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# Molecular analysis and long-term follow-up of patients with different forms of 6-pyruvoyl-tetrahydropterin synthase deficiency

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**Abstract** The outcome of 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, the most common form of tetrahydrobiopterin ($BH_4$) deficiency, depends on factors such as severity of the disease, type of mutation, time of diagnosis, and mode of treatment. We investigated five patients from four different families, four of them presenting with the severe form of PTPS deficiency and one with the mild peripheral form. In this study, missense (L26F, T67M, P87L, V124L, D136G, D136V) and nonsense (R15–16ins) mutations were detected by reverse transcriptase polymerase chain reaction and sequence analysis. Two patients with the severe form were compound heterozygotes (T67M/P87L and D136G/R15–16ins), two siblings were homozygous for the D136V mutation, and in the patient with the mild form, heterozygous L26F/V124L mutations were present. Two patients are on combined therapy with L-dopa/carbidopa/5-hydroxytryptophan plus $BH_4$, the siblings are on monotherapy with $BH_4$, and the patient with the mild form is now off treatment, presenting with normal plasma phenylalanine levels.

**Conclusion** Long-term follow-up shows that the outcome of 6-pyruvoyl-tetrahydropterin synthase deficiency benefits from treatment started in the first months of life and that the phenotype may change with age. Additionally, depending on the type of mutations, prenatal damage to the fetus may multiply the clinical abnormalities and thus worsen the prognosis of the disease. In patients initially diagnosed with the mild peripheral form of the disease, therapy with tetrahydrobiopterin should be stopped after some time to test whether hyperphenylalaninaemia was only a transient condition.

**Key words** Hyperphenylalaninaemia · Mutation analysis · Neurotransmitters · Tetrahydrobiopterin

**Abbreviations** *$BH_4$* tetrahydrobiopterin · 5HIAA 5-hydroxyindoleacetic acid · *HPA* hyperphenylalaninaemia · *5HPT* 5-hydroxytryptophan · *HVA* homovanillic acid · *PTPS* 6-pyruvoyl-tetrahydropterin synthase

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## Introduction

Hyperphenylalaninaemia (HPA) due to tetrahydrobiopterin ($BH_4$) deficiencies represents a heterogeneous group of progressive neurological disorders caused by autosomal recessively inherited mutations affecting enzymes in the biosynthesis or regeneration of $BH_4$ [13]. $BH_4$ is the natural cofactor for phenylalanine (Phe), tyrosine, and tryptophan hydroxylase as well as for all three forms of nitric oxide synthase [29, 34] (Fig. 1). It is synthesised in a three-step pathway from GTP by the enzymes GTP cyclohydrolase 1 (EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS; EC 4.6.1.10), and sepiapterin reductase (EC 1.1.1.153). After coupling as an active cofactor to the aromatic amino acid hydroxylases, it is regenerated by pterin-4a-carbinolamine dehydratase (EC 4.2.1.96) and dihydropteridine reductase (EC 1.6.99.7) [53].

In almost all cases, $BH_4$ deficiency presents with neurological signs linked to impaired catecholamines and serotonin synthesis. Symptoms may become evident in the first weeks of life, but are mostly seen at an average age of 4 months [20, 9]. Most infants are born small for gestational age [46]. Abnormal signs in the neonatal period may include poor sucking, impaired tone, and microcephaly. Frequent symptoms of PTPS deficiency, the most common form of $BH_4$ deficiency, resemble Parkinson disease, indicating a lack of dopamine in the basal ganglia [3]. Extrapyramidal signs include characteristic truncal hypotonia, increased limb tone, postural instability, hypokinesia, choreatic or dystonic limb movements, gait difficulties, hypersalivation due to swallowing difficulties, and oculogyric crises. Ataxia, hyperreflexia, hypothermia as well as episodes of hyperthermia (in the absence of infections), drowsiness, irritability, disturbed sleep patterns, and convulsions (grand mal or myoclonic) are often seen. In addition, ptosis and pinpoint pupils, presumably due to dysfunction of the oculosympathetic pathway, are observed [8, 39].

Among the most prevalent variants of PTPS deficient HPA, at least two different phenotypes are described. The more common severe central (typical) form is accompanied by the above mentioned abnormalities of biogenic amines in CSF, as assessed by CSF measurement of catecholamines and serotonin metabolites [22]. These patients usually require a combined treatment with $BH_4$ and the neurotransmitter precursors L-dopa/carbidopa and 5-hydroxytryptophan (5HPT) [1]. In contrast, the rare mild peripheral (atypical) form of PTPS deficiency is characterised by normal neurotransmitter homeostasis and moderate or transient HPA [36]. These patients need monotherapy with $BH_4$ in order to maintain normal plasma Phe levels.

Reports about the long-term treatment and outcome of patients with $BH_4$ deficiency are still scarce [2, 44, 47, 49]. Many of the patients diagnosed late are retarded and some diagnosed in the first weeks of life did not respond clinically to different treatment protocols. About 9% of all PTPS-deficient patients registered in the international BIODEF database died [8]. Although 33 different mutations have been detected [12, 27, 33, 51, 52, 54], only some have been tested for functionality and in only a few cases data on phenotype-genotype correlation are available [11, 38, 43]. In this paper we describe new mutations and long-term follow-up in five PTPS-deficient patients with various clinical outcomes.

