T. Ohura, H. Iwamoto, S. Suzuki, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, J. Pediatr. 135 (1999) 375–378.
[9] C. Bernegger, N. Blau, High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1919 patients observed from 1988 to 2002, Mol. Genet. Metab. 77 (2002) 304–313.
[10] A.C. Muntau, W. Röschinger, M. Habich, H. Demmelmair, B. Hoffmann, C.P. Sommerhoff, A.A. Roscher, Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria, N. Engl. J. Med. 347 (2002) 2122–2132.
[11] M. Lindner, R. Steinfeld, P. Burgard, A. Schulze, E. Mayatepek, J. Zschocke, Tetrahydrobiopterin sensitivity in German patients with mild phenylalanine hydroxylase deficiency, Hum. Mutat. 21 (2003) 400.
[12] H. Shintaku, S. Kure, T. Ohura, Y. Okano, M. Ohwada, N. Sugiyama, N. Sakura, I. Yoshida, M. Yoshino, Y. Matsubara, K. Suzuki, K. Aoki, T. Kitagawa, Long-term treatment and diagnosis of tetrahydrobiopterin-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene, Pediatr. Res. 55 (2004) 425–430.
[13] B. Perez-Duenas, M.A. Vilaseca, A. Mas, N. Lambruschini, R. Artuch, L. Gomez, J. Pineda, A. Gutierrez, M. Mila, J. Campistol, Tetrahydrobiopterin responsiveness in patients with phenylketonuria, Clin. Biochem. 37 (2004) 1083–1090.
[14] L.R. Desviat, B. Pérez, A. Bèlanger-Quintana, M. Castro, C. Aguado, A. Sánchez, M.J. García, M. Martínez-Pardo, M. Ugarte, Tetrahydrobiopterin responsiveness: results of the BH_4 loading test in 31 Spanish PKU patients and correlation with their genotype, Mol. Genet. Metab. 82 (2004) 157–162.
[15] J.J. Mitchell, B. Wilcken, I. Alexander, C. Ellaway, H. O'Grady, V. Wiley, J. Earl, J. Christodoulou, Tetrahydrobiopterin-responsive phenylketonuria: the New South Wales experience, Mol. Genet. Metab. 86 (2005) 81–85.
[16] L. Fiori, B. Fiege, E. Riva, M. Giovannini, Incidence of BH_4-responsiveness in phenylalanine-hydroxylase-deficient Italian patients, Mol. Genet. Metab. 86 (2005) 67–74.
[17] B. Fiege, L. Bonafé, D. Ballhausen, M. Baumgartner, B. Thöny, D. Meili, L. Fiori, M. Giovannini, N. Blau, Extended tetrahydrobiopterin loading test in the diagnosis of cofactor-responsive phenylketonuria: a pilot study, Mol. Genet. Metab. 86 (2005) 91–95.
[18] V. Leuzzi, C. Carducci, C. Carducci, F. Chiarotti, C. Artiola, T. Giovanniello, I. Antonozzi, The spectrum of phenylalanine variations under tetrahydrobiopterin load in subjects affected by phenylalanine hydroxylase deficiency, J. Inherit. Metab. Dis. 29 (2006) 38–46.
[19] S. Yildirim, A. Tokatli, E. Yilmaz, T. Coskun, Assessment of tetrahydrobiopterin responsiveness in Turkish hyperphenylalaninemic patients, Turk. J. Pediatr. 49 (2007) 1–6.
[20] L. Wang, S. Surendran, K. Michals-Matalon, G. Bhatia, S. Tanskley, R. Koch, J. Grady, S.K. Tyring, R.C. Stevens, F. Guttler, R. Matalon, Mutations in the regulatory domain of phenylalanine hydroxylase and response to tetrahydrobiopterin, Genet. Test. 11 (2007) 174–178.
[21] M.D. Boveda, M.L. Couce, D.E. Castineiras, J.A. Cocho, B. Perez, M. Ugarte, J.M. Fraga, The tetrahydrobiopterin loading test in 36 patients with hyperphenylalaninaemia: evaluation of response and subsequent treatment, J. Inherit. Metab. Dis. 30 (2007) 812.
[22] B.K. Burton, D.K. Grange, A. Milanowski, G. Vockley, F. Feillet, E.A. Crombez, V. Abadie, C.O. Harding, S. Cederbaum, D. Dobbelaere, A. Smith, A. Dorenbaum, The response of patients with phenylketonuria and elevated serum phenylalanine to treatment with oral sapropterin dihydrochloride (6R-tetrahydrobiopterin): a phase II, multicentre, open-label, screening study, J. Inherit. Metab. Dis. 30 (2007) 700–707.
[23] R. Cerone, M.C. Schiaffino, A.R. Fantasia, M. Perfumo, L. Birk Moller, N. Blau, Long-term follow-up of a patient with mild tetrahydrobiopterin-responsive phenylketonuria, Mol. Genet. Metab. 81 (2004) 137–139.
[24] J.B. Hennermann, C. Bührer, N. Blau, B. Vetter, E. Mönch, Long-term treatment with tetrahydrobiopterin increases phenylalanine tolerance in children with severe phenotype of phenylketonuria, Mol. Genet. Metab. 86 (2005) 86–90.
