

# High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1919 patients observed from 1988 to 2002

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## Abstract

Tetrahydrobiopterin (BH<sub>4</sub>)-responsive hyperphenylalaninemia (HPA) is a recently described variant of phenylalanine hydroxylase deficiency. In contrast to patients with classical phenylketonuria, these patients respond to BH<sub>4</sub> loading tests (20 mg/kg) with decrease of plasma phenylalanine levels 4 and 8 h after administration and they can be treated with BH<sub>4</sub> monotherapy. We retrospectively evaluated 1919 loading tests from 33 different countries performed in our laboratory between 1988 and 2002 of which 278 loading tests were performed with 6R-BH<sub>4</sub>, which is about 33% more active than the formerly used 6R,S-BH<sub>4</sub>. The loading tests were performed between the ages of one week and 4.6 years, using 2.6–30.0 mg 6R,S- or 6R-BH<sub>4</sub>/kg. Plasma phenylalanine levels before the test ranged from 121 to 4705 μmol/L. We calculated the phenylalanine “hydroxylation rate” 4 and 8 h after BH<sub>4</sub> administration and plotted the slope of the hydroxylation rate against the phenylalanine levels at time 0. The slope was greater than 3.75 in 65, 74, 33, 17, 0, and 10% of patients with basal phenylalanine levels of 120–400, 400–800, 800–1200, 1200–1600, 1600–2200, and >2200 μmol/L, respectively, when loaded with 20 mg 6R-BH<sub>4</sub>/kg ( $p > 0.0001$ ). This is 5–20 times higher compared with tests using 6R,S-BH<sub>4</sub> or lower doses of BH<sub>4</sub>. More than 70% of patients with mild HPA (<800 μmol/L) are found to be BH<sub>4</sub> responders. Therapy with BH<sub>4</sub> (~10 mg/kg/day) was initiated in several patients instead of a low-phenylalanine diet, resulting in much better treatment compliance. Our data further demonstrate that BH<sub>4</sub> loading tests can only distinguish between BH<sub>4</sub> responders and non-responders. To differentiate between BH<sub>4</sub> and phenylalanine hydroxylase deficiencies additional tests are essential.

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## Introduction

The phenylalanine hydroxylating system consists of the apoenzyme phenylalanine hydroxylase (PAH), the cofactor tetrahydrobiopterin (BH<sub>4</sub>), and two regenerating enzymes, namely, pterin-4a-carbinolamine dehydratase and dihydropteridine reductase [1]. A deficiency of any of these components results in hyperphenylalaninemia (HPA), which can be classified into two groups: (a) PAH deficiency, ranging from mild HPA to classical PKU and (b) BH<sub>4</sub> deficiencies, which can be caused by mutations in one of the genes coding for the enzymes involved in the biosynthesis or regeneration of BH<sub>4</sub>. The

laboratory diagnosis of HPAs is straightforward and starts with newborn screening for PKU by the Guthrie test or tandem mass-spectrometry, followed by analysis of neopterin and biopterin in urine and measurement of dihydropteridine reductase activity in dried blood spots from patients with a positive newborn screening test. Historically, the first BH<sub>4</sub> loading test tried and predicted as the most convenient to selectively screen HPAs, exploited the lowering of plasma phenylalanine in BH<sub>4</sub>-deficient patients after the administration of exogenous BH<sub>4</sub> [2]. Intravenous loading with 2 mg BH<sub>4</sub>/kg body weight was originally proposed by Danks et al., [3] but with the increased purity and availability of the synthesized cofactor, an oral loading test (2.5 mg/kg) was introduced by Niederwieser et al. [4]. This test was later standardized at a dose of 7.5 mg/kg by the same

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group [5]. This very simple test discriminated between patients with PAH and BH<sub>4</sub> deficiencies. However, it soon became evident that some dihydropteridine reductase-deficient patients could be misdiagnosed as their serum phenylalanine levels were not lowered by BH<sub>4</sub> [6]. The observation that BH<sub>4</sub> non-responsiveness in dihydropteridine reductase deficiency correlates with the presence of a mutant enzyme and that it can be overcome by increasing the dose of the administered cofactor to 20 mg/kg, led to a new standard protocol for BH<sub>4</sub> loading [7].

Although it had been suggested in the past that some patients with PAH deficiency responded to BH<sub>4</sub> loading [8,9] not much attention was paid to these cases until the first report by Kure et al. [10]. They described four Japanese patients who were compound heterozygotes for mutations in the PAH gene without evidence of a cofactor deficiency who responded to oral administration of BH<sub>4</sub> (10 mg/kg). Similar observations were subsequently reported by other groups [11–17]. Many different mutations in the PAH gene have been described to be associated with mild HPA and BH<sub>4</sub>-responsiveness (L48S, I65T, V190A, R241C, R261Q, A313T, A373T, E390G, A395P, A403V, P407S, and Y414C). It has been suggested that some mutations result in a  $K_m$  variant of the PAH gene in which residual activity can be stimulated by BH<sub>4</sub> supplementation. Other mutations affecting the catalytic domain of the enzyme (encoded by exons 6–12), may potentially alter the tertiary structure of PAH [18]. The detection of a homozygous mutation (L48S/L48S) located in the regulatory domain of the PAH gene [15] and the fact that some missense mutations may be associated with inconsistent phenotypes [19], suggest that additional factors may be responsible for the PAH-responsiveness by BH<sub>4</sub>. Blau and Trefz [15] proposed that BH<sub>4</sub> may regulate PAH gene expression, as recently reported for the BH<sub>4</sub>-deficient *hph-1* mouse [20].

In contrast to patients with classical PKU (treatment with phenylalanine-low diet) and patients with BH<sub>4</sub> deficiencies (combined therapy with L-Dopa/Carbidopa/5-hydroxytryptophan plus BH<sub>4</sub>), patients with BH<sub>4</sub>-responsive PAH deficiency can be treated with BH<sub>4</sub> alone [11]. Thus, early detection of the latter group of patients has significant implications for the development of a new therapy using BH<sub>4</sub> rather than the classical low-phenylalanine or low-protein diets. In this study, we retrospectively investigated 1919 loading tests performed in our laboratory between 1988 and 2002, in order to determine the frequency of BH<sub>4</sub>-responsiveness in patients with HPA, specifically in those with PAH deficiency. The collected data also demonstrate that the sensitivity of the tests and the responsiveness depended on the amount and quality of the administered BH<sub>4</sub>. Urinary neopterin and biopterin data were tabulated to establish reference ranges for patients with different degrees of HPA.

