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TETRAHYDROBIOPTERIN AND INHERITED HYPERPHENYLALANINEMIAS*

*Nenad Blau PhD***, *Beat Thöny PhD****; *Marco Spada MD*****
*Alberto Ponzone MD******

SUMMARY: Blau N, Thöny B, Spada M, Ponzone A. (Division of Clinical Chemistry, Department of Pediatrics, University of Zürich, Zürich, Switzerland, and Department of Pediatrics, University of Turin, Turin, Italy, and Facolla di Magistero, University of Messina, Messina, Italy). Tetrahydrobiopterin and inherited hyperphenylalaninemias. Turk J Pediatr 1996; 38: 19-35.

Tetrahydrobiopterin deficiency, a variant of hyperphenylalaninemia, may be caused by deficiency of one of the following enzymes: guanosine triphosphate cyclohydrolase 1, 6-pyruvoyltetrahydropterin synthase, dihydropteridin reductase and pterin-4a-carbinolamine dehydratase. The first two enzymes are involved in the biosynthesis of tetrahydrobiopterin, the last two in its regeneration. Although these diseases are rare, early detection by selective screening is essential for the treatment and outcome. Tetrahydrobiopterin deficiencies are very heterogenous ranging from mild forms requiring only marginal if any treatment to severe forms which are in some cases very difficult to treat. All variants of tetrahydrobiopterin deficiency can be differentiated from the classical phenylketonuria (PKU) by measurement of pterin metabolites in patients' urine, tetrahydrobiopterin loading test, and by dihydropteridine reductase activity in erythrocytes from the Guthrie card. *Key words: hyperphenylalaninemia, phenylketonuria, tetrahydrobiopterin, biopterin, neopterin, phenylalanine, neonatal screening, genetics.*

Function of Tetrahydrobiopterin

The best established function of tetrahydrobiopterin (BH_4) in man is as the natural cofactor for phenylalanine-4-hydroxylase (PAH), tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase; the latter two are key enzymes in the biosynthesis of biogenic amines[1]. In addition to the hydroxylation of aromatic amino acids, BH_4 serves as the cofactor for nitric oxide synthase[2] and glyceryl-ether monoxygenase[3] (Fig. 1).

* From the Division of Clinical Chemistry, Department of Pediatrics, University of Zürich, Zürich, and from the Department of Pediatrics, University of Turin, Turin, and from Facolla di Magistero, University of Messina, Messina.

** Biochemist, Department of Pediatrics, University of Zürich.

*** Molecular Biologist, Department of Pediatrics, University of Zürich.

**** Pediatrician, Department of Pediatrics, University of Turin.

***** Pediatrician, Department of Pediatrics, Facolla di Magistero, University of Messina.

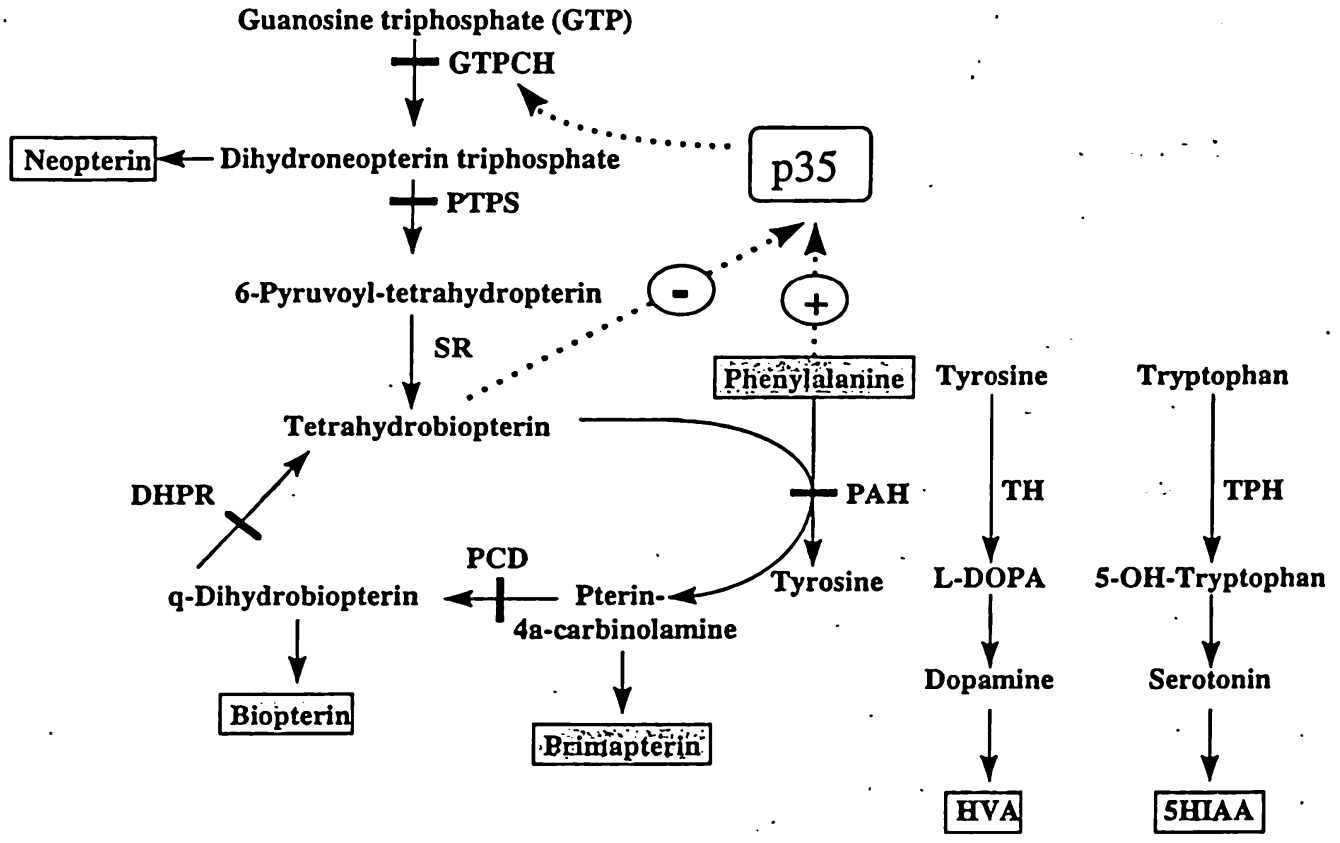

Fig. 1: Biosynthesis and regeneration of tetrahydrobiopterin including hydroxylation of aromatic amino acids. Pathological metabolites used as specific markers in the differential diagnosis are marked in squares.

GTPCH	= GTP cyclohydrolase I	PTPS	= 6-pyruvoyl-tetrahydropterin synthase
SR	= sepiapterin reductase	PCD	= pterin-4a-carbinolamine dehydratase
DHPR	= dihydropteridine reductase	PAH	= phenylalanine-4-hydroxylase
TH	= tyrosine-3-hydroxylase	TPH	= tryptophan-5-hydroxylase
L-DOPA	= 3,4-dihydroxyphenylalanine	HVA	= homovanillic acid
5HIAA	= 5-hydroxyindoleacetic acid	p35	= GTPCH stimulating protein

The function of these reactions derives from the ability of BH_4 to react with molecular oxygen to form an active oxygen intermediate that can hydroxylate substrates. In the hydroxylation process, the co-enzyme loses two electrons and is regenerated in vivo in an NADH-dependent reaction. Although BH_4 was found to be absolutely essential for nitric oxide synthase activity, the exact function in different forms of the enzyme and the mechanism of action are as yet unclear. Furthermore, it has been demonstrated that BH_4 increases the proliferation rate of erythroid cells[4] and that it is active as a dopamine-releasing factor in rat striatum by using an in vivo dialysis technique[5].