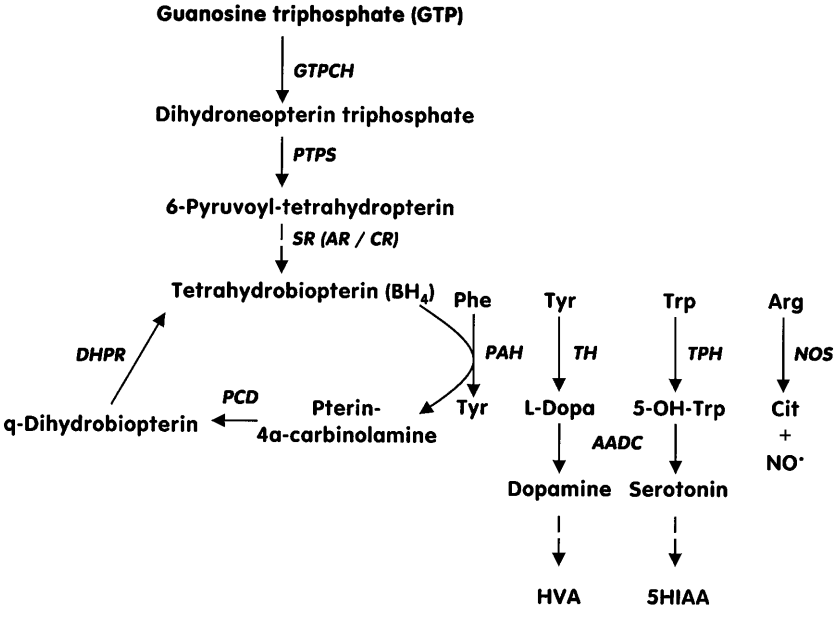


**Fig. 1** Biosynthesis, regeneration, and functions of tetrahydrobiopterin. (*AADC* aromatic amino acid decarboxylase, *AR* aldose reductase, *CR* carbonyl reductase *DHPR* dihydropteridine reductase L-dopa 3,4-dihydroxyphenylalanine, *GTPCH* GTP cyclohydrolase 1,*5HIAA* 5-hydroxyindoleacetic acid, *HVA* homovanillic acid, *5-OH-Trp* 5-hydroxytryptophan, *NOS* nitric oxide synthase, *PAH* phenylalanine-3-hydroxylase, *PCD* pterin-4a-carbinolamine dehydratase, *SR* sepiapterin reductase, *TH* tyrosine-4-hydroxylase, *TPH* tryptophan-5-hydroxylase, *PTPS* 6-pyruvoyl-tetrahydropterin synthase)

## Case reports

All patients are registered in the International Database of $BH_4$ Deficiencies BIODEF [7].

### Case 1 (BIODEF 4)

This girl was born at 36 weeks of gestation to consanguineous Turkish parents. Birth weight was 2210 g, length 49 cm, and during the first 4 weeks she appeared clinically normal. A Guthrie test for PKU was normal (Phe = 80 μmol/l) on the 6th day of life. At the age of 6 months she manifested muscular hypotonia, opisthotonic posture, myoclonic jerks, and a hypsarrhythmic EEG. Her plasma Phe level was then >1200 μmol/l. Three months of a Phe-restricted diet did not improve her clinical status. The diagnosis of a $BH_4$ deficiency was made at the age of 9 months (Table 1) and therapy with $BH_4$ (1.8 mg/kg per day), L-dopa/carbidopa (3.0 mg/kg per day) and 5HTP (2.3 mg/kg per day) was introduced. Therapy was inconsistent for the next few years. The girl had several subsequent neurological crises with ptosis, restlessness, pallor, increasing muscular hypotonia and ataxia. Neurotransmitter precursor substitution failed to improve her condition. A trial with $BH_4$ monotherapy (2.1 mg/kg per day) caused the girl to recover completely within 2 h from a semicomatous state (drowsiness, screaming, hypersalivation, generalised hypotonia and ptosis) to normal. A higher monotherapy dose was initiated (24 mg/kg per day) at the age of 4 years and 9 months and seemed to be adequate since her Phe levels were normal (<80 μmol/l). At the age of 6 years and 9 months, $BH_4$ monotherapy (3.5–8.1 mg/kg per day) was continued despite low levels of 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) in CSF (Fig. 4). However, the patient remained neurologically normal. At 20 years of age the serum prolactin was normal despite low dopamine production. A CT scan of the brain was normal and showed normal white and grey matter without any demarcation of hypodense areas.

### Case 2 (BIODEF 5)

The younger brother of case 1 was born after 39 weeks of gestation. Pregnancy and delivery were uneventful. Birth weight (2750 g) and length (48 cm) were between the 10th and 50th percentiles. A Guthrie test for PKU at the age of 6 and 16 days revealed elevated plasma Phe levels of 363 and 2009 μmol/l, respectively. PTPS deficiency was confirmed (Table 1) and monotherapy with $BH_4$ (10 mg/kg per day) was initiated because of the good experience in his sister. At the age of 6 weeks, short dystonic episodes with opisthotonic head posturing, limb rigidity, and clonus of the feet were observed. Treatment with low dose L-dopa/carbidopa (2.0 mg/kg per day) and 5HTP (1.5 mg/kg per day) for 5 weeks abolished these symptoms. With continuous $BH_4$ monotherapy (10.5 mg/kg per day and later 5.0 mg/kg per day) development was physically and neurologically normal. However, slight mental retardation was noted as in his sister. Despite initially low levels of 5HIAA and HVA in CSF at the age of 6.5 years (Fig. 4) monotherapy was continued, resulting in a stable clinical status of the patient.