## Materials and methods

### Patients

We evaluated BH<sub>4</sub> loading tests performed in 1919 HPA individuals in the period between 1988 and 2002 at the Children's Hospital in Zürich. Tests were done between the ages of one week to 4.6 years (5–95 percentile), but mostly at the age of three weeks (median). Samples were collected from 33 different countries (50.4% Germany, 23.3% Turkey, 6.3% Austria, 6.0% Italy, 4.4% Hungary, 3.2% Switzerland, and 6.4% other countries). Initial plasma phenylalanine concentrations (at the start of loading) ranged from 200 to 2692 µmol/L (5–95 percentile), with a median 1008 µmol/L. A total of 189 patients were diagnosed to be BH<sub>4</sub>-deficient (9 with GTP cyclohydrolase I deficiency, 113 with 6-pyruvoyl-tetrahydropterin synthase deficiency, 61 with dihydropteridine reductase deficiency, and 6 with pterin-4a-carbinolamine dehydratase deficiency). Their plasma phenylalanine levels ranged from 246 to 2437 µmol/L (5–95 percentiles), median 897 µmol/L. The 1730 patients in whom BH<sub>4</sub> deficiency was excluded, presented with plasma phenylalanine levels of 200–2710 µmol/L (5–95 percentile), median 1040 µmol/L.

### Screening for BH<sub>4</sub> deficiency

Screening for BH<sub>4</sub> deficiency was based on the measurement of urinary pterins, the dihydropteridine reductase activity in dried blood spots, and the BH<sub>4</sub> loading tests [21]. Neopterin and biopterin were measured in urine oxidized with manganese dioxide at acidic pH as described elsewhere [22]. Dihydropteridine reductase activity was measured according to Arai et al. [23]. Patients diagnosed as BH<sub>4</sub>-deficient are tabulated in the BIODDEF database ([www.bh4.org](http://www.bh4.org)).

Phenylalanine in serum or plasma was measured in different laboratories using different methods, including ion-exchange chromatography, fluorometry, enzymatic tests, and tandem mass-spectrometry.

### Tetrahydrobiopterin loading test and “phenylalanine hydroxylation” rate

The BH<sub>4</sub> loading test was performed using two different compounds, both produced by Dr. Schircks Laboratories (Jona, Switzerland). Until October 1999 6R,S-BH<sub>4</sub> was used. This product contained 66.6% 6R-BH<sub>4</sub> and 33.3% 6S-BH<sub>4</sub>. Since October 1999, only biologically active 6R-BH<sub>4</sub> is being used. The amount used is 7.4–20.8 mg/kg (5–95 percentile, median 20.0 mg/kg; range 2.6–26.6 mg/kg). Cumulative data for the individual BH<sub>4</sub> dosage (mg/kg) are presented in Fig. 1.

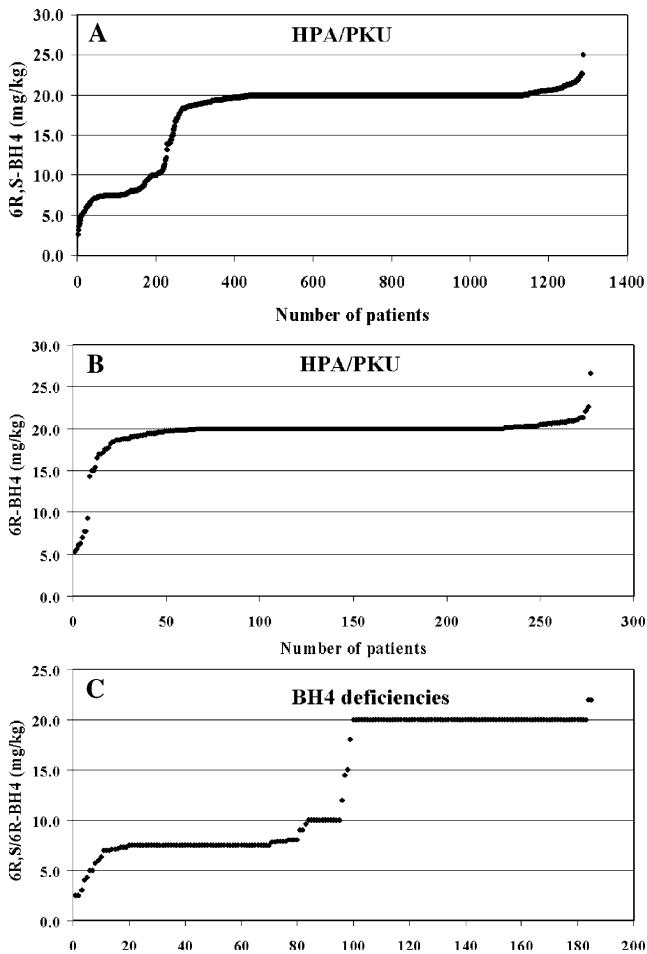


Fig. 1. Cumulative data for BH<sub>4</sub> administered: (A) in patients with HPA/PKU using 6R,S-BH<sub>4</sub>, (B) in patients with HPA/PKU using 6R-BH<sub>4</sub>, and (C) in patients with BH<sub>4</sub> deficiencies using both 6R,S- and 6R-BH<sub>4</sub>.

Tablets of synthetic cofactor were dissolved in 20 ml of water in dim light and administered at least 30 min before a meal.

Table 1  
Summary of the BH<sub>4</sub> loading tests in patients with HPA/PKU and BH<sub>4</sub> deficiencies

	Phe (T <sub>0</sub> ) <sup>*</sup> (μmol/L)		BH <sub>4</sub> (mg/kg)		Age (weeks)	
	5–95 Percentile	Median	5–95 Percentile	Median	5–95 Percentile	Median
HPA/PKU all patients						
6R,S-BH <sub>4</sub> (n = 1452)	200–2702	1074	7.4–20.8	20.0	1–261	3
6R-BH <sub>4</sub> (n = 278)	194–2809	861	17.0–20.8	20.0	1–49	3
HPA/PKU positive <i>S</i> > 0.1 <sup>**</sup>						
6R,S-BH <sub>4</sub> (n = 271)	197–3024	882	7.4–21.1	20.0	1–274	3
6R-BH <sub>4</sub> (n = 135)	193–2317	569	18.3–29.7	20.0	1–30	3
HPA/PKU significant positive <i>S</i> > 3.75 <sup>**</sup>						
6R,S-BH <sub>4</sub> (n = 69)	199–2327	418	10.3–20.6	20.0	1–55	4
6R-BH <sub>4</sub> (n = 99)	183–1271	402	18.8–20.8	20.0	1–23	4
BH <sub>4</sub> deficiencies <sup>***</sup>						
6R,S/6R-BH <sub>4</sub> (n = 189)	249–2437	897	6.1–20.0	10.0	2–325	14

<sup>\*</sup> Blood phenylalanine concentrations at the start of test.