[25] N. Lambruschini, B. Perez-Duenas, M.A. Vilaseca, A. Mas, R. Artuch, R. Gassio, L. Gomez, A. Gutierrez, J. Campistol, Clinical and nutritional evaluation of phenylketonuric patients on tetrahydrobiopterin monotherapy, Mol. Genet. Metab. 86 (2005) 54–60.
[26] F.K. Trefz, D. Scheible, G. Frauendienst-Egger, H. Korall, N. Blau, Long-term treatment of patients with mild and classical phenylketonuria by tetrahydrobiopterin, Mol. Genet. Metab. 86 (2005) 75–80.
[27] L. Levy, A. Milanowski, A. Chakrapani, M. Cleary, P. Lee, F.K. Trefz, C.B. Whitley, F. Feillet, A.S. Feigenbaum, J.D. Bebchuk, H. Christ-Schmidt, A. Dorenbaum, Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH_4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomized placebo-controlled study, Lancet 370 (2007) 504–510.
[28] P. Lee, E.P. Treacy, E. Crombez, M. Wasserstein, L. Waber, J. Wolff, U. Wendel, A. Dorenbaum, J. Bebchuk, H. Christ-Schmidt, M. Seashore, M. Giovannini, B.K. Burton, A.A. Morris, Safety and efficacy of 22 weeks of treatment with sapropterin dihydrochloride in patients with phenylketonuria, Am. J. Med. Genet. A 146A (2008) 2851–2859.
[29] A. Burlina, N. Blau, Effect of BH_4 supplementation on phenylalanine tolerance, J. Inherit. Metab. Dis. 32 (2009) 40–54.
[30] B. Fiege, N. Blau, Assessment of tetrahydrobiopterin (BH_4)-responsiveness in phenylketonuria, J. Pediatr. 150 (2007) 627–630.
[31] H. Levy, B. Burton, S. Cederbaum, C. Scriver, Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH(4)) in phenylketonuria and its use in treatment, Mol. Genet. Metab. 92 (2007) 287–291.
[32] N. Blau, Defining tetrahydrobiopterin (BH_4)-responsiveness in PKU, J. Inherit. Metab. Dis. 31 (2008) 2–3.
[33] N. Blau, A. Belanger-Quintana, M. Demirkol, F. Feillet, M. Giovannini, A. MacDonald, F.K. Trefz, F.J. van Spronsen, Optimizing the use of sapropterin (BH_4) in the management of phenylketonuria, Mol. Genet. Metab. 96 (2009) 158–163.
[34] U. Lässker, J. Zschocke, N. Blau, R. Santer, Tetrahydrobiopterin responsiveness in phenylketonuria. Two new cases and a review of molecular genetic findings, J. Inherit. Metab. Dis. 25 (2002) 65–70.

[35] N. Blau, H. Erlandsen, The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, Mol. Genet. Metab. 82 (2004) 101–111.
[36] F.K. Trefz, D. Scheible, H. Gotz, G. Frauendienst-Egger, Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria, J. Inherit. Metab. Dis. 32 (2009) 22–26.
[37] M.R. Zurflüh, J. Zschocke, M. Lindner, F. Feillet, C. Chery, A. Burlina, R. Stevens, B. Thöny, N. Blau, Molecular genetics of tetrahydrobiopterin responsive phenylalanine hydroxylase deficiency, Hum. Mutat. (2008) 167–175.
[38] M. Zurflüh, M. Giovannini, L. Fiori, B. Fiege, Y. Gokdemir, T. Baykal, L. Kierat, K.H. Gärtner, B. Thöny, N. Blau, Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper, Mol. Genet. Metab. 86 (2005) 96–103.
[39] I. Baric, D. Mardesic, G. Gjuric, V. Sarnavka, B. Gobel-Schreiner, U. Lichter-Konecki, D.S. Konecki, F.K. Trefz, Haplotype distribution and mutations at the PAH locus in Croatia, Hum. Genet. 90 (1992) 155–157.
[40] J. Zschocke, A. Preusse, V. Sarnavka, K. Fumic, D. Mardesic, G.F. Hoffmann, I. Baric, The molecular basis of phenylalanine hydroxylase deficiency in Croatia, Hum. Mutat. 21 (2003) 399.
[41] M. Bik-Multanowski, J.J. Pietrzyk, B. Didycz, A. Szymczakiewicz-Multanowska, Development of a model for assessment of phenylalanine hydroxylase activity in newborns with phenylketonuria receiving tetrahydrobiopter: a potential for practical implementation, Mol. Genet. Metab. 94 (2008) 389–390.
[42] P. Guldberg, F. Rey, J. Zschocke, V. Romano, B. Francois, L. Michiels, K. Ullrich, G.F. Hoffmann, P. Burgard, H. Schmidt, C. Meli, E. Riva, I. Dianzani, A. Ponzone, J. Rey, F. Güttler, A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype, Am. J. Hum. Genet. 63 (1998) 71–79.
[43] L.J. Spaapen, M. Estela Rubio-Gozalbo, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state of the art, Mol. Genet. Metab. 78 (2003) 93–99.
[44] P. Guldberg, V. Romano, N. Ceratto, P. Bosco, M. Ciuna, A. Indelicato, F. Mollica, C. Meli, M. Giovannini, E. Riva, et al., Mutational spectrum of phenylalanine hydroxylase deficiency in Sicily: implications for diagnosis of hyperphenylalaninemia in southern Europe, Hum. Mol. Genet 2 (1993) 1703–1707.