### Case 3 (BIODEF 49)

This Turkish infant was born in 1977 and recognised at the age of 11 months because of progressive neurological symptoms resembling Parkinson disease and suggestive of a disorder of neurotransmitter metabolism: muscular hypotonia changing to hypertonia during activity, bradykinesia, poor head control, and hyperreflexia. She was diagnosed late because there was no newborn screening programme in Turkey. A selective metabolic work-up at the age of 7 years was performed because of progressive psychomotor retardation, oculogyric crises, and epileptic seizures,

**Table 1** Phenotype, biochemical parameters, and mutation analysis. (*CRM* cross-reacting material on Western blot, *ND* not done)

| Patient | Age at diagnosis | Phenotype | Phe (μmol/l)[a] | Neopterin (mmol/mol creatinine) | Biopterin (mmol/mol creatinine) | %Bio | PTPS activity (RBC) μU/g Hb (% of controls) | PTPS activity (fibroblasts) μU/mg protein (% of controls) | CRM | Mutant designation (exon) | Nucleotide aberration |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Case 1 | 9 Months | Severe | 80 | 1.60 | 0.13 | 7.5 | 6[b] | 0.040 | + | D136V/D136V | 407A > T/ |
| BIODEF #4 | | Central | | | | | | (0.29) | | (6/6) | 407A > T |
| Case 2 | 18 Days | Severe | 847 | 32.8 | 0.07 | 0.2 | 3[b] | ND | ND | D136V/D136V | 407A > T/ |
| BIODEF #5 | | Central | | | | | | | | (6/6) | 407A > T |
| Case 3 | 7 Years | Severe | 310 | 8.29 | 0.21 | 2.5 | 0.78 | 0.039 | (+) | R15–16ins/D136G | 45–46insGGC/ |
| BIODEF #49 | | Central | | | | | (4.4) | (0.28) | | (6/1) | 407A > G |
| Case 4 | 3 Months | Mild | 541 | 18.70 | 0.65 | 3.0 | 0.92 | 0.063 | – | L26F/V124L | 78G > T/ |
| BIODEF #247 | and 1 week | Peripheral | | | | | (5.0) | (0.45) | | (1/6) | 370G > T |
| Case 5 | 1 Month | Severe | 1398 | 26.70 | 0 | 0 | 0.45 | 0.038 | – | T67M/P87L | 200C > T/ |
| BIODEF #341 | | Central | | | | | (2.6) | (0.28) | | (4/5) | 260C > T |
| Controls | | | < 120 | 1.1–4.0 | 0.5–3.0 | 44–77 | 34–64 (100) | 1.9–2.6 (100) | + | | |

[a] At the time of diagnosis of HPA
[b] In liver biopsy

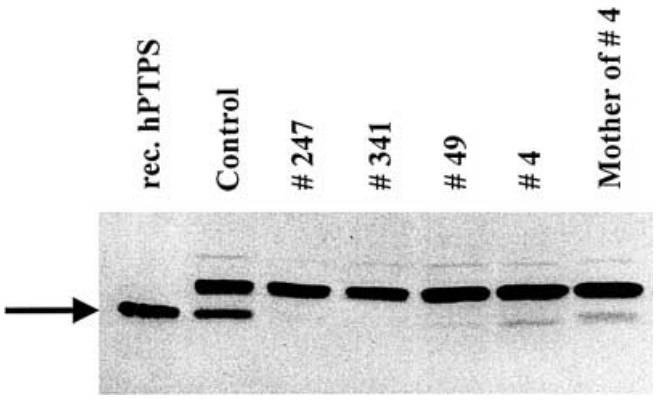


**Fig. 2** Western blot analysis of PTPS in primary fibroblasts from four patients and from the mother of case 1. As controls, a fibroblast protein extract from a healthy subject (control) and purified human recombinant PTPS (rec. hPTPS) were used. Note that amount of immunoreactive PTPS protein is reduced in heterozygote cases when compared with control fibroblasts

revealing HPA, abnormal urinary pterins (Table 1), and a positive $BH_4$ loading test (6.3 mg/kg; plasma Phe 590 μmol/l before and 48 μmol/l after administration) leading to the diagnosis of PTPS deficiency. $BH_4$ monotherapy (6.2 mg/kg per day) resulted in a slight improvement in clinical status where ocular deviation and sudden falling were no longer observed. Because of very low levels of 5HIAA and HVA in CSF, a combined therapy with neurotransmitter substitution (L-dopa 3.3 mg/kg per day, carbidopa 0.3 mg/kg per day, 5HTP 1.6 mg/kg per day) was started at the age of 7.5 years. Even though choreoathetosis and rigor-like movements improved somewhat, slowing down of motor and speech development persisted with low concentrations of 5 HIAA and HVA in CSF. Doses of L-dopa (up to 15.9 mg/kg per day) and 5HTP (up to 11.9 mg/kg per day) were increased stepwise resulting in marked choreatic and dystonic movements, hyperkinesia and persistent agitation. The coincidence of not adequately increasing levels of biogenic amines in CSF on high dose therapy as well as an elevated prolactin concentration in plasma, again reflecting the inversely correlated hypothalamic low dopamine level, might be due to incomplete compliance. Episodes of steady intake of L-dopa, however, led to critical symptoms of overdose. During the following 10 years, different therapeutic protocols were attempted without achieving a consistent success. Neither Pergolid (a dopamine agonist) nor Sertralin (a selective serotonin re-uptake inhibitor) were able to modify the clinical course significantly. The patient is now 23 years old and shows a severe clinical course that led her into increased social isolation and finally to reactive depression.