<sup>\*\*</sup> Slope of the "hydroxylation rate," for details see "Section 2."

<sup>\*\*\*</sup> Except for three DHPR-deficient patients, all positive.

Plasma phenylalanine was measured before, and 4 and 8 h after oral administration of BH<sub>4</sub>. In a very few patients additional measurements were performed at 12 and 24 h post loading, however, these data are not included in this study.

Only those tests were considered positive where plasma phenylalanine concentrations decreased constantly (by at least 5%) between 0 and 4 h and between 4 and 8 h after administration of BH<sub>4</sub>. The "hydroxylation rate" was estimated from the percentage of phenylalanine eliminated between 0 and 4 h and between 4 and 8 h after loading.

A slope (*S*) was calculated for the "hydroxylation rates" at 0, 4, and 8 h using the following equation:

$$S = \frac{\sum (y_i - \bar{y}_i) \times x_i}{\sum (x_i - \bar{x}_i)^2},$$

where *y<sub>i</sub>*, percentage of phenylalanine elimination at time 0, 4, and 8 h (*x<sub>i</sub>*).

All loading tests with *S* > 3.75, corresponding to "hydroxylation rates" of at least 30% between 0 and 4 h and between 4 and 8 h were considered significantly positive.

#### Statistical methods

$\chi^2$  and *t* tests (Winstat, Kalmia Company) were used to compare the samples variance. Differences were considered significant when *p* values were <0.05.

## Results

Table 1 summarizes the biochemical data from patients investigated between 1988 and 2002. A total of 1919 loading tests were performed in this period at the Children's Hospital in Zürich and 189 patients were diagnosed as BH<sub>4</sub>-deficient. BH<sub>4</sub> deficiency was confirmed

by the measurement of urinary neopterin and biopterin, and dihydropteridine reductase activity in blood (data not shown).

#### Loading test in patients with phenylalanine hydroxylase deficiency

The 1730 patients in whom BH<sub>4</sub> deficiency was excluded were loaded with either 6R,S-BH<sub>4</sub> (before October 1999) or with the new product, 6R-BH<sub>4</sub>. The loading test was considered positive when  $S > 0.1$  or significantly positive when  $S > 3.75$ . Although the amount of BH<sub>4</sub> used was similar in both groups (median 20 mg/kg), the slope of the “hydroxylation rate” ( $S$ ) was greater than 3.75 in 65, 74, 33, 17, 0, and 10% of patients with initial phenylalanine levels of 120–400, 400–800, 800–1200, 1200–1600, 1600–2200, and >2200 μmol/L, respectively, in the group loaded with 6R-BH<sub>4</sub>/kg. This is 5–20 times higher compared with tests using 6R,S-BH<sub>4</sub> ( $p < 0.0001$ ) (Fig. 2). Fig. 3 shows how patients with different degrees of HPA respond to different amounts of BH<sub>4</sub>. Again, it is obvious that loading with higher amount (18–23 mg/kg) and using 6R-BH<sub>4</sub> gives

the best response. Only very few patients responded to lower dosages of BH<sub>4</sub> (8–18 mg/kg) (Figs. 3A–D) and there was no significant response when using <8 mg BH<sub>4</sub>/kg in patients with initial plasma phenylalanine levels >800 μmol/L (Figs. 3B and D).

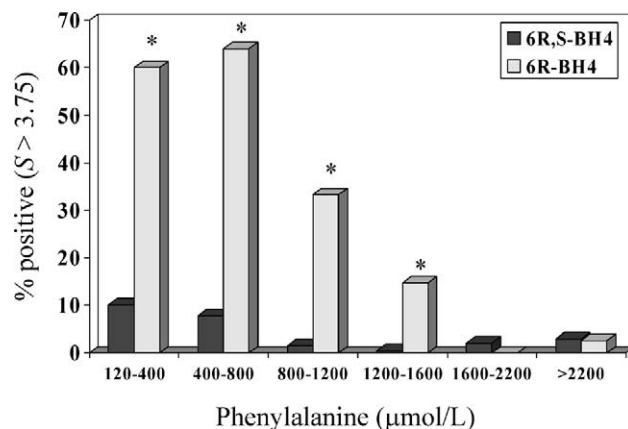


Fig. 2. Percentage of positive loading tests ( $S > 3.75$ ) in patients with different severity of HPA loaded with 6R,S-BH<sub>4</sub> or 6R-BH<sub>4</sub>. \* $p < 0.0005$ .