Metabolism of Tetrahydrobiopterin

BH_4 is synthesized from guanosine triphosphate (GTP) in at least four enzymatic steps by the action of three different enzymes (Fig. 1)[6-9]. GTP cyclohydrolase

I (GTPCH), the first enzyme in BH_4 biosynthesis, catalyzes the formation of 7,8-dihydroneopterin triphosphate from GTP in a single reaction step[10]. GTPCH is subject to feedback inhibition by BH_4. Inhibition occurs through a BH_4-dependent complex formation between a newly discovered p35 protein and GTPCH[11]. Furthermore, the inhibition is specifically reversed by phenylalanine. These may explain the high neopterin and biopterin contents observed in patients with hyperphenylalaninemia (HPA)[12].

In the following step, the 6-pyruvoyl-tetrahydropterin synthase (PTPS) catalyzes the conversion of 7,8-dihydroneopterin triphosphate to 6-pyruvoyl-tetrahydropterin[13].This conversion is magnesium-and zinc-dependent and involves the elimination of triphosphate and an intermolecular redox rearrangement reaction[14]. Whereas GTPCH is found to be highly regulated by the p35 protein, for PTPS no such regulatory processes are described. Nevertheless, PTPS needs post-translational modification(s), i.e., phosphorylation, in order to be fully active in vivo[15]. Furthermore, it has been suggested that PTPS is the rate-limiting enzyme for BH_4 biosynthesis, at least in the human liver[16].

Sepiapterin reductase (SR) has been shown to be an NADPH oxidoreductase required for the final two-step reduction of the diketo intermediate 6-pyruvoyl-tetrahydropterin to BH_4[17].

During the enzymatic hydroxylation of aromatic amino acids, molecular oxygen is consumed and tetrahydrobiopterin is peroxidated and oxidized. The pterin intermediate is subsequently reduced back to BH_4 by two enzymes and a reduced pyridine nucleotide (NADH) in a complex recycling reaction (Fig. 1). Molecular oxygen is first bound to BH_4 to form an unstable 4a-peroxy-tetrahydrobiopterin. The monooxygenation of aromatic amino acids is thus concomitant with oxidation of BH_4 to 4a-hydroxy-tetrahydrobiopterin (pterin-4a-carbinolamine)[18]. Pterin-4a-carbinolamine is subsequently dehydrated to quinonoid-dihydrobiopterin (q-dihydrobiopterin) and water by the specific and highly efficient pterin-4a-carbinolamine dehydratase (PCD)[19, 20]. The PCD was originally detected as a contaminant in a preparation of the rat PAH as a consequence of its ability to stimulate the BH_4-dependent hydroxylation of phenylalanine[21]. PCD activity was also shown to be present in human liver, and its absence is concomitant with the formation of 7-substituted pterins[22]. Unexpectedly, the primary structure of PCD is identical with a protein of the cell nucleus, named dimerization cofactor of hepatocyte nuclear factor 1a (DCoH), reported to have a general transcriptional function[23-25]. However, the dual function of regulation of PAH activity and transcription activation in a single protein is unprecedented.

In the last step of BH_4 recycling, the q-dihydrobiopterin is reduced back to BH_4 by the NADH-dependent dihydropteridine reductase (DHPR). There is no

evidence that DHPR has regulatory properties; however, it has been shown that a number of drugs can inhibit the enzyme's activity, both in vivo and in vitro. For instance, methotrexate, an antineoplastic drug commonly used in the treatment of cancer, can significantly inhibit DHPR activity[26].

Tetrahydrobiopterin Deficiency

The first patients with BH_4 deficiency were identified in 1969 by Tada et al.[27] Two siblings with mild HPA were, at that time, described as "a genetic variant of phenylketonuria" (PKU), but later were characterized as DHPR-deficient[28]. In 1974 in London, Smith et al.[29] described three children with "PKU" who had an unusual clinical course. Despite early diagnosis and treatment with a low-phenylalanine diet, these patients developed progressive neurological disease and died. Independently, Bartholomé[30] in Heidelberg reported a similar case of "atypical" HPA unresponsive to dietary treatment. The atypical course, high tolerance for phenylalanine and normal PAH activity in liver biopsy in Bartholomé's patient, led to the speculation that this syndrome was a new form of HPA, probably due to a defect in the metabolism of BH_4. Smith et al.[29] reasoned that a defect in the metabolism of BH_4 in brain tissue would also result in defective turnover of the neurotransmitters L-DOPA, noradrenaline, and serotonin. In the following years a number of cases of BH_4 deficiency were described, and it was suggested that all these patients suffered from a defect in BH_4 metabolism[31-39]. Because all untreated patients show severe cerebral deterioration and most of them die at an early age, it was suggested that this clinical syndrome should be called "malignant" HPA[40, 41].

Based on the speculation that pterins might be used in the treatment of PKU, Smith et al.[31] proposed that patients with BH_4 deficiency might benefit from substitution with reduced pterins. Indeed, Danks et al.[36] showed that intravenous administration of synthetic BH_4 decreases the serum phenylalanine levels and, therefore, can function as a cofactor subsituting for hepatic PAH in vivo. Meanwhile, therapy with L-DOPA, carbidopa, and 5-hydroxytryptophan alone or in combination with BH_4, was shown to be beneficial for patients with various forms of BH_4 deficiency[42-49].

Because BH_4 deficiency may cause a severe disease but is treatable, it became necessary to develop selective screening tests for detection early in infancy. Every newborn with even slight but persistent HPA should be tested for a BH_4 deficiency. Such tests have been introduced in many developed countries, but even today older children are invariably detected because of the appearance of clinical symptoms, such as hypotonia of the trunk, hypertonia of the extremities, and often myoclonic seizures, which are unresponsive to a low-phenylalanine diet. According to the literature the frequency of BH_4 deficiency is estimated to

be one to two percent among cases with HPA[50-52], although in some countries, such as Turkey and Saudi Arabia, because of consanguinity the incidence is even higher.

Nomenclature of Tetrahydrobiopterin Deficiency

The BH_4 deficiencies are a heterogeneous group of diseases, and different phenotypes, ranging from very mild to severe forms, define the variants. The terms severe/general or mild/peripheral/partial can be used according to the actual need for treatment[51]. Accordingly the following nomenclature applies (Table I). Older terms such as "atypical PKU" (non-PKU hyperphenylalaninemia) or "malignant PKU" should be avoided.

Table I: Overview of Tetrahydrobiopterin Deficiencies and Treatment

Defect	Phenotype	Neonatal HPA Phe (µmol/L)	BH_4 Loading (20 mg/kg)	Residual Enzyme Activity (%)	Neurotransmitters and/or BH_4 Therapy
GTPCH deficiency	severe	120-1200	+	< 1*	+
PTPS deficiency	severe	250-2500	+	0-20**	+
	mild	240-2200	+	8-31**	–
DHPR deficiency	severe	180-2500	+•	< 1***	+
	mild	280-600	+	< 5***	–
PCD	transient	180-1200	+	?	–

* liver. ** erythrocytes. *** fibroblasts.
• at least two patients with "non-responder" phenotype are documented.

