

## Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Dutch neonates

L. J. M. SPAAPEN<sup>1\*</sup>, J. A. BAKKER<sup>2</sup>, C. VELTER<sup>2</sup>, W. LOOTS<sup>2</sup>,  
M. E. RUBIO-GONZALBO<sup>1,3</sup>, P. P. FORGET<sup>3</sup>, L. DORLAND<sup>4</sup>,  
T. J. DE KONING<sup>4</sup>, B. T. POLL-THE<sup>4</sup>, H. K. PLOOS VAN AMSTEL<sup>4</sup>,  
J. BEKHOF<sup>5</sup>, N. BLAU<sup>6</sup> and M. DURAN<sup>4</sup>

*Departments of*<sup>1</sup>*Biochemical Genetics and*<sup>2</sup>*Molecular Genetics, Stichting Klinische Genetica Zuid-Oost Nederland;* <sup>3</sup>*Department of Pediatrics, Academic Hospital Maastricht;* <sup>4</sup>*University Medical Center Utrecht;* <sup>5</sup>*Department of Pediatrics, Academic Hospital Groningen, The Netherlands;* <sup>6</sup>*Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zurich, Switzerland*

*\* Correspondence: Department of Biochemical Genetics, Stichting Klinische Genetica Zuid-Oost Nederland, Joseph Bechlaan 113, 6229 GR Maastricht, The Netherlands. E-mail: leo.spaapen@gen.unimaas.nl*

*MS received 26.09.00 Accepted 01.12.00*

**Summary** Four neonates with a positive phenylalanine screening test (Phe concentrations between 258 and 1250  $\mu\text{mol/L}$ ) were investigated further to differentiate between phenylalanine hydroxylase (PAH) deficiency and variant hyperphenylalaninaemia (HPA) forms. In patients 1 and 2 a tetrahydrobiopterin ( $\text{BH}_4$ ) load caused a significant decrease of the plasma Phe levels. A combined phenylalanine/ $\text{BH}_4$  loading test was performed in patients 2, 3 and 4. In the latter two patients, plasma Phe concentrations completely normalized within 8 h after the  $\text{BH}_4$  load (20 mg/kg). Basal urinary pterins were normal in all four patients. The activity of dihydropteridine reductase (DHPR) was normal in patients 1, 2 and 3 and 50% of control values in patient 4 (not in the range of DHPR-deficient patients). In patient 3 a subsequent phenylalanine loading test with concomitant analysis of plasma bipterins revealed a normal increase of bipterin, excluding a  $\text{BH}_4$  biosynthesis defect. Pterins and neurotransmitter metabolites in CSF of patients 1, 3 and 4 were normal. DNA mutations detected in the PAH gene of patients 1–4 were A313T, and L367fsinsC; V190A and R243X; A300S and A403V; R241C and A403V. The results are suggestive for mutant PAH enzymes with decreased affinity for the cofactor  $\text{BH}_4$ .

Hyperphenylalaninaemia (HPA) is a disorder caused by a deficient or a decreased activity of phenylalanine-4-hydroxylase (PAH, EC 1.14.16.1) due either to a

mutated enzyme protein or to a deficiency of its obligatory cofactor tetrahydrobiopterin (BH<sub>4</sub>). The latter group comprises defects in the biosynthesis and in the regeneration of BH<sub>4</sub> (Scriver et al 1995). Detection of HPA is included in the newborn mass screening programme. Differential diagnostic investigations are necessary, however to detect BH<sub>4</sub> deficiencies even if phenylalanine (Phe) concentrations are only slightly elevated (Ponzone et al 1993). Screening for BH<sub>4</sub> deficiency is performed by analysis of pterins in urine and measurement of dihydropteridine reductase (DHPR) activity in erythrocytes or skin fibroblasts (Blau and Blaskoviks 1996). In the Netherlands as well as in several other countries, a BH<sub>4</sub> loading test is included in the screening protocol of HPA. However, if the initial Phe concentration is below 400 µmol/L, a combined Phe/BH<sub>4</sub> loading is performed (Ponzone et al 1993). Recently, Kure and colleagues (1999) reported that serum Phe concentrations in four patients with mild HPA decreased after a BH<sub>4</sub> challenge. Urinary pterins in their patients and DHPR activities in blood appeared to be normal. In addition, mutations were detected in the PAH genes of those patients.