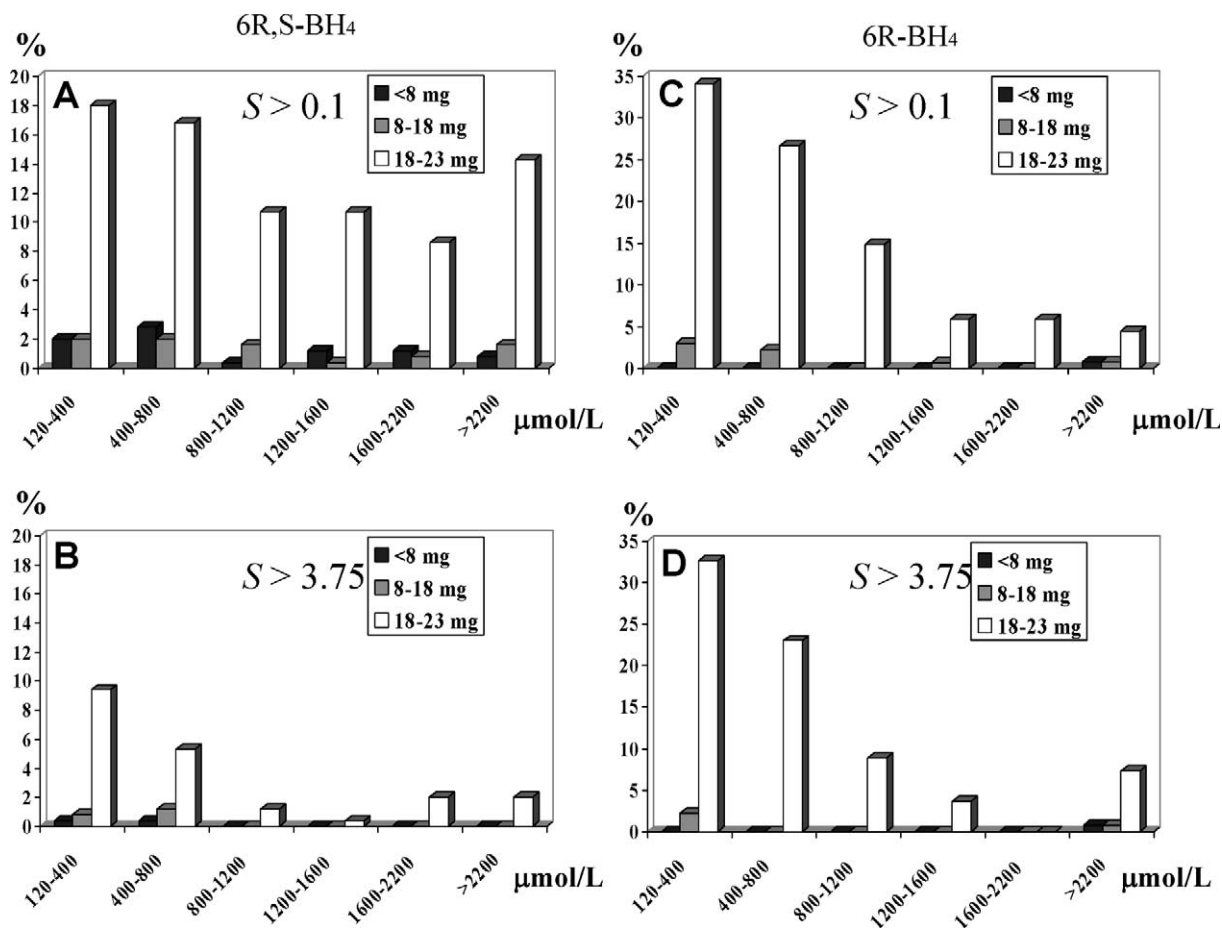


Fig. 3. Distribution of positive ( $S > 0.1$ ) and significantly positive ( $S > 3.75$ ) loading tests according to the amount of administered BH<sub>4</sub> and severity of HPA using 6R,S-BH<sub>4</sub> (A and B) and 6R-BH<sub>4</sub> (C and D).

The “hydroxylation rate” was calculated from the difference in plasma phenylalanine concentrations 4 and 8 h after administration of BH<sub>4</sub> and the slope ( $S$ ) of the “hydroxylation rate” was used as an indicator for the BH<sub>4</sub> responsiveness (Fig. 4). In patients tested with the old product (6R,S-BH<sub>4</sub>), which contained 33.3% of the biologically inactive substance, the percentage of positive tests ( $S > 0.1$ ) was much lower compared with tests using the fully active product 6R-BH<sub>4</sub> ( $p < 0.02$ ) (Figs. 4A and B). The highest positive slopes ( $S$ ) were obtained in patients with mild HPA (phenylalanine  $< 1200 \mu\text{mol/L}$ ), regardless which product was used. However, the percentage of positive and significantly positive tests ( $S > 3.75$ ) was much higher in patients loaded with 6R-BH<sub>4</sub> ( $p < 0.001$ ) (Figs. 2 and 4C–D).

Although the slope ( $S$ ) discriminates between the BH<sub>4</sub>-responders and non-responders, the effectiveness of BH<sub>4</sub> to reduce phenylalanine concentrations to  $360 \mu\text{mol/L}$  (cut-off level accepted to be safe in newborns) depends on the initial concentrations. Thus, patients with the same slope ( $S$ ) but with higher initial plasma phenylalanine concentrations need longer to

reach the cut-off levels (Fig. 5). While patients with initial phenylalanine levels of  $551 \mu\text{mol/L}$  reach the cut-off level 8 hours after loading with  $20 \text{ mg BH}_4/\text{kg}$ , patients with the same slope ( $S \sim 4.3$ ) but initial phenylalanine levels of  $1312$  and  $3394 \mu\text{mol/L}$  need 19 and 23 h, respectively.

In order to estimate the approximate time required for the reduction of plasma phenylalanine concentrations to  $360 \mu\text{mol/L}$  after administration of BH<sub>4</sub> ( $20 \text{ mg/kg}$ ) a ratio of initial phenylalanine concentration and slope ( $S$ ) was plotted against the time. By a simple interpolation between the  $y$ -axis, correlation curve, and  $x$ -axis the time required for the test can be estimated (Fig. 6). Thus, a patient with a basal phenylalanine level of  $841 \mu\text{mol/L}$  and with  $S = 4.6$  (Phe/ $S = 184$ ) requires about 12 h to lower plasma phenylalanine concentrations to  $360 \mu\text{mol/L}$ .

#### *Loading test in patients with tetrahydrobiopterin deficiencies*

With exception of the three patients with dihydropyridine reductase deficiency (loaded with  $7.5 \text{ mg BH}_4/$