Selective Screening and Ultimate Diagnosis

Because BH_4 deficiencies are a group of diseases that can be detected but not identified through neonatal mass screening for HPA, selective screening for a BH_4 deficiency is essential in every newborn with even slightly elevated phenylalanine levels[53, 54]. Screening for a BH_4 deficiency should be done in all newborns with plasma phenylalanine levels higher than 120 µmol/L, as well as in older children with neurological signs and symptoms. The following tests are recommended:

1. Analysis of pterins in urine (see Appendix).
2. Measurement of DHPR activity in blood from Guthrie card (see Appendix).
3. Loading test with BH_4 (see Appendix).

The first two tests are essential and allow differentiation between all BH_4 defects. With some limitations, the BH_4 loading test is an additional, useful diagnostic tool for rapid differentiation between calissical PKU and the BH_4 variants. A diagnostic flow-chart is shown in Figure 2.

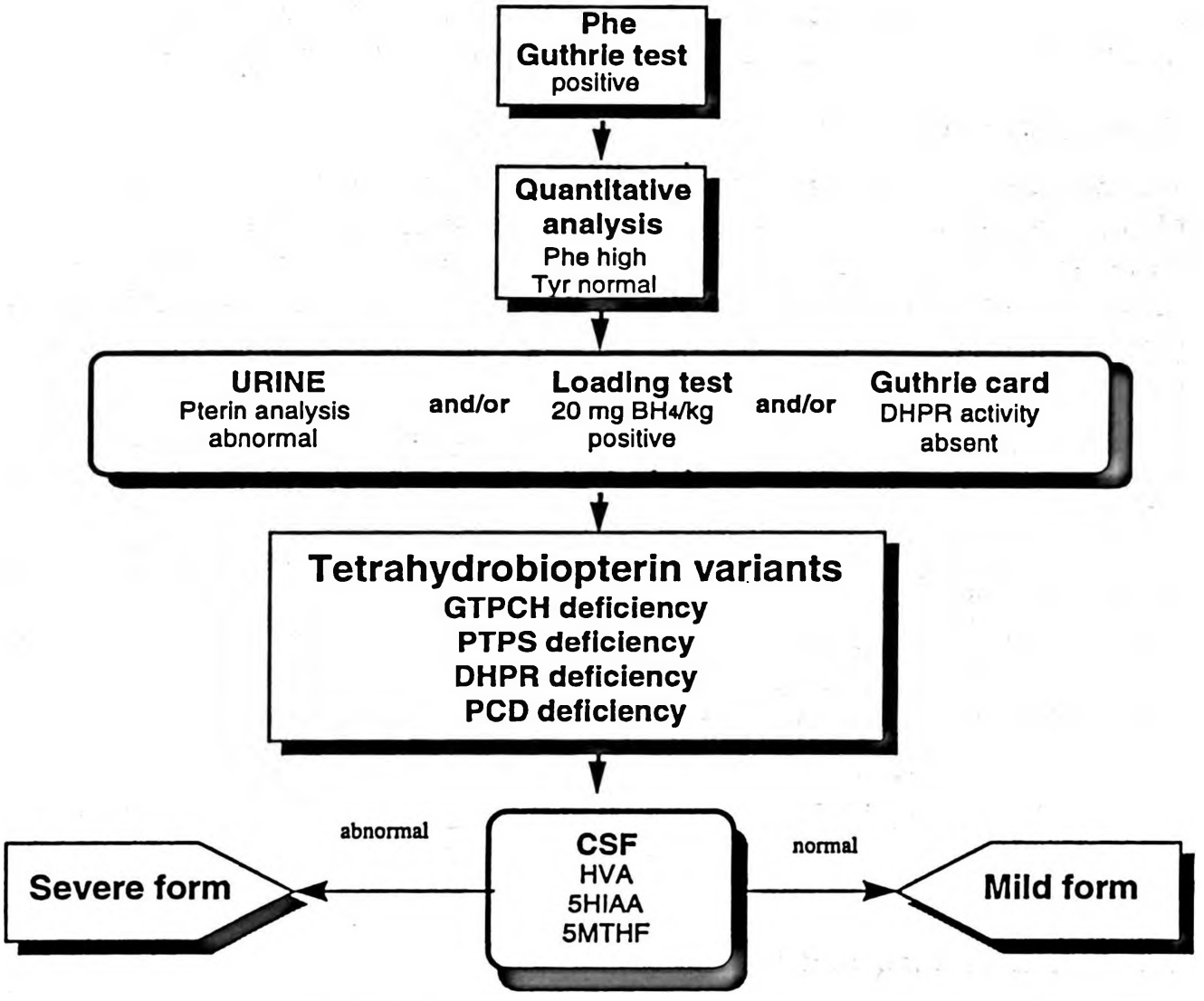

Fig. 2: Diagnostic flow-chart for the differentiation of hyperphenylalaninemias.

Phe = phenylalanine
Tyr = tyrosine
GTPCH = GTP cyclohydrolase I
PTPS = 6-pyruvoyl-tetrahydropterin synthase
PCD = pterin-4a-carbinolamine dehydratase
DHPR = dihydropteridine reductase
HVA = homovanillic acid
5HIAA = 5-hydroxyindoleacetic acid
5MTHF = 5-methyltetrahydrofolic acid

Analysis of Pterins

Liquid urinary samples or even better a random urine specimen dried on filter paper can be used[55]. Reduced urinary pterins are extremely unstable and are affected by daylight, temperature and storage time. The recovery of pterins in urine dried on filter paper and stored at room temperature for one and two weeks showed good correlation with standard methods. A significant advantage of this method is that the samples can be mailed unfrozen in an envelope, speeding delivery and minimizing costs.

Typical urinary pterin profiles are as follows: in GTPCH deficiency, neopterin and biopterin are very low; in PTPS deficiency, neopterin and monapterin (isomer of neopterin) are very high and there are only traces of biopterin; in DHPR deficiency, neopterin is normal or slightly increased and biopterin is very high; and in PCD deficiency, neopterin is initially high, biopterin is in the subnormal range and primapterin (7-substituted biopterin) is present. Patients with classical PKU excrete generally more neopterin and biopterin in urine compared with the normal controls.

By two-dimensional plotting of total biopterin versus percentage of biopterin, of the sum of neopterin plus biopterin, variants of HPA can be separated (Fig. 3).

