Nomenclature and laboratory diagnosis of tetrahydrobiopterin deficiencies

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Because tetrahydrobiopterin (BH₄) deficiency may cause a severe but treatable disease, it became necessary to develop selective screening tests for detection early in infancy. Every newborn with even slight but persistent hyperphenylalaninemia (HPA) should be tested for BH₄ 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, unresponsive to a low-phenylalanine diet. Although according to the literature the frequency of BH₄ deficiency is estimated to be 1 to 2 percent among cases with HPA, in some countries, like Turkey and Saudi Arabia the incidence is even higher because of consanguinity.

Nomenclature of Tetrahydrobiopterin Deficiencies

The BH₄ deficiencies are a very heterogeneous group of diseases, and different clinical and biochemical criteria define and characterize the variants [1]. The primary enzyme defect, its severity, outcome of the BH₄ challenge, type of mutation, and responses to therapy are some of the criteria used to define a specific defect. The terms severe or mild/peripheral should be used according to the actual need for treatment with neurotransmitter precursors. Accordingly, the nomenclature given in Table 1 applies. Older terms such as “atypical PKU” or “malignant PKU” should be avoided.

In addition to “classical” BH₄ deficiencies, e.g. autosomal recessive GTP cyclohydrolase I (arGTPCH) deficiency, 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, carbino-lamine-4a-dehydratase (PCD) deficiency and dihydropteridine reductase (DHPR) deficiency, there are a number of genetic disorders related to BH₄ deficiency presenting without HPA, e.g. dopa-responsive dystonia (DRD, autosomal dominant, adGTPCH deficiency), and sepiapterin reductase (SR) deficiency [2].

Screening and Diagnosis

Because “classical” BH₄ deficiencies are a group of diseases that can be detected but not identified through neonatal mass screening for HPA, selective screening for a BH₄ deficiency is essential in every newborn with even slightly elevated phenylalanine levels [3].
Screening for BH₄ 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 [4]. The following tests are recommended:

1. Analysis of pterins in urine
2. Measurement of DHPR activity in blood from Guthrie card
3. Loading test with BH₄
4. Analysis of pterins, folates, and neurotransmitter metabolites in cerebrospinal fluid (CSF)
5. Enzyme activity measurement

The first two tests are essential and enable all BH₄ defects presenting with HPA to be differentiated. With some limitations, the BH₄ loading test is an additional, useful diagnostic tool for the rapid differentiation between classic PKU and BH₄ variants. This test alone can not differentiate between some patients with mild forms of PKU/HPA and BH₄ variants. Analysis of neopterin, biopterin, 5-methyltetrahydrofolic acid, and the neurotransmitter metabolites, 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA), allows for differentiation between severe and mild forms of BH₄ deficiencies. A diagnostic flowchart is shown in Figure 1.

### Table 1: Nomenclature of BH₄ deficiencies

<table>
<thead>
<tr>
<th>Enzyme defect</th>
<th>Phenotype</th>
<th>HPA</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>arGTPCH def.</td>
<td>severe</td>
<td>+</td>
<td>NT + BH₄</td>
</tr>
<tr>
<td>adGTPCH def.</td>
<td>severe</td>
<td>-</td>
<td>L-Dopa/Carbidopa</td>
</tr>
<tr>
<td>PTPS def.</td>
<td>severe</td>
<td>+</td>
<td>NT + BH₄</td>
</tr>
<tr>
<td>mild</td>
<td>+</td>
<td>BH₄</td>
<td></td>
</tr>
<tr>
<td>SR def.</td>
<td>severe</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>PCD def.</td>
<td>mild</td>
<td>+</td>
<td>BH₄ *</td>
</tr>
<tr>
<td>DHPR def.</td>
<td>severe</td>
<td>+</td>
<td>NT + diet + folinic acid</td>
</tr>
<tr>
<td>mild</td>
<td>+</td>
<td>Diet</td>
<td></td>
</tr>
<tr>
<td>NT: L-Dopa/Carbidopa/5-OH-Trp; Diet: low Phe</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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### Pterins in Urine

Oxidized pterins are highly fluorescent and can be detected with specificity and sensitivity after high-performance liquid chromatography (HPLC) in urine, blood, CSF, and amniotic fluid. The method of Niederwieser et al. [5] utilizes an automatic HPLC system with the capability for column switching. This method involves minimal pretreatment of the bio-
logic sample (i.e., oxidation with MnO₂), separates complex mixtures of pterins in a short time, and uses fluorometric detection [6].

Either native urine or urine dried on filter paper may be used for selective screening [7]. In instances of long shipping times or extreme temperatures, the reduced pterins must be stabilized by oxidation for BH₄ deficiency to be differentiated from classic PKU.

Urinary pterins should be analyzed at elevated plasma phenylalanine levels and not during treatment with a low-phenylalanine diet. Healthy newborns excrete relatively higher levels of pterins than children or adults. Patients with classic PKU excrete generally more neopterin and biopterin in urine than normal controls [8]. This is due to the activation of GTPCH by phenylalanine via the GTPCH feedback regulatory protein (GFRP).

Typical urinary pterin profiles are as follows (Figure 1): neopterin and biopterin are very low in GTPCH deficiency; neopterin and monapterin (isomer of neopterin) are very high in

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**Figure 1: Flow chart in the differential diagnosis of hyperphenylalaninemia variants.**

*N = neopterin; M = monapterin; IX = isoxanthopterin; B = biopterin; 7-B = primapterin; P = pterin.*
PTPS deficiency and there are only traces of biopterin; neopterin is normal or slightly increased and biopterin is very high in DHPR deficiency; and neopterin is initially high, biopterin is in the subnormal range, and primapterin (7-substituted biopterin) is present in PCD deficiency. By two-dimensional plotting of total urinary biopterin versus percentage of biopterin, of the sum of neopterin plus biopterin, variants of HPA can be differentiated.