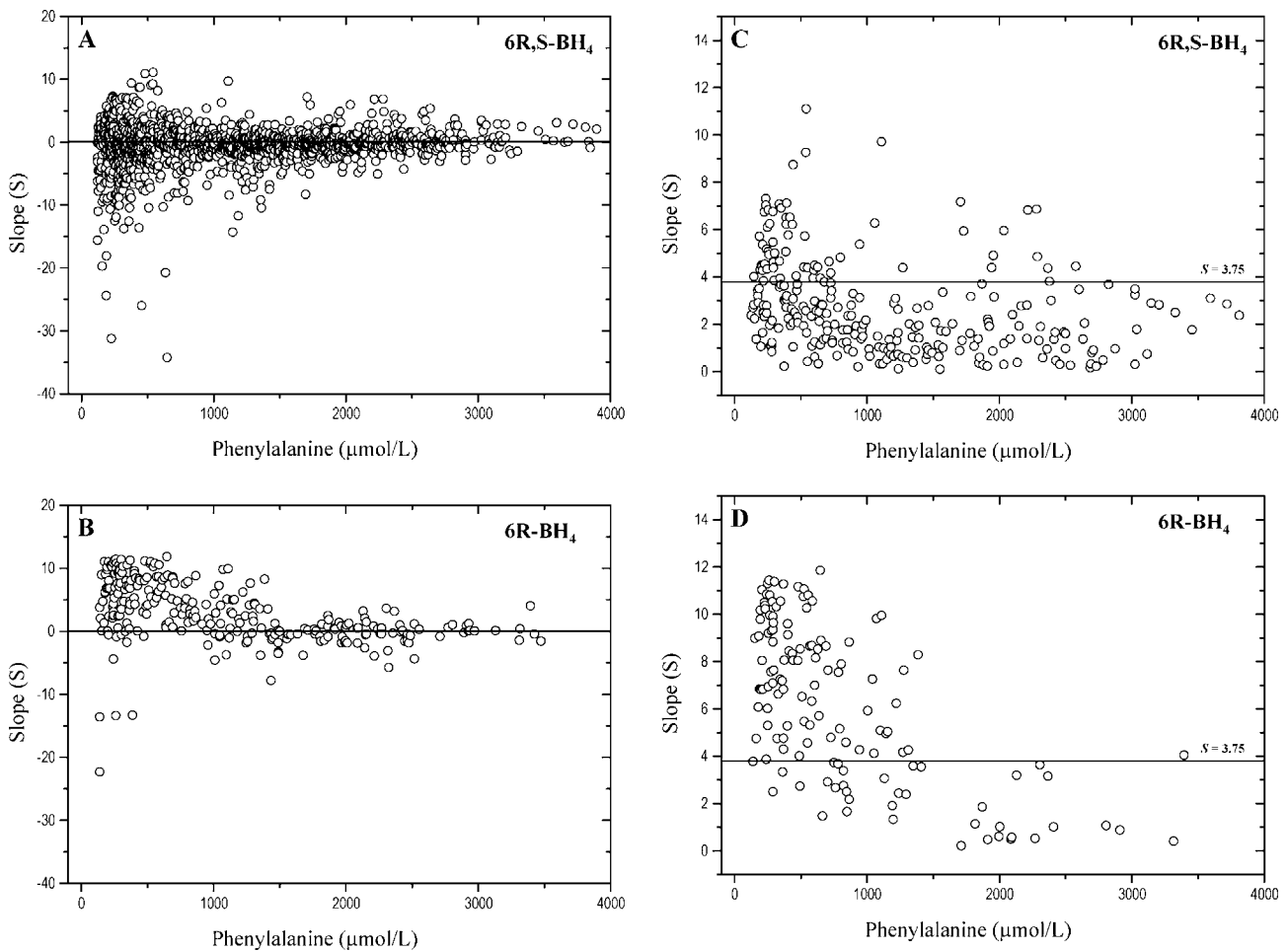


Fig. 4. BH<sub>4</sub>-responsiveness in patients with HPA loaded with (A) 6R,S-BH<sub>4</sub> and (B) 6R-BH<sub>4</sub>. Positive loading tests in patients tested with (C) 6R,S-BH<sub>4</sub> and (D) 6R-BH<sub>4</sub>. Positive are all tests with the slope ( $S$ ) greater than 0.1.  $S > 3.75$  is considered significantly positive ( $p < 0.001$ ).

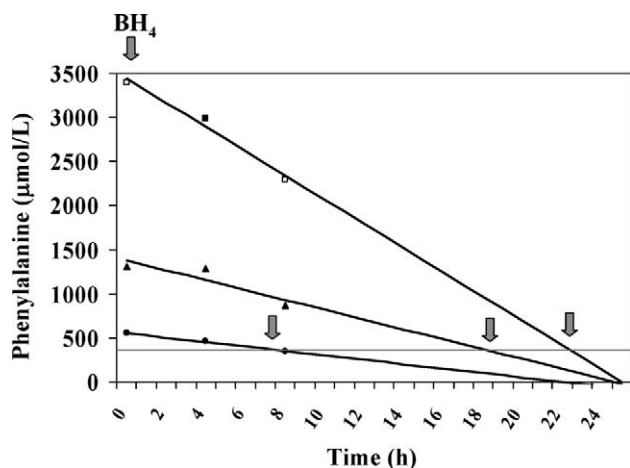


Fig. 5.  $\text{BH}_4$  loading test in three patients with different degrees of HPA but with similar slopes ( $S = 4.1\text{--}4.6$ ). Arrow indicates the time needed to reach the therapeutic phenylalanine level of  $360\ \mu\text{mol/L}$ . Not corrected for  $\text{BH}_4$  half-life (ca. 8 h).

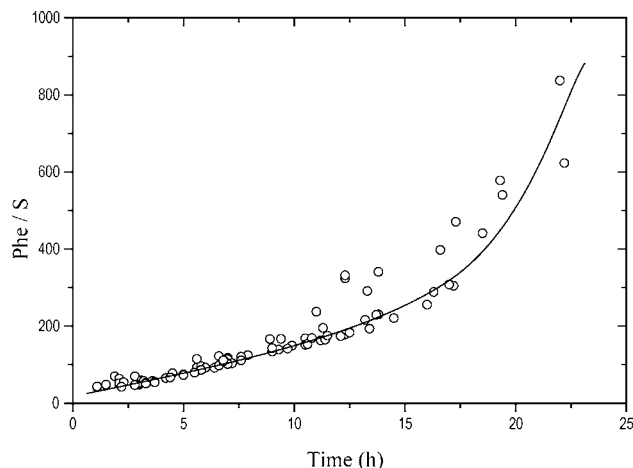


Fig. 6. Plot of initial phenylalanine/slope ( $S$ ) ratios against time. Data generated using the loading tests with  $6\text{R,S-BH}_4$  and  $6\text{R-BH}_4$  in patients with initial phenylalanine concentrations  $>400\ \mu\text{mol/L}$  ( $n = 85$ ).

kg) all patients with different forms of  $\text{BH}_4$  deficiency responded to the loading test (Fig. 7), regardless of the product used. The median dosage used was  $10.0\ \text{mg/kg}$  (Table 1). Because in most patients with defects in the biosynthesis of  $\text{BH}_4$  (6-pyruvoyl-tetrahydropterin syn-

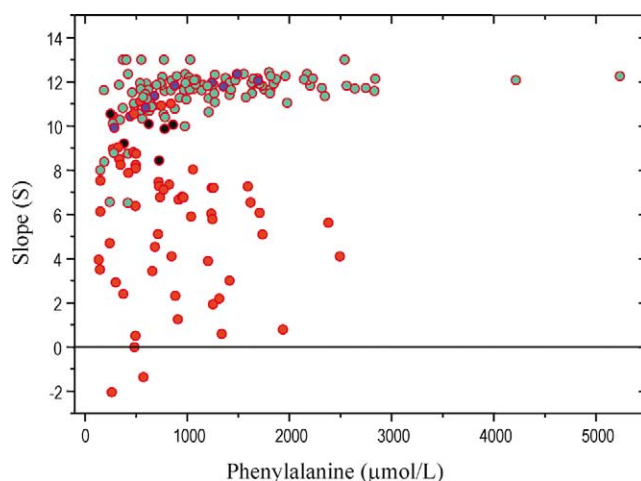


Fig. 7.  $\text{BH}_4$ -responsiveness in patients with  $\text{BH}_4$  deficiencies loaded with  $6\text{R,S-BH}_4$  or  $6\text{R-BH}_4$ .  $S = 3.75$  is considered significantly positive ( $p < 0.001$ ). Green: PTPS deficiency ( $n = 113$ ), blue: GTPCH deficiency ( $n = 9$ ), black: PCD deficiency ( $n = 6$ ), and red: DHPR deficiency ( $n = 61$ ).

thase and GTP cyclohydrolase deficiency) plasma phenylalanine levels normalize within the first 4 h after  $\text{BH}_4$  administration, the “hydroxylation rate” decreases considerably between 4 and 8 h and the slope ( $S$ ) is no longer discriminative. When calculating the slope ( $S$ ) for the first hour after  $\text{BH}_4$  administration in patients with 6-pyruvoyl-tetrahydropterin synthase and GTP cyclohydrolase deficiency values ranged between 10.6 and 24.3 (data not shown). Patients with dihydropteridine reductase deficiency are definitely slow responders with slopes ( $S$ ) below 10 and diagnosis is possible only by measurement of DHPR activity in red blood cells.