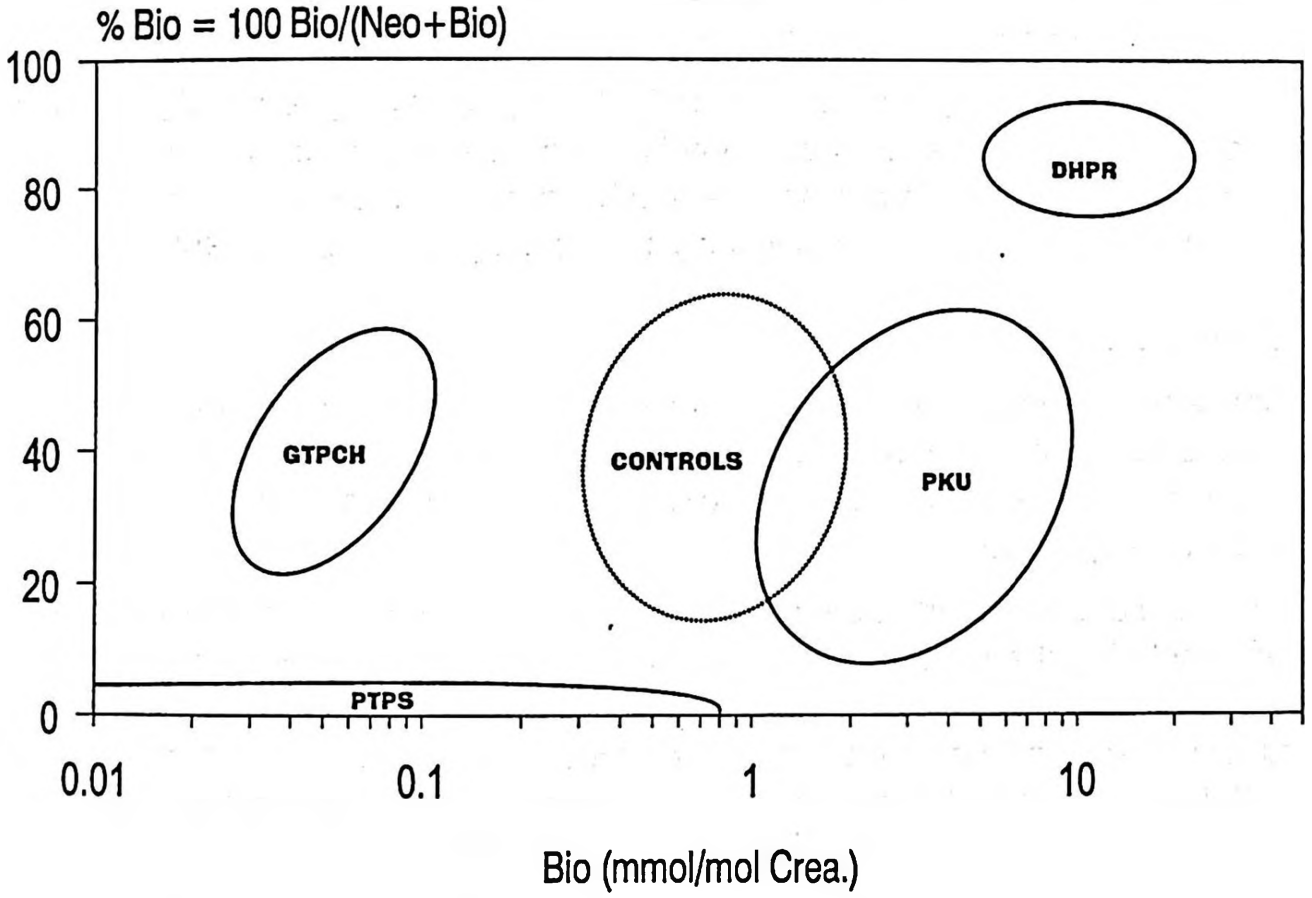

Fig. 3: Two-dimensional semilogarithmic plotting of total urinary biopterin and percentage of biopterin. Percentage of biopterin is calculated from the sum of neopterin and biopterin.

Loading Tests

Two types of loading tests have been used in the differential diagnosis of BH_4 deficiencies:

1. A simple oral BH_4 load
2. A combined phenylalanine and BH_4 load

Historically, the first method tried[36] which was predicted as the most convenient to screen selectively among HPAs, exploited the lowering of plasma phenylalanine in BH_4-deficient patients after the administration of exogenous BH_4. Intravenous

loading with 2 mg/kg BH_4 was originally proposed by Danks et al.[37] With the increased purity and availability of this synthesized cofactor (BH_4), an oral loading test (2.5 mg/kg bw) was introduced by Niederwieser et al.[56] and was standardized at a dose of 7.5 mg/kg by the same group[48]. This very simple test discriminated between patients with PAH and BH_4 deficiencies. However, it soon became obvious that some DHPR-deficient patients could be misdiagnosed, in as much as their serum phenylalanine levels were not lowered by BH_4[57], thus introducing an unacceptable limitation if this was the sole test performed. The observation that BH_4 nonresponsiveness in DHPR deficiency correlates with the presence of a mutant enzyme[58] and that it can be overcome by increasing the dose (20 mg/kg bw) of the administered synthetic cofactor, led to a new standard protocol for BH_4 loading.

Phenylalanine (100 mg/kg bw) plus BH_4 (20 mg/kg bw) given orally enables selective screening of all BH_4 deficiencies when pterin analysis is not available or when a clear diagnosis has not previously been made[59]. In this simplest form, this test can be interpreted using only four blood spots from a Guthrie card.

Additional Tests

Ultimate diagnosis can be established by enzymatic studies and by measurement of the cerebrospinal fluid (CSF) concentrations of the major dopamine and serotonin catabolites, homovanillic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA), respectively.

PTPS[60] and DHPR[61] activities are both expressed in erythrocytes and skin fibroblasts, thus simplifying the patients' investigations. GTPCH activity is measurable in the liver[62] and in cytokine-stimulated mononuclear cells[63] or fibroblasts[64, 65], while PCD activity is measurable only in the liver[19]. As reported below, the human genes and/or cDNAs coding for all these enzymes have been cloned, and mutation analysis is in progress to establish a genotype-phenotype correlation.

The measurement of CSF neurotransmitter metabolites is of capital importance for clinical and treatment follow-up, as it allows separation of typical from atypical cases of BH_4 deficiency (the latter are phenotypically defined by the absence of clinical signs and symptoms and do not need any treatment for biogenic amine deficiency).

Treatment and Prognosis

With the exception of cases with PCD deficiency, which is generally a benign and transitory form of HPA[66, 67], untreated patients with typical BH_4 deficiency show a virtually similar picture of progressive neurological deterioration, regardless of the different enzyme defects[68, 69]. The median age of onset of the clinical presentations is between four and five months[68]; however, abnormal signs (microcephaly, poor suckling, hypotonia) may be present since birth, particularly

in PTPS deficiency, suggesting that in utero damage may occur[70]. Within a few months, patients develop a typical neurological syndrome characterized by disturbances of tone and posture (truncal hypotonia with limb hypertonia), tremors, hypokinesis, drowsiness, irritability, swallowing difficulties, abnormal movements, oculogiric crises, grand mal or myoclonic convulsions, and recurrent episodes of hyperthermia without infections. These symptoms are due to the combined amine deficiency (Table II), fluctuate diurnally in their intensity, and can even be completely nullified by treatment. With time, untreated patients develop progressive microcephaly associated with electroencephalogram (EEG) paroxysmal activity as well as brain atrophy on computerized tomography scan or magnetic resonance imaging. If HPA has not been treated soon after birth[71], treatment becomes ineffective and death occurs in infancy or childhood. In addition to the above symptoms, patients suffering from DHPR deficiency may develop long-term signs associated with perivascular calcifications located in the basal ganglia and in the areas of the white and gray matter[73]. These changes are irreversible and are thought to occur as a result of inhibition of folate metabolism when folinic acid is not substituted[73, 74].