Case 4 (BIODEF 247)

This girl was born at term as the third child to non-consanguineous parents of German origin. Birth weight was 2670 g, length 48 cm, and no abnormal symptoms were observed post-partum. Newborn screening performed at 5 days of age indicated mild HPA (Phe = 363 μmol/l). Repeated studies at 8 days and 1 month of age confirmed HPA with plasma Phe concentrations of 182 and 466 μmol/l, respectively. Introduction of a Phe-restricted diet reduced Phe levels to 121 μmol/l. Differential diagnosis for HPA variants was performed at the age of 3 months and 1 week. The loading test with $BH_4$ normalised plasma Phe levels from 541 μmol/l to 101 μmol/l and 60 μmol/l after 4 and 8 h, respectively. Elevated concentrations of neopterin and only traces of biopterin in urine indicated a PTPS deficiency (Table 1). Neurotransmitter metabolites in CSF were normal at all times (Table 1). Repeatedly studied plasma prolactin levels were always in the normal range (165–285 mU/l; controls: 34–480 mU/L). At the age of 5 months, monotherapy with $BH_4$ (2.0 mg/kg per day) was introduced resulting in normalisation of plasma Phe (<120 μmol/l). The infant developed normally (sitting at 5 months, free standing at 12 months, walking at 14 months, and speaking more than ten single words at 16 months of age). $BH_4$ was reduced to 0.8 mg/kg per day and withdrawn at the age of 5 years. Pterin excretion in urine remained almost normal, while plasma Phe was in the borderline range. The girl is now 8 years old; physical and neurological development are normal.

Case 5 (BIODEF 341)

This girl was born to non-consanguineous parents of German origin. Delivery was premature (37 weeks), birth weight 2180 g (below the 10th percentile), and length 43 cm (below the 3rd percentile). Hyperphenylalaninaemia was noted at the age of 5 days (Phe = 605 μmol/l). A loading test with $BH_4$ (19 mg/kg) normalised the plasma Phe from 1694 μmol/l to 212 μmol/l and 51 μmol/l after 4 and 8 h, respectively. CSF analysis of neurotransmitter metabolites and urinary excretion of neopterin and biopterin at the age of 1 month (Table 1) suggested a PTPS deficiency. Reduced PTPS activity in erythrocytes (0.45 μU/g Hb; 2.6% of controls) confirmed the initial diagnosis. No abnormal clinical or neurological signs were seen at this time.

$BH_4$ monotherapy was started at a dose of 3.6 mg/kg per day, without improvement in CSF neurotransmitter monoamine concentrations (Fig. 4). At the age of 4 months, muscular hypotonia, movement disorders, and psychomotor retardation were noticed despite an increased $BH_4$ dosage (8.8 mg/kg per day). Plasma Phe concentrations were in the normal range (< 60 μmol/l). Low neurotransmitter precursors supplementation was introduced at the age of 5 months because of very low CSF concentrations of 5HIAA and HVA (Fig. 4) and marked extrapyramidal signs (intention tremor and hypotonia) in addition to persistent psychomotor retardation. L-Dopa/10% carbidopa and 5HTP were both carefully titrated upwards from 1 mg/kg per day to 1.9 mg/kg per day, resulting in consequent improvement in tremor, general attention, and truncal hypotonia. Plasma prolactin levels, which before therapy were continuously over 1000 mU/l, decreased to 451 mU/l. At the age of 6 months, swallowing difficulties and extension seizures were noticed and therapy was increased to 4.6 mg/kg per day L-dopa/10% carbidopa and 3.4 mg/kg per day 5HTP. CSF monoamines were now in the normal range and prolactin normalised to 272 mU/l. Both hypotonia and the clinical status improved markedly. Subsequent progress in psychomotor development (adequate head control at 1 year, grasping of objects at 13 months, independent walking at 19 months, speaking single words at 26 months) was noted with stepwise increasing doses of L-dopa/10% carbidopa ranging between 5.9 and 7.5 mg/kg per day, and 5HTP between 3.6 and 4.3 mg/kg per day. There was only mild speech retardation. At the age of 4.5 years, the girl showed an almost age-appropriate mental development.

## Materials and methods

$BH_4$ and other pterins were purchased from Dr. Schircks Laboratories (Jona, Switzerland).

Determination of pterins and neurotransmitter metabolites

Pterins in urine and CSF were measured by HPLC and fluorescence detection after oxidation with manganese dioxide at acidic pH [17]. 5HIAA and HVA acid in CSF were measured by HPLC and electrochemical detection [10].