#### Urinary neopterin and biopterin

In order to exclude  $\text{BH}_4$  deficiency and to monitor GI absorption of the administered  $\text{BH}_4$  all loading tests performed were accompanied by the measurement of urinary neopterin and biopterin. Table 2 summarizes neopterin and biopterin concentrations in urine from patients with PAH deficiency and different degrees of HPA. These data clearly document that patients with

Table 2  
Neopterin and biopterin excretion in urine from patients with phenylalanine hydroxylase deficiency

Phenylalanine* ( $\mu\text{mol/L}$ )	$n$	Neopterin** (mmol/mol creat.)	Biopterin** (mmol/mol creat.)
<400	401	1.00–7.90	0.50–4.60
400–800	321	1.20–13.22	0.60–5.10
800–1200	244	1.20–14.50	0.70–5.30
1200–1600	249	1.20–15.50	0.75–7.30
1600–2200	276	1.40–18.71	0.89–7.60
>2200	239	1.40–19.80	0.90–7.90

\* Basal blood levels.

\*\* 5–95 Percentile.

higher blood phenylalanine levels excrete more neopterin and biopterin in urine.

## Discussion

The BH<sub>4</sub> loading test was initially developed as a practical tool for the diagnosis of BH<sub>4</sub> deficiencies [9]. Different protocols, using 7.5–20.0 mg BH<sub>4</sub>/kg body weight, were used in the past to discriminate between defects in the BH<sub>4</sub> cofactor metabolism and classical PKU. The main advantage of the BH<sub>4</sub> loading test was the fact that amino acid analysis is more frequently available than measurement of urinary pterins. In most clinical centers blood phenylalanine and tyrosine can be measured within 24 h after the challenge. Urinary pterins analysis is available only in a few specialized laboratories and it takes longer to receive a final report. Information of a positive loading test will speed up further investigations and patient management. An important precondition for the BH<sub>4</sub> loading test is high enough plasma phenylalanine levels (>400 μmol/L) [24]. Patients with lower plasma phenylalanine levels or those already on low-phenylalanine administration need to be preloaded (3 h before BH<sub>4</sub> administration) with phenylalanine (100 mg/kg). Although it is generally assumed that the BH<sub>4</sub> loading test differentiates between BH<sub>4</sub> defects and classical PKU, patients with dihydropteridine reductase deficiency are rather slow responders and in some the test is negative when performed with lower doses of 7.5 mg BH<sub>4</sub>/kg [6]. Data from our study demonstrate clearly that higher doses of 20.0 mg/kg are more effective in these patients.

For many years we have detected a number of patients in whom BH<sub>4</sub> deficiency was excluded and who responded positively to the BH<sub>4</sub> loading test and it has been suggested that this may be due to a hypothetical *K<sub>m</sub>* mutant of PAH. However, only since the recent publication by Kure et al. [10] have such cases been investigated in more detail. Thus, the BH<sub>4</sub> loading test seems to be helpful in the diagnosis of this new group of patients with PAH deficiency. Our retrospective study with 1730 patients documents that most of the BH<sub>4</sub>-responsive PAH deficiencies belongs to mild forms of HPA. Almost 70% of patients with plasma phenylalanine levels between 400 and 800 μmol/L responded to oral administration of 20 mg 6R-BH<sub>4</sub>/kg.

In the past commercially available BH<sub>4</sub> was a mixture of 66.6% biologically active 6R-BH<sub>4</sub> and 33.3 inactive 6S-BH<sub>4</sub>. Thus, loading protocols before October 1999 used much lower concentrations of the active substance; i.e., in a protocol designed for the loading test with 20 mg/kg only 13.2 mg/kg active BH<sub>4</sub> was available. This may be one of the reasons that in the last few years more positive tests are accounted for. It may also explain why only one out of three patients with the common R408W/

Y414C mutation responded to the loading with 20 mg BH<sub>4</sub>/kg body weight [12]. When comparing loading tests performed with 6R,S-BH<sub>4</sub> before October 1999 with those performed with 6R-BH<sub>4</sub> after October 1999, we found the percentage of positives to be much higher in the second group (Fig. 2). The greatest difference was found in the group of mild HPAs. Patients with plasma phenylalanine levels <1200 μmol/L responded more frequently to BH<sub>4</sub>-loading than patients with higher phenylalanine levels. As already mentioned, patients with levels between 400 and 800 μmol/L are the best responders and a potential target population for treatment with BH<sub>4</sub>. In patients with phenylalanine levels <1600 μmol/L, 6R-BH<sub>4</sub> was 5–20 times more effective than the less active 6R,S-BH<sub>4</sub>.

Our study was limited to loading tests performed within 8 h after BH<sub>4</sub> administration (>99.4% of all tests performed) and it was obvious that some patients responded very slowly. BH<sub>4</sub>-responsiveness was calculated as “phenylalanine hydroxylation” at 4 and 8 h after loading and was expressed as the percentage of phenylalanine eliminated. The slope (*S*) of “hydroxylation rates” at 0, 4, and 8 h was compared for different BH<sub>4</sub> products and for different groups of patients presenting with different initial plasma phenylalanine concentrations. The slope (*S*) discriminates between non-responders, slow responders, and responders. Fig. 5 demonstrates that slow responders with a lower slope (*S*) need more time to reach the cut-off values of 360 μmol/L and that the effectiveness of administered BH<sub>4</sub> depends on the initial phenylalanine levels. For some patients with phenylalanine levels <800 μmol/L and for most of those with plasma levels >1200 μmol/L, additional phenylalanine measurement at 24 h after loading is recommended. A plot of the ratio Phe/*S* against time can be used to estimate the approximate time needed to reach the therapeutic phenylalanine values of <360 μmol/L. This calculation is only relative as the half-life of BH<sub>4</sub> is approximately 8 h [25] and the second BH<sub>4</sub> administration after 12 h may be necessary in order to maintain a constant blood BH<sub>4</sub> concentration. However, from a practical point of view the single loading test is sensitive enough to detect both responsive PAH and BH<sub>4</sub> deficiencies.