In the follow-up of a positive newborn PKU-screening test we found four children to be responsive after either a BH<sub>4</sub> challenge or a combined Phe/BH<sub>4</sub> loading test. DHPR deficiency was excluded and urinary pterins appeared to be normal. Mutations were found in the PAH genes of all these patients; thus this group of patients belongs to a new variant of BH<sub>4</sub>-responsive PAH deficiency.

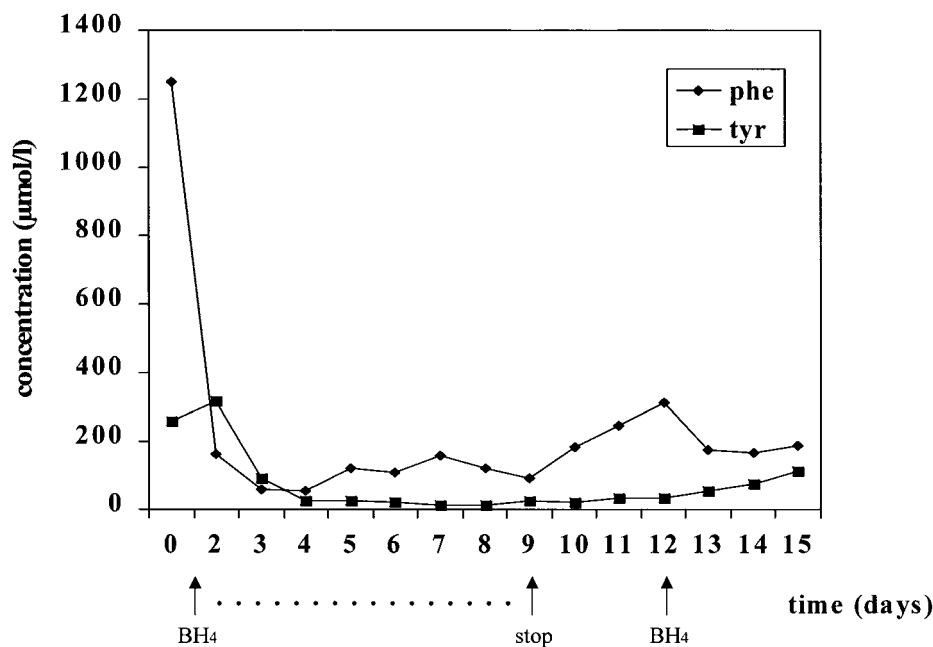
## PATIENTS AND METHODS

Patient 1, a girl, was born prematurely after a pregnancy of 28 weeks because of maternal complications. Her birth weight was 750 g, she was dysmature and suffered from severe neonatal complications. Patients 2, 3 and 4 were born at term after uncomplicated pregnancies. They were admitted to the academic hospital for evaluation of a positive newborn PKU screening test. Combined Phe/BH<sub>4</sub> loading tests were performed according to Ponzone and colleagues (1993).

Amino acids were analysed by means of automated ion-exchange chromatography with postcolumn ninhydrin derivatization (Biochrom 20, Amersham Pharmacia Biotech). DHPR activity in erythrocytes was measured as described previously (Surplice et al 1990) or in cultured skin fibroblasts according to Bonafé and colleagues (2000). Urinary pterins were analysed by a HPLC procedure adapted from Fukushima and Nixon (1980) and Nixon and colleagues (1980). Neurotransmitter metabolites were analysed in CSF of patients 1, 3 and 4 as described (Blau et al 1999). Mutations in the PAH gene were detected by means of single-strand conformational analysis and subsequent sequence analysis (van der Sijs-Bos et al 1996).