**Table 2** Amplification and analysis of coding regions of the PTS gene (gDNA)

| Exon | Sense primer | Antisense primer | Annealing temperature | Primers for sequencing |
|---|---|---|---|---|
| 1 | PTPS 206B | PTPS 207 | 63 °C | PTPS 207 |
| 2 | PTPS 347 | PTPS 330 | 54 °C | PTPS 330 |
| 3 and 4 | PTPS 349 | PTPS 319 | 54 °C | PTPS 323 for exon 3<br>PTPS 320 for exon 4 |
| 5 and 6 | PTPS 318 | PTPS 14B | 54 °C | PTPS 208 for exon 5<br>PTPS 209 for exon 6 |

### Assay for PTPS activity and Western blot analysis in fibroblasts

Preparation of whole cell lysates from fibroblasts for Western blot analysis and PTPS activity measurement was carried out as described previously [38]. Enzymatic production of $BH_4$ derived from the substrate dihydroneopterin triphosphate in the presence of NADH, $Mg^{2+}$, and sepiapterin reductase was measured according to Bonafé et al. [14].

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [31]. Proteins were blotted onto a nitrocellulose sheet (Biorad) and stained with the anti-human PTPS polyclonal antibody F3878 [37] (1:10,000 dilution) followed by a second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate from Biorad).

### Direct cDNA sequence analysis of reverse transcriptase-PCR products

Primary skin fibroblasts were cultured in Dulbecco modified Eagle medium (Gibco) containing 10% fetal bovine serum, 50 U/ml penicillin (Gibco) and 50 µg/ml streptomycin (Gibco). Total RNA was extracted from confluent fibroblasts and cultured following a protocol using guanidinium thiocyanate from cell lysis and centrifuged in caesium chloride solution [41]. First strand cDNA was synthesised from this RNA by Superscript RTII using oligodeoxythymidine as primer according to Thöny et al. [52]. The cDNA was PCR-amplified (primer PTPS 206 and PTPS 14B) and directly sequenced with primers PTPS 7, PTPS 11, PTPS 208, and PTPS 209, using the ABI PRISM (Perkin Elmer) sequence analyser.

### Exon-specific PCR amplification and sequence analysis of genomic DNA

Genomic DNA from patients was isolated from cultured primary skin fibroblasts using the Genome DNA kit Bio 101 (Qiagen). PCR amplification of genomic DNA of patients and parents (gDNA from whole blood cells) with exon-specific primers (Table 2) was performed according to Oppliger et al. [38] and subsequently sequenced (ABI PRISM sequence analyser).

### Transient expression of mutant PTPS alleles in COS-1 cells

In order to express recombinant PTPS mutant proteins in COS-1 cells, the corresponding cDNAs were cloned into the eukaryotic expression vector pSCT1 as described previously [52]. Briefly, mutant PTPS-cDNA was PCR amplified using primer pair PTPS 21/102. The PCR product was ligated into the pSCT1 vector. Correct inserts were verified by DNA sequence analysis (ABI PRISM) using vector- and PTPS-specific primers. For transfection controls, we used expression plasmids with the wild-type PTPS sequence either in the correct (pHSY2013) or reverse orientation (pHSY2008; 38). Activities were normalised to $\beta$-galactosidase activity expressed from the co-transfected plasmid pRSV$\beta$gal. Transfection was performed by applying a modified DEAE-dextran protocol [52].

## Results

### Mutation analysis

The two siblings (cases 1 and 2) with the severe, central type of PTPS deficiency were homozygous for a A to T missense mutation in the second position of codon 136, responsible for a Asp to Val exchange (Table 1). Both parents showed this D136V mutation. Although enzymatic activity in fibroblasts from case1 was not detectable, the PTPS protein was weakly present on Western blot analysis. This suggests that the D136V mutation, a change from a negative to a positive charged amino acid, located in the basic pore of the PTPS homohexamere, does not completely abolish the stability of the protein but might be involved in the catalytic process.

In case 3 an insertion of the triplet GGC coding for Arg after nucleotide position 45 was found heterozygously. An A to G transition in the second position of codon 136 in exon 6, indicating an amino acid change from Asp to Gly, was present on the other allele (Table 1). Residue 136 could also be a mutation hotspot since other mutations were detected at this place in other patients [38, 55]. As PTPS protein was hardly visible in the patient's fibroblasts (Fig. 2), the PTPS expressed from these alleles might be degraded and not active.

In case 4 two heterozygous missense mutations, designated as L26F and V124L, were detected. The first one

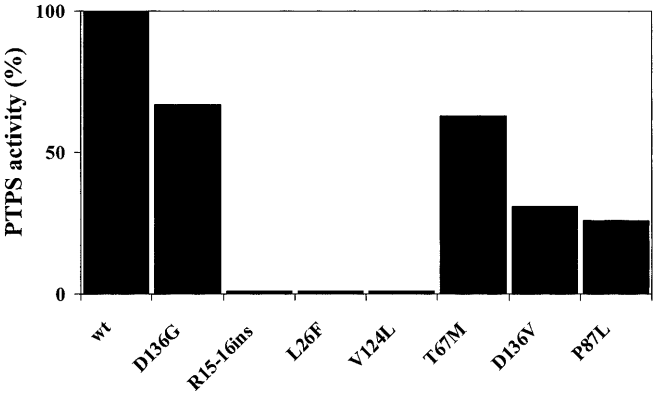


**Fig. 3** Wild-type and mutant PTPS activities of transiently transfected COS-1 cells. Relative PTPS activity was normalised for $\beta$-galactosidase activity expressed from the co-transfected reporter gene vector. Wild-type activity was defined as 100%, whereas expression of PTPS-cDNA in the reverse orientation had no activity (data not shown)