Based on our investigations, the following protocol is recommended for the loading test in patients with plasma phenylalanine levels >400 μmol/L [26]: The test is carried out after at least 3 h of fasting. Urine samples for neopterin and biopterin should be collected before the beginning of the test. After oral application of 6R-BH<sub>4</sub> (20 mg/kg body weight) normal food intake is continued during the entire testing period. Blood samples for phenylalanine and tyrosine measurement are taken at 0, 4, 8, and 24 h. The second urine sample is collected between 4 and 8 h. A blood spot for dihydropteridine reductase activity measurement can be

collected anytime during the test. In patients with plasma phenylalanine levels  $<400 \mu\text{mol/L}$  or in patients already on a low-phenylalanine diet, a combined phenylalanine-BH<sub>4</sub> test is recommended. In these patients 100 mg phenylalanine/kg body weight should be given orally 3 h before the BH<sub>4</sub> administration [27].

Detection of patients with BH<sub>4</sub>-responsive PAH deficiency may have important therapeutic implications as these patients can be treated with BH<sub>4</sub> instead of phenylalanine or a protein-low diet. Preliminary results document that BH<sub>4</sub> supplementation is effective and compliance is very good [11,13,19]. In most studies an initial dosage of 10 mg BH<sub>4</sub>/kg/day was used. Due to individual variations and residual activity of PAH, each patient should be titrated with BH<sub>4</sub> and the dosage should be increased or decreased accordingly. The relative short half-life of BH<sub>4</sub> (ca. 8 h) requires the daily amount of BH<sub>4</sub> to be given in at least two doses. In patients with classical PKU (phenylalanine  $>1200 \mu\text{mol/L}$ ) phenylalanine tolerance can be significantly increased by a combined BH<sub>4</sub>-PKU diet treatment [28]. Thus, BH<sub>4</sub> therapy is indicated in all BH<sub>4</sub>-responsive variants of PAH deficiency regardless of the severity of HPA. The main problem in treatment with BH<sub>4</sub> is the relative high price. While the approximate cost for BH<sub>4</sub> treatment per year in newborns is similar as for the low-phenylalanine diet (US\$ ~2500, for 10 mg BH<sub>4</sub>/kg/day), it increases with age (at 2 years: US\$ 7000 for BH<sub>4</sub> vs. US\$ 3000 for the diet; at 7 years: US\$ 13,000 for BK vs. US\$ 5600 for the diet).

DNA analysis was done in only a few patients included in this retrospective study and it was not possible to correlate particular mutations with BH<sub>4</sub>-responsiveness. However, based on previously published data it is evident that more than one mechanism is responsible for this phenotype [11,12,14–17,28–30]. Table 3 summarizes the mutations found on at least one allele in patients with BH<sub>4</sub>-responsive HPA. Most of these are located in the catalytic domain of PAH, some are in the regulatory domain, and one is in the tetramerization domain. Many of the missense mutations found show considerable residual activity ( $>25\%$ ) when expressed recombinantly in eukaryotic cell systems. Most of the patients were found to be compound heterozygotes, two patients were homozygotes for the L48S/L48S and Y414C/Y414C mutations [15,16]. Thus, in addition to the initially proposed  $K_m$  mutants affecting the affinity of the enzyme for BH<sub>4</sub>, other mechanisms such as protein stabilization or *PAH* gene expression have also been proposed. It has been demonstrated that BH<sub>4</sub> can increase both *PAH* mRNA and enzyme activity in the *hph-1* mouse (GTP cyclohydrolase deficiency) by 50% within 30 min [20]. Responsiveness to BH<sub>4</sub> may exert a combined effect of increasing the affinity for BH<sub>4</sub>, tetramer stabilization, and short term gene expression. More in vitro studies are required to clarify the exact mechanism of PAH activation.

This study demonstrates the importance of performing a BH<sub>4</sub> loading test in all patients with HPA. Beside its importance in the diagnosis of BH<sub>4</sub> deficiencies, it is

Table 3  
Mutations in the *PAH* gene associated with BH<sub>4</sub>-responsiveness

Amino acid substitution	Exon	PAH domain	PAH enzyme activity*	Reference
F39L	2	Regulatory	46	[30]
L48S	2	Regulatory	39	[15]
I65T	3	Regulatory	nd	[19]
R68S	3	Regulatory	76	[30]
A104D	3	Regulatory	nd	[16]
S110C	3	Regulatory	nd	[28]
D129G	4	Regulatory	nd	[28]
E178G	5	Catalytic	nd	[30]
V190A	5	Catalytic	nd	[14]
P211T	5	Catalytic	72	[28]
R241C	6	Catalytic	25	[10,14]
R261Q	6	Catalytic	27	[19]
A300S	7	Catalytic	nd	[14]
L308F	8	Catalytic	nd	[30]
A313T	8	Catalytic	nd	[14]
K320N	8	Catalytic	nd	[16]
A373T	10	Catalytic	nd	[10]
V388M	10	Catalytic	23	[31]
E390G	10	Catalytic	70	[11,17]
A395P	10	Catalytic	nd	[19]
A403V	11	Catalytic	32	[14]
P407S	11	Catalytic	nd	[10]
Y414C	11	Tetramerization	28	[10,12,16,17]

nd, not done.

\* As percentage of wild type (in cell lysates).

essential for the diagnosis of BH<sub>4</sub>-responsive variants of PAH deficiency. More than 60% of patients with mild forms of PAH deficiency may benefit from BH<sub>4</sub> therapy and in patients with classical PKU additional supplementation of BH<sub>4</sub> may increase phenylalanine tolerance. The new protocol for the BH<sub>4</sub> loading test using 20 mg/kg and blood sampling for phenylalanine and tyrosine at 0, 4, 8, and 24 h differentiates between BH<sub>4</sub>-responders and non-responders. In BH<sub>4</sub>-responders cofactor defects should be excluded by additional investigations.