Table II: Symptoms Related to Specific Biogenic Amine Deficiency

Dopamine	Serotonin	Noradrenaline
Immobility	Depression	Axial hypotonia
Parkinsonism	Altered thermogenesis	Cerebellar symptoms
Sleepiness	Insominia	
Dystonia		
Eye rolling		
Swallowing difficulties		

The prognosis of severity in BH_4 deficiency is closely related to the degree of HPA and to impaired biogenic amine production. Therefore a combination of therapies to correct this metabolic clutter must be applied promptly after diagnosis. Control of HPA can be achieved either by a phenylalanine-restricted diet or by administration of synthetic BH_4. The dietary tolerance to phenylalanine is higher in BH_4 deficiency (300-700 mg/d) than in classical phenylketonuria, but normal serum phenylalanine levels should be attained to avoid interference with neurotransmitter metabolism[75]. The diet can be calculated following the lines used in phenylketonuria, also supplementing tyrosine. The administration of synthetic BH_4 is always effective in the control of HPA. A single, small daily dose (1-5 mg/kg) is sufficient in synthesis defects, while larger and fractionated daily doses (20 mg/kg, given in 3-4 doses) are necessary in some DHPR-deficient patients[76]. It should be noted, however, that the dietary phenylalanine tolerance increases markedly with age in these patients (Ponzone A, Spada M, personal communication).

Despite substantial amounts of peripherally administered BH_4 found in CSF[77, 78], only a few patients respond at the central level to BH_4 monotherapy by normalizing their neurotransmitter production[44, 45, 76, 79]. Most patients need neurotransmitter substitutive therapy[80-83]. This can be realized by administering the precursors L-dopa and 5-hydroxytryptophan in conjunction with carbidopa, an inhibitor of peripheral aromatic amino acid decarboxylase. Daily doses and the number of administrations must individually adjusted according to the patient's age and requirements (Table III).

Table III: Therapy of Tetrahydrobiopterin Deficiencies

Therapy	Age	Doses / day	PTPS / GTPCH Deficiency	DHPR Deficiency
		Combined Therapy		
Phenylalanine-low diet or	initially	–	no	yes no
BH_4 (mg/kg/d)	initially	1	1-5	no 20
L-DOPA (mg/kg/d) + 10%	initially	3-4	1-2	1-2
Carbidopa	< 2y	3-4	5	5
	> 2y	3-4	8-10	8-10
5-Hydroxytryptophan (mg/kg/d)	initially	3-4	1-2	1-2
	< 2y	3-4	5	5
	> 2y	3-4	6-8	6-8
Folinic acid (mg/d)	initially	1	no	10-20
		BH_4 Monotherapy		
BH_4 (mg/kg/d)	initially	1-2	5-20	no 20

Monitoring of treatment can be implemented either clinically, by evaluating the minimal dose effective in relieving cardinal symptoms, or biochemically, by periodically measuring the CSF concentration of HVA and 5-HIAA[71]. Unfortunately, when higher doses of neurotransmitter precursors are necessary, patients may show prominent overdose effects or on-off phenomena[84]. Good results have recently been obtained in similar cases with the concurrent administration of L-deprenyl, a selective monoamine oxidase (MAO) B inhibitor, allowing dosage reduction of administered precursors by curtailing their catabolism[85].

Folinic acid administration (10-20 mg per day) is mandatory only in DHPR deficiency to avoid folate depletion.

The clinical outcome of BH_4 deficiency depends largely on early treatment intervention and compliance. Fetal damage and delay in treating HPA, with subsequent irreversible damage, worsen the prognosis.

Incidence and Genetics

All variants of BH_4 deficiencies have an autosomal recessive trait. The frequency of all BH_4 deficiencies is uncertain, but, assuming they represent altogether about

one to two percent of PKU patients, the combined frequency of BH_4 deficiencies is approximately 1:10^6 live births. Data from newborn and selective screening programs reveal regional (demographic) variations in the frequency of BH_4 deficiencies[52]. In the Piemonte region as well as in southern parts of Italy, 10 percent of all patients with HPA are accounted for by BH_4 deficiencies (Ponzone A, personal communication). In Turkey, the incidence is even higher (15%)[86]. In Taiwan, it is percent 19 percent[87], and Saudi Arabia has the highest incidence (66%)[88, 89].

GTPCH, PTPS, PCD, and DHPR are each encoded by single genes, and the corresponding loci are mapped to chromosomes 14q21q-q22, 11q22.3-q23.3, 10q22, and 4p15.3, respectively[90-93]. Phenotypically, BH_4 deficiencies are a very heterogeneous group of disorders. Cloning of the wild-type genes and/or corresponding cDNAs now enables characterization of mutations on the nucleotide level, which extends the initially limited studies by enzymatic assays of cultured primary cells or patients' lymphocytes. So far, only a limited number of patients with GTPCH deficiency[16, 94], PTPS deficiency[15, 95-98], DHPR deficiency[99-101], and PCD deficiency[20, 102] have been investigated for DNA mutations that result in RNA splicing defects or in abberrant mutant proteins. Studies of the consequences on the enzymatic activity and/or stability of mutations resulting in amino acid substitutions, deletions or insertions, and truncations were performed by recombinant expression of the corresponding mutant proteins. These combined analyses will help to understand the different clinical phenotypes of BH_4 deficiencies.

Appendix

Collection of Liquid Urine Specimens: Pipette 5 ml of fresh random urine into a centrifugation vial and adjust pH to 1.0-1.5 with 6M HCl, Add 100 mg of manganese dioxide (MnO_2, No: 5957, Merck, Darmstadt, Germany) and shake for 5 min at room temperature. Centrifuge for 10 min at 4000 rpm. Immediately transfer 1 ml of the clear supernatant into a vial for shipment. Wrap the vial in aluminum foil to protect from light.

Collection of Dried Urine Specimens: Fresh random urine was processed as described for liquid specimen. Filter paper strips (3x5 cm, filter paper backing 165-0921, Bio-Rad, Richmond, USA) were dipped into the clear supernatant of the oxidized urine up to 1 cm below the upper edge. Excess urine was wiped off and the filter paper was left to dry at room temperature in dim light. The filter strip was then covered with aluminum foil and sent to the laboratory in an envelope by express mail.

Loading Test with BH_4 (20 mg/kg bw): A simple oral BH_4 loading test was performed at least 30 min before the meal. BH_4 tablets (No: 2501.00.00, Milupa AG, Friedrichsdorf, Germany, or Dr. Schircks Lab., Jona, Switzerland, or Suntory

Pharmaceuticals, Tokyo, Japan) were dissolved in orange juice or water (ca. 10 ml per 10 mg BH_4) and administered immediately at a dose of 20 mg/kg body weight. Blood for the analysis of phenylalanine and tyrosine was collected before, and 4 and 8 hours after administration.

Combined Loading Test with Phenylalanine (Phe) (100 mg/kg bw) and BH_4 (20 mg/kg bw): Tablets of synthetic cofactor (20 mg/kg body weight) were dissolved as described above and administered 3 hours after the Phe load (100 mg/kg body weight). Blood was collected before, and 3, 7, and 11 hours after Phe administration. Store at -20 °C.

Blood Collection for DHPR Activity: Collect 2-4 drops of blood on a Guthrie card. Protect the card from humidity.

Collection of CSF Specimen: Discard the first 0.5 ml of the CSF and collect the next 1.0 ml in an EDTA tube. Keep immediately frozen at -20 °C.