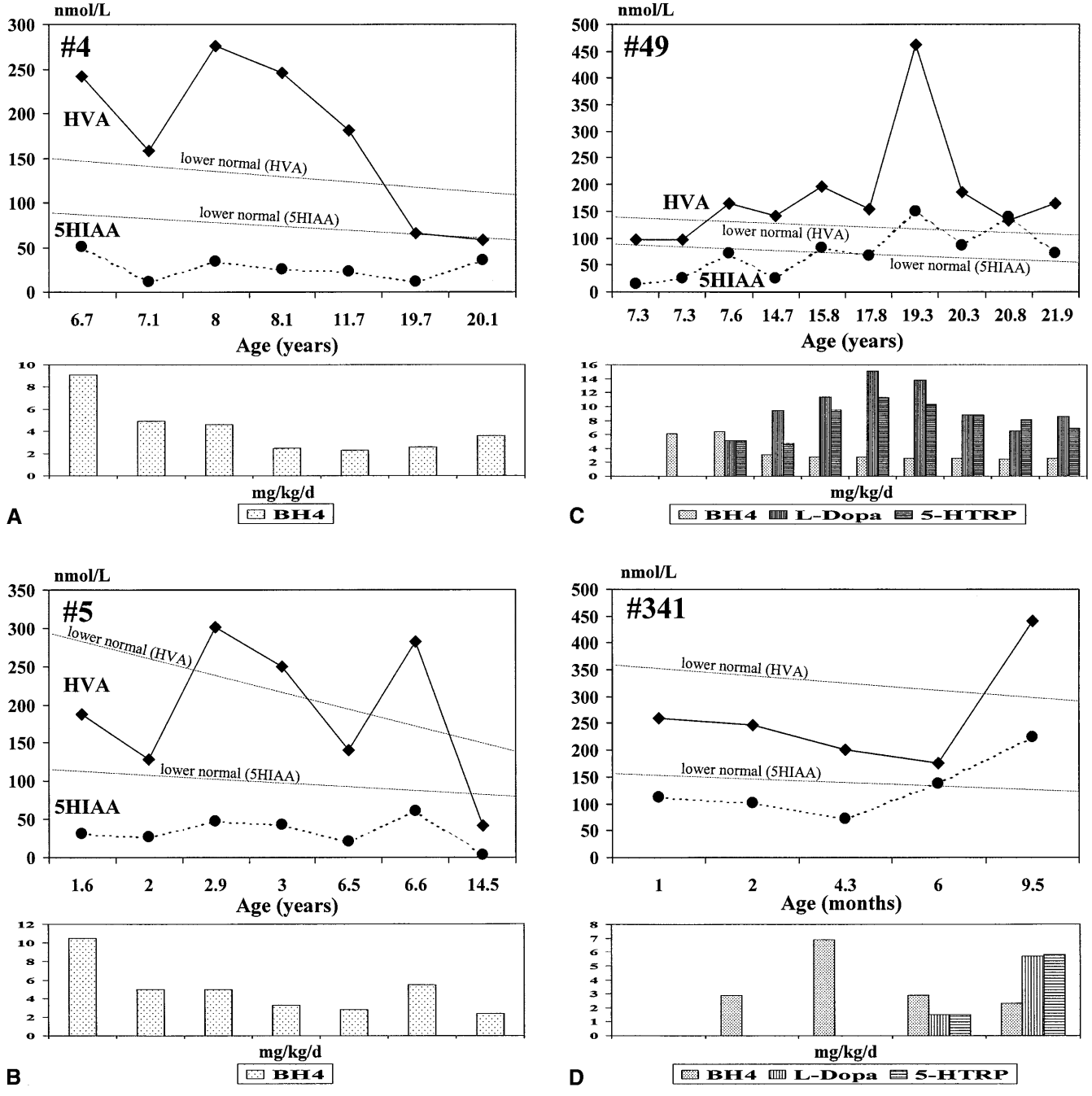


**Fig. 4** Long-term follow-up of CSF neurotransmitter metabolites 5HIAA and HVA in response to different treatment protocols in two patients (case 1 BIODEF #4 and case 2 BIODEF #5) on $BH_4$ monotherapy (**A** and **B**) and two patients (case 3 BIODEF #49 and case 5 BIODEF #341) on combined $BH_4$/L-dopa/carbidopa/5HTP therapy (**C** and **D**). *Dotted lines* illustrating the lower normal range are linearised age-dependent cut-off values for both metabolites [6]

was found in the patient's mother on exon 1, due to a G to T transition at nucleotide position 78. The second mutation was inherited from her father and is the result of a G to T transition at nucleotide 370, causing a Lys to Phe exchange. No cross-reactive material was found in fibroblasts of the patient and no enzyme activity was detectable (Fig. 2).