## RESULTS AND DISCUSSION

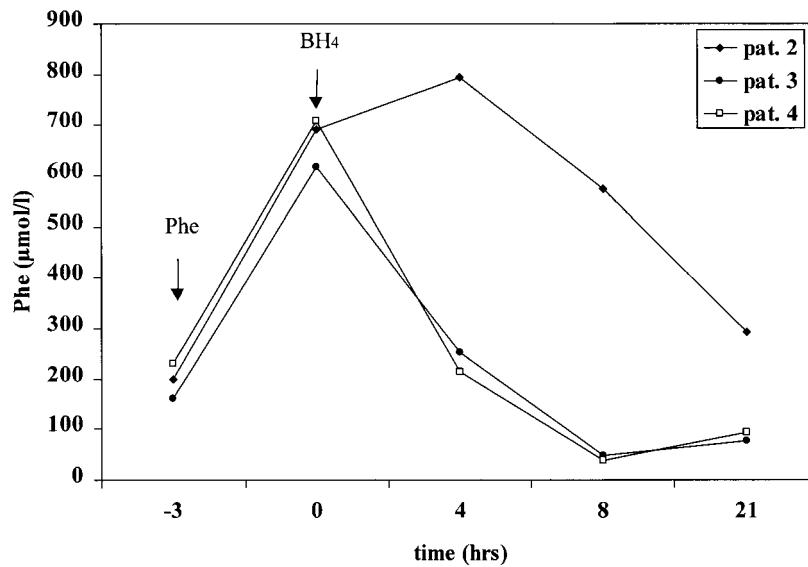
The positive PKU screening test in patient 1 was followed by a BH<sub>4</sub> load of 20 mg/kg body weight. Figure 1 shows the response of plasma Phe to the BH<sub>4</sub> load. Because of the rapid decrease of the plasma Phe concentration, treatment with BH<sub>4</sub> (5 mg/kg per day) was continued during the next 8 days. From day 9 the treatment was stopped



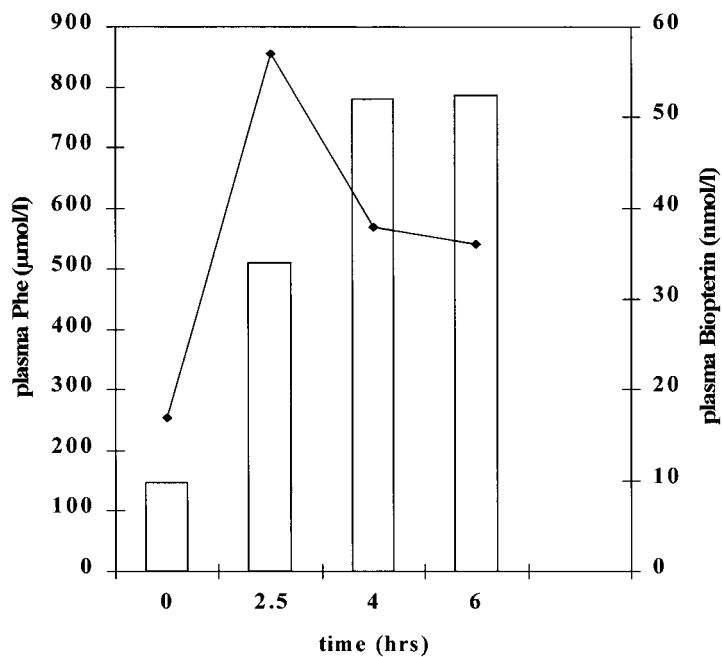
**Figure 1** Course of plasma phenylalanine and tyrosine concentrations in patient 1 after BH<sub>4</sub> supplementation: day 1, 20 mg/kg; days 2–9, 5 mg/kg per day; days 9–12 stop supplementation; from day 12 on continuation of treatment with BH<sub>4</sub> 5 mg/kg per day

for 3 days, resulting in an increase of the Phe level. Subsequently, the treatment was instituted again. The initial Phe concentration in patient 2 was 450 μmol/L. A single BH<sub>4</sub> load of 20 mg/kg resulted in a decrease of Phe from 445 μmol/L before load to 251 μmol/L and 26 μmol/L, 8 and 33 h following load, respectively.