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REFERENCES

1. Scriver CR, Eisensmith RC, Woo SLC, et al. The hyperphenylalaninemias of man and mouse. Annu Rev Genet 1994; 28: 141-165.
2. Marletta MA. Nitric oxide synthase structure and mechanism. J Biol Chem 1993; 268: 12231-12234.
3. Kaufman S, Pollock RJ, Summer GK, Das AK, Hajra AK. Dependence of an alkyl glycolether monooxygenase activity upon tetrahydropterins. Biochim Biophys Acta 1990; 1040: 19-27.
4. Tanaka K, Kaufman S, Milstien S. Tetrahydrobiopterin, the cofactor for aromatic amino acid hydroxylases, is synthesized by and regulates proliferation of erythroid cells. Proc Natl Acad Sci USA 1989; 86: 5864-5867.
5. Koshimura K, Miwa S, Lee K, Fujiwara M, Watanabe Y. Enhancement of dopamine release in vivo from the rat striatum by dialytic perfusion of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin. J Neurochem 1990; 54: 1391-1397.
6. Nichol CA, Smith GK, Duch DS. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. Annu Rev Biochem 1985; 54: 729-764.
7. Curtius CH, Heintel D, Ghisla S, Kuster TH, Leimbacher W, Niederwieser A. Biosynthesis of tetrahydrobiopterin in man. J Inherit Metab Dis 1985; 8: 28-33.
8. Duch DS, Smith GK. Biosynthesis and function of tetrahydrobiopterin. J Nutr Biochem 1991; 2: 411-423.
9. Kaufman S. New tetrahydrobiopterin-dependent systems. Annu Rev Nutr 1993; 13: 261-286.
10. Blau N, Niederwieser A. GTP-cyclohydrolases: a review. J Clin Chem Clin Biochem 1985; 23: 169-176.
11. Harada T, Kagamiyama H, Hatakeyama K. Feedback regulation mechanisms for the control of GTP cyclohydrolase I activity. Science 1993; 260: 1507-1510.

12. Ponzone A, Guardamagna O, Spada M, et al. Hyperphenylalaninemia and pterin metabolism in serum and erythrocytes. Clin Chim Acta 1993; 216: 63-71.

13. Takikawa SI, Curtius CH, Redweik U, Leimbacher W, Ghisla S. Biosynthesis of tetrahydrobiopterin. Purification and caharacterization of 6-pyruvoyl-tetrahydropterin synthase from human liver. Eur J Biochem 1986; 161: 295-302.

14. Bürgisser D, Thöny B, Redweik U, et al. 6-pyruvoyl tetrahydropterin synthase, an enzyme with a novel type of active site involving both zinc binding and an intersubunit catalytic triad motif; Site-directed mutagenesis of the proposed active center, characterization of the metal binding site and modelling of substrate binding. J Mol Biol 1995; 253: 358-369.

15. Oppliger T, Thöny B, Nar H, et al. Structural and functional consequences of mutations in 6-pyruvoyl tetrahydropterin synthase causing hyperphenylalaninemia in humans. J Biol Chem 1995; 270: 1-9.

16. Ichinose H, Ohye T, Matsuda Y, et al. Characterization of mouse and human GTP cyclohydrolase I genes-Mutations in patients with GTP cyclohydrolase I deficiency. J Biol Chem 1995; 270: 10062-10071.

17. Smith GK. On the role of sepiapterin reductase in the biosynthesis of tetrahydrobiopterin. Arch Biochem Biophys 1987; 255: 254-266.

18. Lazarus RA, Benkovic SJ, Kaufman S. Phenylalanine hydroxylase stimulator protein is a 4a-carbinolamine dehydratase. J Biol Chem 1983; 258: 10960-10962.

19. Rebrin I, Bailey SW, Boerth SR, Ardell MD, Ayling JE. Catalytic characterization of 4a-hydroxytetrahydropterin dehydratase. Biochemistry 1995; 34: 5801-5810.

20. Köster S, Thöny B, Macheroux P, et al. Human pterin-4a-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1-alpha: characterization and kinetic analysis of wild-type and mutant enzymes. Eur J Biochem 1995; 231: 414-423.

21. Kaufman S. A protein that stimulates rat liver phenylalanine hydroxylase. J Biol Chem 1970; 254: 4751-4759.

22. Curtius HC, Matasovic A, Schoedon G, et al. 7-Substituted pterins. A new class of mammalian pteridines. J Biol Chem 1990; 265: 3923-3930.

23. Citron BA, Davis MD, Milstien S, et al. Identity of 4a-carbinolamine dehydratase, a component of the phenylalanine hydroxylation system, and DCoH, a transregulator of homeodomain proteins. Proc Natl Acad Sci USA 1992; 89: 11891-11894.

24. Hauer CR, Rebrin I, Thöny B, et al. Phenylalanine hydroxylase-stimulating protein/pterin-4alpha-carbinolamine dehydratase from rat and human liver. Purification, characterization, and complete amino acid sequence. J Biol Chem 1993; 268: 4828-4831.

25. Mendel DB, Khavari PA, Conley PB, et al. Characterization of a cofactor that regulates dimerization of mammalian homeodomain protein. Science 1991; 254: 1762-1767.

26. Netter JC, Dhondt JL, Rance F, Petrus M. Early neurotoxicity of high-dose of methotrexate and tetrahydrobiopterin deficiency. Arch Fr Pediatr 1991; 48: 719-722.

27. Tada K, Yoshida T, Mochizuki K, et al. Two siblings of hyperphenylalaninemia: suggestion to a genetic variant of phenylketonuria. Tohoku J Exp Med 1969; 100: 249-253.

28. Tada K, Narisawa K, Arai N, Ogasawara Y, Ishizawa S. A sibling case of hyperphenylalaninemia due to a deficiency of dihydropteridine reductase: biochemical and pathological findings. Tohoku J Exp Med 1980; 132: 123-131.

29. Smith I. Atypical phenylketonuria accompanied by a severe progressive neurological illness unresponsive to dietary treatment. Arch Dis Child 1974; 49: 245.

30. Bartholomé K. A new molecular defect in phenylketonuria. Lancet 1974; 2: 1580.

31. Smith I, Clayton BE, Wolff OH. New variant of phenylketonuria with progressive neurological illness unresponsive to phenylalanine restriction. Lancet 1975; 1: 1108-1111.

32. Bartholomé K, Byrd DJ, Kaufman S, Milstien S. Atypical phenylketonuria with normal phenylalanine hydroxylase and dihydropteridine reductase activity in vitro. Pediatrics 1977; 59: 757-761.

33. Bartholomé K, Lutz P, Bickel H. Determination of phenylalanine hydroxylase activity in patients with phenylketonuria and hyperphenylalaninemia. Pediatr Res 1975; 9: 899-903.

34. Brewster TG, Abroms IF, Kaufman S, et al. Atypical PKU; seizures, and developmental delay with dihydropteridine reductase deficiency. Pediatr Res 1976; 10: 446.

35. Butler IJ, Holtzman NA, Kaufman S, Koslow SH, Krumholz A. Phenylketonuria due to deficiency of dihydropteridine reductase. Pediatr Res 1975; 9: 349.

36. Danks DM, Cotton RGH, Schlesinger P. Tetrahydrobiopterin treatment of variant form of phenylketonuria. Lancet 1975; 2: 1043.

37. Danks DM, Cotton RGH, Schlesinger P. Variant forms of phenylketonuria. Lancet 1976; 1: 1236-1237.

38. Kaufman S, Milstien S, Bartholomé K. New forms of phenylketonuria. Lancet 1975; 2: 708.

39. Rey F, Blandin-Savoja F, Rey J. Atypical phenylketonuria with normal dihydropteridine reductase activity. N Engl J Med 1976; 295: 1138-1139.