Mutation analysis in case 5 revealed compound heterozygosity with two missense mutations. A C to T transition at nucleotide position 260 in exon 5 leading to a Pro 87 to Leu substitution was also heterozygously present in her father's genomic DNA. This single point mutation is already known from other cases and confirms the assumption that the locus at codon 87 is a mutation hotspot. Like her mother, the patient carries a T67M mutation in exon 4, caused by C to T transition at nucleotide position 200, which has also been detected previously in another case. No cross-reactive material was found on Western blot and no enzyme activity was measurable in fibroblasts (Fig. 2).

### PTPS activity of transiently expressed recombinant mutant proteins

Upon recombinant expression of mutant alleles in COS-1 cells, only the D136G allele was active (67% of wild-type activity). All other alleles, L26F, V124L, and R15–16ins showed no measurable activity in COS-1 cell extracts (Fig. 3). The three other alleles T67M and D136V [38], and P87L [37] were tested previously, and were known to be only partially active (63%, 31%, and 26%, respectively).

### Laboratory diagnosis and treatment follow-up

All patients were initially diagnosed by the abnormal urinary pterin excretion typical for PTPS deficiency (high neopterin and very low or not detectable biopterin) (Table 1). Percentage of biopterin was lower than 7.5% (of the sum of neopterin and biopterin). Diagnosis was confirmed by very low or absent PTPS activity in red blood cells and cultured skin fibroblasts (Table 1). Parents showed red blood cell PTPS activity in the heterozygote range (3.4–5.3 μU/g Hb; 19–30% of controls). Neurotransmitter metabolites 5HIAA and HVA in CSF before and during treatment are summarised in Fig. 4. In cases 1 and 2, both on monotherapy with $BH_4$, 5HIAA was always below the lower normal range and HVA was fluctuating between normal and very low (Fig. 4). Cases 3 and 5, with the severe form of PTPS deficiency, responded to a combined therapy with $BH_4$, L-dopa/carbidopa/5HTP by increasing both 5HIAA and HVA levels in CSF (Fig. 4). Several attempts to normalise CSF neurotransmitters by $BH_4$ alone failed in case 5. Neurotransmitter metabolites 5HIAA and HVA were normal on two occasions (before treatment and on $BH_4$ monotherapy) in CSF from the patient (case 4) with the mild peripheral form (data not shown).

## Discussion

Selective screening for $BH_4$ deficiencies is today an integral part of the newborn screening for PKU. Thus, every newborn with even slight HPA can be detected as $BH_4$-deficient by urinary pterin analysis and DHPR activity test in Guthrie card [19]. Although in most cases laboratory diagnosis is straightforward, only a few specialised clinical centres are offering these tests (for more details see the $BH_4$ Home Page at http://www.bh4.org). During the last 21 years we investigated in our unit 133 patients with PTPS deficiency, 68 with dihydropteridine reductase deficiency, 15 with pterin-4a-carbinolamine dehydratase deficiency, and nine with GTP cyclohydrolase 1 deficiency, which accounts for more than 50% of all patients diagnosed worldwide [7]. Out of the patients diagnosed with PTPS deficiency, only a small portion (20%) belongs to the so-called mild peripheral phenotype. While treatment and follow-up of these patients is relatively simple ($BH_4$ substitution in order to control plasma Phe levels) [21, 30, 42], infants with severe forms of PTPS deficiency need a combined therapy with $BH_4$ and neurotransmitter precursors [4, 35]. Several case reports have been published; however, it must be stressed that they differ with regard to the time of diagnosis, treatment, clinical outcome, and mode of follow-up [5, 16, 23, 25, 28, 38, 50].

### Therapy and follow-up

In most cases repeated measurements of blood Phe levels and urinary excretion of neopterin and biopterin seem to be informative for the $BH_4$ dosage required to control hepatic phenylalanine hydroxylase activity. However, the crucial point in monitoring the therapy is to record the actual concentrations of catecholamines and serotonin in the CNS. Lumbar punctures are unfortunately not always possible and in some cases levels of 5HIAA and HVA do not correlate well with the clinical status of the patient. Despite low CSF neurotransmitters concentrations, some patients (cases 1 and 2) display no neurological abnormalities (Fig. 4). Similar findings were already documented in another PTPS-deficient patient who was initially diagnosed as a mild peripheral phenotype but with age turned out to have a central form [40]. However, in this particular infant HPA was only transient and her CSF neurotransmitter metabolites dropped below the lower normal range without treatment. She was never neurologically abnormal. Theoretically there are several different explanations for these findings. Early dysfunction of aminergic neurons due to the lack of $BH_4$ could result in a (post-synaptic) receptor supersensitivity so that lower concentrations of monoamines are actually required. This may also explain a tendency to overdose effect (in case 3) and fluctuating responses, due to a narrowing of the therapeutic window and steepening of the dose-response curve as a result of receptor adaptation. Furthermore, not all areas of aminergic neurons might be similarly affected by neurotransmitter depletion. Since PTPS activity has been shown to be cell-dependent [53], certain cell groups may still have sufficient amounts of monoamines and contribute to sustain important functions. Balanced loss of dopamine and serotonin may not have as devastating effect as the disruption of just one of the two, as the metabolism of monoamines is normally carefully integrated.