Because of the moderately increased Phe concentrations in patients 3 and 4, combined Phe/BH<sub>4</sub> loading tests were performed. Patient 2 was retested in the same way. Figure 2 shows the courses of the plasma Phe concentrations during the tests. Urinary pterins were normal in all four patients. DHPR activity in cultured fibroblasts of patient 1 and in erythrocytes of patients 2 and 3 were found normal, whereas in patient 4 DHPR activity was 46% of control (data not shown). Phenylalanine-induced biopterin synthesis was studied in patient 3 following an oral Phe load (100 mg/kg). The normal increase of biopterin (Figure 3) excluded a BH<sub>4</sub> biosynthesis defect. Analysis of neurotransmitter metabolites in CSF of patients 1, 3 and 4 did not reveal deficiencies of neurotransmitters (Table 1); CSF of patient 2 was not analysed. In addition, CSF pterins were found normal (data not shown). These normal findings prompted us to analyse the PAH gene. Mutations in both alleles of the PAH gene were identified in all four patients (Table 2). Patients 3 and 4 share the same mutation (A403V) although they are not related. They showed a similar rapid response on BH<sub>4</sub> loading, resulting in normalization of plasma Phe



**Figure 2** Course of plasma phenylalanine concentrations during a combined Phe/BH<sub>4</sub> loading test in patients 2, 3 and 4



**Figure 3** Course of plasma biopterin (—◆—) and phenylalanine (bars) concentrations in patient 3 after a phenylalanine load (100 mg/kg)

**Table 1 Biogenic amines in CSF (nmol/L)**

	<i>5-HIAA</i>	<i>HVA</i>	<i>HVA/5-HIAA</i>	<i>DOPAC</i>	<i>MHPG</i>	<i>3-OMD</i>	<i>5-OH-Trp</i>	<i>L-Dopa</i>
Patient 1	697	758	1.1	16.3		335	17	12.5
Patient 3	212	563	2.7			75.0	8.7	12.3
Patient 4	316	523	1.7	29.9	67.2	71.3	<5	38.5
Controls <sup>a</sup>	150–800	310–1100	1.5–3.5	8–18	98–168	<300	<10	<25

<sup>a</sup> Age range: 0–0.5 years

5-HIAA, 5-hydroxyindoleacetic acid; *HVA*, homovanillic acid; *DOPAC*, 3,4-dihydroxyphenylacetic acid; *MHPG*, 3-methoxy-4-hydroxyphenylglycol; *3-OMD*: 3-*O*-methyl-dopa; *5-OH-Trp*, 5-hydroxytryptophan

**Table 2** Mutations in the PAH gene

	<i>Allele 1</i>	<i>Allele 2</i>
Patient 1	A313T ex 9	L367fsinsC ex 11
Patient 2	V190A ex 6	R243X ex 7
Patient 3	A300S ex 8	A403V ex 12
Patient 4	R241C ex 7	A403V ex 12

within 8 h (Figure 2). The R241C mutation found in patient 4 was detected earlier in two other BH<sub>4</sub>-responsive HPA patients. In contrast to our patient 4, plasma Phe concentrations in those two patients apparently did not decrease to normal values within 24 h after the BH<sub>4</sub> load. However, they received a lower dose of 10 mg/kg BH<sub>4</sub> (Kure et al 1999). The A300S and A403V mutations have been found in non-PKU HPA, though no BH<sub>4</sub>-responsiveness was reported (Mallolas et al 1999). The A313T mutation due to a 937G>A transition, detected in patient 1, has not been reported before.