40. Danks DM. Pteridines and phenylketonuria: report of a workshop: introductory comments. J Inherit Metab Dis 1978; 1: 47-48.

41. Danks DM, Schlesinger P, Firgaira et al. Malignant hyperphenylalaninemia-clinical features, biochemical findings, and experience with administration of biopterins. Pediatr Res 1979, 13: 1150-1155.

42. Bartholomé K, Byrd DJ. L-dopa and 5-hydroxytryptophan therapy in phenylketonuria with normal phenylalanine-hydroxylase activity. Lancet 1975; 2: 1042-1043.

43. Butler IJ, O'-Flynn ME, Seifert WE, Howell RR. Neurotransmitter defects and treatment of disorders of hyperphenylalaninemia. J Pediatr 1981; 98: 729-733.

44. Endres W, Niederwieser A, Curtius HCH, Wang M, Ohrt B, Schaub J. Atypical phenylketonuria due to biopterin deficiency. Early treatment with tetrahydrobiopterin and neurotransmitter precursors, trials of monotherapy. Helv Paediatr Acta 1982; 37: 489-498.

45. Endres W, Niederwieser A, Curtius HCH, Ohrt B, Schaub J. Dihydrobiopterin deficiency: monotherapy with tetrahydrobiopterin (BH4) and diacetyl BH4. Pediatr Res 1982; 16: 694.

46. Kaufman S, Kapatos G, McInnes RR, Schulman JD, Rizzo WR. Use of tetrahydropterins in the treatment of hyperphenylalaninemia due to defective synthesis of tetrahydrobiopterin: evidence that peripherally administered tetrahydropterins enter the brain. Pediatrics 1982; 70: 376-380.

47. McInnes RR, Kaufman S, Warsh JJ, et al. Biopterin synthesis defect. Treatment with L-dopa and 5-hydroxytryptophan compared with therapy with a tetrahydropterin. J Clin Invest 1984; 73: 458-469.

48. Niederwieser A, Curtius HCH, Wang M, Leupold D. Atypical phenylketonuria with defective biopterin metabolism. Monotherapy with tetrahydrobiopterin or sepiapterin, screening and study of biosynthesis in man. Eur J Pediatr 1982; 138: 110-112.

49. Schaub J, Daumling S, Curtius HCH, et al. Tetrahydrobiopterin therapy of atypical phenylketonuria due to defective dihydrobiopterin biosynthesis. Arch Dis Child 1978; 53: 674-676.

50. Dhondt JL. Tetrahydrobiopterin deficiencies: preliminary analysis from an international survey. J Pediatr 1984; 104: 501-508.

51. Blau N, Thöny B, Heizmann CW, Dhonat JL. Tetrahydrobiopterin deficiency: from phenotype to genotype. Pteridines 1993; 4: 1-10.

52. Blau N, Barnes I, Dhondt JL. International database of tetrahydrobiopterin deficiencies. J Inherit Metab Dis 1996 (in press).

53. Blau N. Guidelines for the screening for hyperphenylalaninemia due to tetrahydrobiopterin deficiency. Cro Med J 1992; 33: 17-22.

54. Dhondt JL. Strategy for the screening of tetrahydrobiopterin deficiency among hyperphenylalaninaemic patients: 15-years experience. J Inherit Metab Dis 1991; 14: 117-127.

55. Blau N, Kierat L, Heizmann CW, Endres W, Giudici T, Wang M. Screening for tetrahydrobiopterin deficiency in newborns using dried urine on filter paper. J Inherit Metab Dis 1992; 15: 402-404.

56. Niederwieser A, Curtius HCH, Viscontini M, Schaub J, Schmidt H. Phenylketonuria variants. Lancet 1979; 1: 550.

57. Lipson A, Yu J, O Halloran M, Potter M, Wilken B. Dihydropteridine reductase deficiency: non-response to oral tetrahydrobiopterin load test. J Inherit Metab Dis 1984; 7: 69-71.

58. Cotton RGH, Jennings I, Bracco G, Panzone A, Guardamagna O. Tetrahydrobiopterin non-responsiveness in dihydropteridine reductase deficiency is associated with the presence of mutant protein. J Inherit Metab Dis 1986; 9: 239-243.

59. Ponzone A, Guardamagna O, Spada M, et al. Differential diagnosis of hyperphenylalaninemia by a combined phenylalanine-tetrahydrobiopterin loading test. Eur J Pediatr 1993; 152: 655-661.

60. Shintaku H, Niederwieser A, Leimbacher W, Curtius HCH. Tetrahydrobiopterin deficiency: assay for 6-pyruvoyl-tetrahydropterin synthase activity in erythrocytes, and detection of patients and heterozygous carriers. Eur J Pediatr 1988; 147: 15-19.

61. Arai N, Narisawa K, Hayakawa H, Tada K. Hyperphenylalaninemia due to dihydropteridine reductase deficiency: diagnosis by enzyme assays on dried blood spots. Pediatrics 1982; 70: 426-430.

62. Blau N, Niederwieser A. Guanosine triphosphate cyclohydrolase-I assay in human and rat liver using high-performance liquid chromatography of neopterin phosphates and guanine nucleotides. Anal Biochem 1983; 128: 446-452.

63. Blau N, Joller P, Atarés M, Cardesa-Garcia J, Niederwiesser A. Increase of GTP cyclohydrolase-I activity in mononuclear blood cells by stimulation: detection of heterozygotes of GTP cyclohydrolase-I deficiency. Clin Chim Acta 1985; 148: 47-52.

64. Werner ER, Werner Felmayer G, Fuchs D, HausenA, Reibnegger G, Wachter H. Parallel induction of tetrahydrobiopterin biosynthesis and indoleamine 2,3-dioxygenase activity in human cells and cell lines by interferon-gamma. Biochem J 1989; 262: 861-866.

65. Milstienn S, Kaufman S, Sakai N. Tetrahydrobiopterin biosynthesis defects examined in cytokine-stimulated fibroblasts. J Inherit Metab Dis 1993; 16: 975-981.

66. Dhondt JL, Guibaud P, Rolland MO, et al. Neonatal hyperphenylalaninaemia presumably caused by a new variant of biopterin synthetase deficiency. Eur J Pediatr 1988; 147: 153-157.

67. Blaskovics M, Giudici TA. A new variant of biopterin deficiency. N Engl J Med 1988, 319: 1611-1612.

68. Dhondt JL. Tetrahydrobiopterin deficiencies: lessons from the compilation of 200 patients. Dev Brain Dysfunct 1993; 139: 141-157.

69. Hyland K. Abnormalities of biogenic amine metabolism. J Inherit Metab Dis 1993; 16: 676-690.

70. Smith I, Dhondt JL. Birthweight in patients with defective biopterin metabolism. Lancet 1985; 1: 818.

71. Ponzone A, Ferroro GB, Guardamagna O, Ferroris S, Curtius HCH, Blau N. Screening and treatment of tetrahydrobiopterin deficiency. In: Curtius HC, Ghisla S, Blau N (eds). Chemistry and Biology of Pteridines 1989. Berlin: Walter de Gruyter, 1990: 393-401.

72. Coşkun T, Besim A, Özalp I, et al. Intracranial calcification in dihydropteridine reductase deficiency. Turk J Pediatr 1990; 32: 259-264.

73. Woody RC, Brewster MA, Glasier C. Progressive intracranial calcification in dihydropteridine reductase deficiency prior to folinic acid therapy. Neurology1989; 39: 673-675.