Prolactin in blood seems to inversely correlate with actual dopamine levels in the tuber infundibular nucleus of the hypothalamus. Since measurement of prolactin is easier to perform than a lumbar puncture, it may be introduced as an additional monitoring procedure to CSF investigations [48]. However, prolactin does not reflect serotonin turnover and normal large fluctuations in plasma concentrations during the day due to episodic secretion, stress, exercise, and food are reported.

The minimal dose of neurotransmitters effective in relieving cardinal symptoms should be determined pri-

marily on the basis of clinical symptoms. Case 3 demonstrates how difficult it is to find the optimal dosage that allows control of symptoms of $BH_4$ deficiency and does not evoke overdose effects. Careful examination of neurological symptoms is required, as adverse effects of medication and signs of amine deficiency or excess are very similar (e.g. sleeping difficulties, rigidity, depressions, affective disorders).

## Clinical outcome

Very few reports on the long-term outcome of patients with PTPS deficiency document that early diagnosis and introduction of adequate therapy prevent mental retardation [24,47]. A number of patients documented in the international BIODEF database developed normally either on $BH_4$ monotherapy or on combined neurotransmitters substitution [7]. The major neurological symptoms improved and could be controlled. In cases 1 and 2 monotherapy with $BH_4$ seems to be beneficial. Unfortunately, some infants do not respond to conventional therapy despite early diagnosis and some of them died [49].

Some unusual clinical observations were made in infants studied in this work. In cases 1 and 3, fallen arches have been diagnosed, suggesting that muscular dystonia contributes to orthopedic problems. In case 1 menarche appeared at the age of 11 years and 8 months, and a regular menstrual cycle was observed in case 3 (menarche at 12 years and 7 months). This finding is remarkable since hyperprolactinaemia due to a loss of dopamine could affect the reproductive system. High concentrations of prolactin could cause anovulation/amenorrhoea through reduction of granulosa cells, inhibition of 17-oestradiol production by interfering with FSH action, and suppression effect on GnRH pulsative release. Severe hyperprolactinaemia with disruption of the pulsatile and circadian secretion pattern of hormones was documented previously in a girl with PTPS deficiency [5]. Case 1 also suffered from repeated nocturnal enuresis, starting at the age of 11 years, that could not be explained by sonography of the renal and ureal ducts. Nocturia, often seen in Parkinson patients, might also be present in PTPS-deficient patients since it can be precipitated by the loss of dopaminergic output from the substantia nigra which appears to increase detrusor hyperreflexia.

One prominent persistent symptom, observed in many cases of severe PTPS deficiency, is mental retardation, including impaired cognitive and verbal skills. As this neurological impairment is not very amenable to therapy, it must be considered that prenatal or early development of the brain is affected. A possible reason for this finding is the involvement of nitric oxide synthase in long-term potentiation in the hippocampus by acting as a retrograde messenger [18]. Lack of nitric oxide synthase activity due to $BH_4$ deficiency could severely affect learning and cognitive functions in these patients. Studies on an animal model (hph-1 mouse) have shown that depletion of $BH_4$ influences both nitric oxide production and neurotransmitter biosynthesis [15, 26]. Furthermore, aminergic neurons and their corresponding neurotransmitters play an important role in the functional maturation of their targets in the brain and for synaptogenesis of the cortex in the early ages of life. High Phe concentrations during the prenatal period may also inhibit the rate of protein synthesis, which may affect early dendritic proliferation and myelinisation. A moderate tendency of PTPS deficiency associated with a broad range of behavioural disorders, sleep disturbance, fluctuation of alertness, depression, affective disorders, anxiety or social withdrawal was noted in siblings cases 1, 2 and 3. However, it is difficult to explain these symptoms by amine deficiency alone, since they could be precipitated by effects of the therapy and the mental strain of the disease itself.

Several reasons can explain the poor outcome despite treatment in some patients with the severe form of PTPS deficiency such as a higher incidence of low birth weight, microcephaly, and prematurity [20]. Also, iatrogenic damage cannot be excluded as it is not known whether neurotransmitter precursors or the inhibitor of peripheral decarboxylases, carbidopa, might have side-effects during early brain development. It has been further suggested that $BH_4$ stimulates dopamine release in striatum directly (independent of its cofactor activity for tyrosine hydroxylase) or via nitric oxide synthase [32]. Thus, insufficient oral administration of $BH_4$ would explain the limited success of neurotransmitter substitution, as dopamine release is decreased independently of sufficient concentrations of neurotransmitter precursors.

## Genotype-phenotype correlation

Although 33 different mutations have so far been detected in patients with different forms of PTPS deficiency, there is no clear correlation between the type of mutation and the clinical phenotype [12]. Functional investigations of recombinant mutant proteins revealed that some missense mutations detected in patients with severe PTPS deficiency produced a significantly lower PTPS activity. One particular mutation (K129E), found in a patient with transient HPA who changed with age from peripheral to central form of PTPS deficiency, was fully active when expressed recombinantly in COS-1 cells [38], and a dominant negative allele (N47D) detected in compound heterozygotes for a variant of PTPS deficiency was found to cause transient HPA [43]. A dominant negative effect of some particular mutations may also be responsible for the low erythrocyte PTPS activity found in obligate heterozygotes [45].

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