Normal human PAH protein is present as homopolymer produced by a single genetic locus (Scriver et al 1995). The HPA patients described in this study all bear two different allele mutations, leading to the presence of various heteropolymeric and homopolymeric PAH proteins. Increasing intracellular BH<sub>4</sub> concentrations by oral supplementation apparently increases residual PAH activity, possibly as result of a decreased affinity of the enzyme for this cofactor. In the patients who showed a fast normalization of plasma Phe within 8 h after BH<sub>4</sub> load, both heteropolymeric and homopolymeric PAH subunits may be involved in the restoration of enzymic activity. In patients who responded partially to BH<sub>4</sub> supplementation, only homopolymeric PAH molecules bearing one of the mutations may be activated by the cofactor, leading to a partially restored enzyme activity. So far, PAH mutations specifically affecting the BH<sub>4</sub> binding site of the enzyme are unknown.

The patients presented above belong to a group with a new variant of PAH deficiency, a BH<sub>4</sub>-responsive one. Patient 1 had Phe and Tyr concentrations in the 120–150 µmol/L range on BH<sub>4</sub> monotherapy. In patients 2 and 4, plasma Phe levels remain well controlled (<350 µmol/L) on protein restriction and a PKU formula. Without protein restriction, Phe concentrations in patient 3 remain mostly below 250 µmol/L. In principle this form of PAH deficiency is treatable with BH<sub>4</sub>. Institution of BH<sub>4</sub> supplementation in our patients is a point at issue. These novel BH<sub>4</sub>-responsive subtypes of PAH deficiency can only be detected if a BH<sub>4</sub> loading test is included in the differential diagnostic investigations of HPA patients.

#### ACKNOWLEDGEMENTS

The authors thank Connie Diepstraten and Jan van de Worp for excellent technical assistance. This work is supported in part by the Swiss Science National Foundation grant no. 31-54183-98 (to N.B.)

## REFERENCES

- Blau N, Blaskovics ME (1996) Hyperphenylalaninemia. In Blau N, Duran M, Blaskovics ME, eds. *Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases*. London: Chapman and Hall Medical, 65–78.
- Blau N, Thöny B, Renneberg A, et al (1999) Variant of dihydropteridine reductase deficiency without hyperphenylalaninemia: effect of oral phenylalanine loading. *J Inherit Metab Dis* **22**: 216–220.
- Bonafé L, Thöny B, Leimbacher W, Kierat L, Blau N. (2001) Diagnosis of Dopa-responsive dystonia and other tetrahydrobiopterin disorders by the study of biopterin metabolism in fibroblasts. *Clin Chem* **47**: 477–485.
- Fukushima T, Nixon JC (1980) Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal Biochem* **102**: 176–188.
- Kure S, Hou D-C, Ohura T, et al (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *J Pediatr* **135**: 375–378.
- Mallolas J, Vilaseca MA, Campistol J, et al (1999) Mutational spectrum of phenylalanine hydroxylase deficiency in the population resident in Catalonia: genotype–phenotype correlation. *Hum Genet* **105**: 468–473.
- Nixon JC, Lee Ch-L, Milstien S, Kaufman S, Bartholome K. (1980). Neopterin and biopterin levels in patients with atypical forms of phenylketonuria. *J Neurochem* **35**: 898–904.
- Ponzone A, Guardamagna O, Spada M, et al (1993) Differential diagnosis of hyperphenylalaninaemia by a combined phenylalanine-tetrahydrobiopterin loading test. *Eur J Pediatr* **152**: 655–661.
- Scriver CR, Kaufman S, Eisensmith RC, Woo SLC (1995) The hyperphenylalaninemia. In Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease*, 7th edn. New York: McGraw-Hill, 1015–1075.
- Surplice IM, Griffiths PD, Green A, Leeming RJ (1990). Dihydropteridine reductase activity in eluates from dried blood spots: automation of an assay for a national screening service. *J Inherit Metab Dis* **13**: 169–177.
- van der Sijs-Bos CJ, Diepstraten CM, Juyn JA, et al 1996. Phenylketonuria in the Netherlands: 93% of the mutations are detected by single-strand conformational analysis. *Hum Hered* **46**: 185–190.