74. Biasucci G, Valsasina R, Giovannini M, Brioschi M, Saleri L, Riva E. Neuroradiological improvement after one year of therapy in a case of DHPR deficiency. In Curtius HC, Ghisla S, Blau N (eds). Chemistry and Biology of Pteridines 1989. Berlin: Walter de Gruyter, 1990: 438-444.

75. Ponzone A, Guardamagna O, Ferraris S, Biasetti S, Bracco G, Niederwieser A. Neurotransmitter therapy and diet in malignant phenylketonuria. Eur J Pediatr 1987; 146: 93-94.

76. Ponzone A, Guardamagna O, Dianzani I, et al. Catalytic activity of tetrahydrobiopterin in dihydropteridine reductase deficiency and indications for treatment. Pediatr Res 1993; 33: 125-128.

77. Ponzone A, Biasetti S, Ferraris S, et al. Differential entrance of tetrahydrobiopterin into the brain of patients with 6-pyruvoyl tetrahydropterin synthase deficiency. In: Levine RA, Milstien S, Kuhn DM, et al (eds). Pterins and Biogenic Amines in Neuropsychiatry, Pediatrics and Immunology. Grosse Pointe: Lakeshore Publishing Co, 1989: 325-334.

78. Kapatos G, Kaufman S. Peripherally administered reduced pterins do enter the brain. Science 1981; 212: 955-956.

79. Leupold D. Tetrahydrobiopterin monotherapy in two siblings wtih dihydrobiopterin deficiency. In Wachter H, Curtius HC, Pfleiderer W (eds). Biochemical and Clinical Aspects of Pteridines. Berlin: Walter de Gruyter, 1982: 307-317.

80. Giugliani R, Costa da-Costa J, Dutra-Filho CS, et al. Successful therapy of hyperphenylalaninemia due to defective tetrahydrobiopterin metabolism in two siblings. Rev Brasil Genet 1986; 9: 685-692.

81. Snyderman SE, Sansaricq C, Pulmones MT. Successful long term therapy of biopterin deficiency. J Inherit Metab Dis 1987; 10: 260-266.

82. Endres W, Blau N, Curtius HC. Newborn screening and treatment of hyperphenylalaninemia due to tetrahydrobiopterin deficiency. In: Schmidt BJ, Diamenti AJ, Loghin-Grosso NS (eds). Current Trends in Infant Screening. Amsterdam: Excerpta Medica, 1989: 77-82.

83. Endres W. Differential diagnosis and therapy of various forms of hyperphenylalaninemia: facts and fiction. Wien Klin Wochenschr 1992, 104: 503-509.

84. Tanaka Y, Matsuo N, Tsuzak S, Araki K, Tsuchiya Y, Niederwieser A. On-off phenomenon in a child with tetrahydrobiopterin deficiency due to 6-pyruvoyl tetrahydropterin synthase deficiency (BH4 deficiency). Eur J Pediatr 1989; 148: 450-452.

85. Schuler A, Blau N, Ponzone A. Monoamine oxidase inhibitors in tetrahydrobiopterin deficiency. Eur J Pediatr, 1995 (in press).

86. Coşkun T, Özalp I, Tokatlı A, Blau N, Niederwieser A. Hyperphenylalaninemia due to tetrahydrobiopterin deficiency. A report of 16 cases. J Inherit Metab Dis 1993; 16: 605-607.

87. Hsiao KJ, Chiang SH, Liu TT, Chiu PC, Wuu KT. Tetrahydrobiopterin deficient phenylketonuria detected by neonatal screening in Taiwan. In: Curtius HC, Ghisla S, Blau N (eds). Chemistry and Biology of Pteridines 1989. Berlin: Walter de Gruyter 1990: 402-407.

88. Al-Aqeel A, Ozand PT, Gascon G, et al. Biopterin-dependent hyperphenylalaninemia due to deficiency of 6-pyruvoyl tetrahydropterin synthase. Neurology 1991; 41: 730-737.

89. Al-Aqeel A, Ozand PT, Gascon G, et al. Response of 6-pyruvoyl-tetrahydropterin synthase deficiency to tetrahydrobiopterin. J Child Neurol 1992; 7 (Suppl): 526-530.

90. Thöny B, Heizmann CW, Mattei MG. Chromosomal localization of two human genes encoding tetrahydrobiopterin-metabolizing enzymes: 6-pyruvoyl-tetrahydropterin synthase maps to 11q22.3-q23.3 and pterin-4a-carbinolamine dehydratase maps to 10q22. Genomics 1994; 19: 365-368.

91. Thöny B, Heizmann CW, Mattei MG. Human GTP cyclohydrolase I gene and sepiapterin reductase gene map to region 14q21-q22 and 2p14-p12, respectively, by in situ hibridization. Genomics 1995; 20: 168-170.

92. Brown RM, Dahl HH. Localization of the human dihydropteridine reductase gene to band p15.3 of chromosome 4 by in situ hybridization. Genomics 1987; 1: 67-70.

93. Thöny B, Neuheiser F, Blau N, Heizmann CW. Characterization of the human PCBD gene encoding the bifunctional protein pterin-4α-carbinolamine dehydratase/dimerization cofactor for the transcription factor HNF-1α. Biochem Biophys Res Commun 1995; 210: 966-973.

94. Blau N, Ichinose H, Nagatsu T, Heizmann CW, Zacchello F, Burlina B. A missense mutation in a patient with guanosine triphosphate cyclohydrolase I deficiency missed in the newborn screening program. J Pediatr 1995; 126: 401-405.

95. Thöny B, Leimbacher W, Blau N, Harvie A, Heizmann CW. Hyperphenylalaninemia due to defects in tetrahydrobiopterin metabolism: molecular characterization of mutations in 6-pyruvoyl-tetrahydropterin synthase. Am J Hum Genet 1994; 54: 782-792.

96. Imamura T, Okano Y, Sawada Y, et al. A missense mutation of 6-pyruvoyl-tetrahydropterin synthase deficiency in Japanese. Pteridines 1994; 5: 31.

97. Ashida A, Owada M, Hatakeyama K. A missense mutation (A to G) of 6-pyruvoyltetrahydropterin synthase in tetrahydrobiopterin-deficient form of hyperphenylalaninemia. Genomics 1994; 24: 408-410.

98. Oppliger T, Thöny B, Leimbacher W, et al. Mutation analysis in patients with 6-pyruvoyl-tetrahydropterin synthase deficiency. Pteridines 1995; 6: 141-143.

99. Smooker PM, Cotton RGH. Molecular basis of dihydropteridine reductase deficiency. Hum Mutat 1995; 5: 279-284.

100. Smooker PM, Howells DW, Dianzani I, Cotton RGH. The spectrum of mutations in dihydropteridine reductase deficiency. In: Ayling JE, Nair MG, Baugh CM (eds). Chemistry and Biology of Pteridines and Folates. New York: Plenum Press, 1993: 135-138.

101. Blau N, Heizmann CW, Sperl W, et al. Atypical (mild) forms of dihydropteridine reductase deficiency: neurochemical evaluation and mutation detection. Pediatr Res 1992; 32: 726-730.

102. Citron BA, Kaufman S, Milstien S, Naylor EW, Greene CL, Davis MD. Mutation in the 4a-carbinolamine dehydratase gene leads to mild hyperphenylalaninemia with defective cofactor metabolism. Am J Hum Genet 1993; 53: 768-774.