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### References

- [1] N. Blau, B. Thöny, R.G.H. Cotton, K. Hyland, Disorders of tetrahydrobiopterin and related biogenic amines, 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*, eighth ed., McGraw-Hill, New York, 2001, pp. 1725–1776.
- [2] D.M. Danks, R.G.H. Cotton, P. Schlesinger, Tetrahydrobiopterin treatment of variant form of phenylketonuria, *Lancet* 2 (1975) 1043.
- [3] D.M. Danks, R.G.H. Cotton, P. Schlesinger, Variant forms of phenylketonuria, *Lancet* 1 (1976) 236–237.
- [4] A. Niederwieser, H.C. Curtius, M. Viscontini, J. Schaub, H. Schmidt, Phenylketonuria variants, *Lancet* 1 (1979) 550.
- [5] A. Niederwieser, H.C. Curtius, M. Wang, D. Leupold, Atypical phenylketonuria with defective biopterin metabolism. Monotherapy with tetrahydrobiopterin or sepiapterin, screening and study of biosynthesis in man, *Eur. J. Pediatr.* 138 (2) (1982) 110–112.
- [6] A. Lipson, J. Yu, M. O Halloran, M. Potter, B. Wilken, Dihydropteridine reductase deficiency: non-response to oral tetrahydrobiopterin load test, *J. Inherit. Metab. Dis.* 7 (1984) 69–71.
- [7] A. Ponzzone, O. Guardamagna, S. Ferraris, G. Bracco, R.G.H. Cotton, Screening for malignant phenylketonuria, *Lancet* 1 (1987) 512–513.
- [8] 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.
- [9] A. Ponzzone, O. Guardamagna, S. Ferraris, G.B. Ferrero, I. Dianzani, R.G.H. Cotton, Tetrahydrobiopterin loading test in hyperphenylalaninemia, *Pediatr. Res.* 30 (1991) 435–438.
- [10] S. Kure, D.C. Hou, T. Ohura, H.S.S. Iwamoto, N. Sugiyama, O. Sakamoto, K. Fujii, Y. Matsubara, K. Narisawa, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, *J. Pediatr.* 135 (3) (1999) 375–378.
- [11] F.K. Trefz, C. Aulehla-Scholz, N. Blau, Successful treatment of phenylketonuria with tetrahydrobiopterin, *Eur. J. Pediatr.* 160 (2001) 315.
- [12] M. Lindner, D. Haas, J. Zschocke, P. Burgard, Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype, *Mol. Genet. Metab.* 73 (1) (2001) 104–106.
- [13] J.M. Nuoffer, B. Thöny, A. Romstad, N. Blau, A patient with phenylketonuria successfully treated with tetrahydrobiopterin, *J. Inherit. Metab. Dis.* 24 (Suppl. 1) (2001) 29.
- [14] L.J.M. Spaapen, J.A. Bakker, C. Velter, W. Loots, M.E. Rubio, P.P. Forget, L. Dorland, T.J. de Konig, B.T. Poll-The, H.K. Ploos van Amstel, J. Bekhof, N. Blau, M. Duran, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Dutch neonates, *J. Inherit. Metab. Dis.* 24 (2001) 325–358.
- [15] N. Blau, F.K. Trefz, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency: possible regulation of gene expression in a patient with the homozygous L48S mutation, *Mol. Genet. Metabol.* 75 (2002) 186–187.
- [16] R. Steinfeld, A. Kohlschütter, J. Zschocke, M. Lindner, K. Ullrich, Z. Lukacs, Tetrahydrobiopterin monotherapy for phenylketonuria patients with common mild mutations, *Eur. J. Pediatr.* 161 (7) (2002) 403–405.
- [17] J. Weglage, M. Grenzebach, A. v. Teeffelen-Heithoff, T. Marquardt, R. Feldmann, J. Denecke, D. Gödde, H.G. Koch, Tetrahydrobiopterin responsiveness in a large series of phenylketonuria patients, *J. Inherit. Metab. Dis.* 25 (2002) 321–322.
- [18] H. Erlandsen, R.C. Stevens, A structural hypothesis for BH<sub>4</sub> responsiveness in patients with mild forms of hyperphenylalaninemia and phenylketonuria, *J. Inherit. Metab. Dis.* 24 (2001) 213–230.
- [19] 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.
- [20] K. Hyland, T.L. Munk-Martin, Tetrahydrobiopterin regulates tyrosine hydroxylase and phenylalanine hydroxylase gene expression in dominantly inherited GTP cyclohydrolase deficiency, *J. Inherit. Metab. Dis.* 24 (Suppl. 1) (2001) 30.
- [21] N. Blau, B. Thöny, M. Spada, A. Ponzzone, Tetrahydrobiopterin and inherited hyperphenylalaninemia, *Turk. J. Pediatr.* 38 (1996) 19–35.
- [22] H.C. Curtius, N. Blau, T. Kuster, Pterins, in: F.A. Hommes (Ed.), *Techniques in Diagnostic Human Biochemical Genetics*, Wiley-Liss, New York, 1991, pp. 377–396.
- [23] N. Arai, K. Narisawa, H. Hayakawa, K. Tada, Hyperphenylalaninemia due to dihydropteridine reductase deficiency: diagnosis by enzyme assays on dried blood spots, *Pediatrics* 70 (1982) 426–430.
- [24] N. Blau, L. Bonafé, M. Blaskovics, Disorders of phenylalanine and tetrahydrobiopterin, in: N. Blau, M. Duran, M. Blaskovics, K.M. Gibson (Eds.), *Physician' Guide to the Laboratory Diagnosis of Metabolic Disease*, Springer, Heidelberg, 2002, pp. 89–106.
- [25] A. Ponzzone, O. Guardamagna, M. Spada, R. Ponzzone, M. Sartore, L. Kierat, C.W. Heizmann, N. Blau, Hyperphenylalaninemia and pterin metabolism in serum and erythrocytes, *Clin. Chim. Acta.* 216 (1993) 63–71.
- [26] N. Blau, A. Muntau, Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, in: *EMG Workshop Results*, Milupa, Friedrichsdorf, 2002.
- [27] A. Ponzzone, O. Guardamagna, M. Spada, S. Ferraris, R. Ponzzone, L. Kierat, N. Blau, Differential diagnosis of hyperphenylalaninemia by a combined phenylalanine-tetrahydrobiopterin loading test, *Eur. J. Pediatr.* 152 (1993) 655–661.
- [28] J.B. Hennermann, B. Vetter, A.E. Kulozik, E. Mönch, Partiel und total tetrahydrobiopterin-responsiveness in classical and mild

- phenylketonuria (PKU), *J. Inherit. Metab. Dis.* 25 (Suppl. 1) (2002) 21.
- [29] R. Koch, G. Flemming, N. Blau, Mental illness, mild hyperphenylalaninemia, and intellectual loss responsive to tetrahydrobiopterin therapy, *Mol. Genet. Metab.* 75 (2002) 284–286.
- [30] R. Matalon, R. Koch, K. Michals-Matalon, K. Mosley, R. Stevens, Tetrahydrobiopterin-responsive phenylalanine hydroxylase mutations, *J. Inherit. Metab. Dis.* 25 (Suppl. 1) (2002) 23.
- [31] P. Leandro, I. Rivera, M.C. Lechner, I.T. de Almeida, D. Konecki, The V388M mutation results in a kinetic variant form of phenylalanine hydroxylase, *Mol. Genet. Metab.* 69 (3) (2000) 204–212.