Pterin excretion is normal in patients with DRD and SR deficiency.

**Dihydropteridine Reductase Activity in Blood**

Measurement of DHPR activity in dry blood spots of Guthrie cards is a widely used and routine method for the diagnosis of DHPR deficiency. The method of Arai et al. [9] uses the reduction of BH₄ to quinonoid dihydropterin in the presence of ferricytochrome C. In the presence of NADH, quinonoid dihydropterin is reduced back to BH₄ by DHPR. The formation of ferrocytochrome C is monitored spectrophotometrically. Only two spots of blood are required for the assay.

**Tetrahydrobiopterin Loading Tests**

The simple BH₄ loading test (20 mg/kg body weight) has been used in more than 3,000 patients with different forms of HPA. The blood phenylalanine level needs to be elevated (more than 400 µM) for this test to be interpretable. Patients with BH₄-responsive PKU (PAH deficiency) respond slowly to BH₄ administration [10]. It is customary for a PKU diet to be stopped 2–3 days before a loading test. A BH₄ test kit is now available and distributed by Dr. Schircks Laboratories (Jona, Switzerland) and should be used at the recommended dosage of 20 mg/kg body weight about 30 min before a regular meal. Blood samples should be collected at times 0, 4, 8, and 24 hours after BH₄ administration. An extended BH₄ loading test (2 x 20 mg over 48 hours) is used to detect some “slow” BH₄-responsive HPA/PKU patients.

**Pterins, Folates, and Neurotransmitter Metabolites in Cerebrospinal Fluid**

To distinguish between the different variants of BH₄ deficiency, i.e., severe and mild (peripheral, partial) forms, quantification of neopterin, biopterin, and the neurotransmitter metabolites, 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA), in CSF is essential. In patients with DHPR deficiency, measurement of 5-methyltetrahydrofoleric acid is important due to cerebral folate deficiency.
5HIAA, HVA and 5-methyltetrahydrofolate (5MTHF) are easily quantified by using HPLC with electrochemical detection [11]. Except for the mild/peripheral forms of GTPCH, PTPS, and PCD deficiencies, 5HIAA and HVA are dramatically decreased in the CSF of patients with the severe form of BH₄ deficiency. The ratio of HVA/5HIAA is much lower in patients with GTPCH deficiency and DRD than in those with PTPS deficiency, DHPR deficiency, or SR deficiency. In controls, this ratio is between 1.5 and 3.5. The levels of both metabolites decrease with age in all BH₄ deficiencies, and a similar correlation was found in control children. In the mild forms of a DHPR deficiency, 5HIAA is usually decreased, while HVA levels are normal.

Figure 2: HPLC of neurotransmitter metabolites 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) in CSF of a control person and patient with PTPS deficiency.
Concentrations of neopterin and biopterin in CSF are also found to be age-related. Particularly in neonatal samples, biopterin concentration is much higher in the first days of life than at the age of 1 month. Generally, in patients with BH4 deficiencies, the pterin pattern is similar to that found in urine except that, in GTPCH deficiency and DRD, biopterin is always slightly higher than neopterin. Patients with SR deficiency present, similar to patients with DHPR deficiency, with high total biopterin and dihydrobiopterin concentrations in CSF.

Supportive evidence for folate deficiency in the central nervous system (CNS) includes low CSF levels of 5-methyltetrahydrofolic acid (5MTHF) and frequent occurrence of basal ganglia calcifications similar to those found in congenital folate malabsorption. Measurement of 5-methyltetrahydrofolic acid in CSF with HPLC and electrochemical detection [12] is, therefore, important in the differential diagnosis of DHPR deficiency.

**GTPCH Deficiency**

In GTPCH deficiency, biosynthesis of pterins is blocked at a very early stage and almost no pterins can be formed [13]. The urinary excretion of neopterin, biopterin, isoxan-
thopterin, and pterin is very low, although the relative proportion of pterins is normal. In CSF, neopterin and biopterin as well as the neurotransmitter metabolites, 5HIAA and HVA, are also very low. The biopterin content is always slightly higher (Table 2).

PTPS Deficiency

Dihydroneopterin triphosphate cannot be converted to 6-pyruvoyl-tetrahydropterin, resulting in an accumulation of dihydroneopterin triphosphate in the tissue of patients with this defect [14]. 6-Pyruvoyl-tetrahydropterin is readily dephosphorylated by pyrophosphatase and excreted as dihydroneopterin and its oxidation product, neopterin. High concentrations of neopterin, monapterin (an isomer of neopterin), and 3’-hydroxysepiapterin, and only traces of biopterin, are found in the urine of patients with PTPS deficiency. These patients present with the highest urinary neopterin levels, and the ratio of neopterin to biopterin is the highest among BH₄ deficiencies. With regard to HPA, phenylalanine concentrations in plasma are generally around 1200 µM (Table 2).

In the severe (typical) form of PTPS deficiency, the neurotransmitter status is similar to that in patients with GTPCH deficiency. In mild (peripheral/partial) forms of PTPS deficiency, neopterin levels are almost as high as in the typical form, both in urine and in CSF, and the biopterin level is slightly higher but still just under the lower normal range [15]. It appears that the degree of HPA is significantly lower, with a median concentration of around 500 µM. Some infants have been recognized because of a reinvestigation of their mild HPA.

The most striking biochemical difference between the atypical and typical forms of PTPS deficiency is that in the atypical form normal levels of neurotransmitter metabolites can be detected in CSF (Table 2).

PCD Deficiency

Most patients with PCD deficiency were initially diagnosed as having mild forms of PTPS deficiency [16]. In the newborn period, they present with variably elevated phenylalanine levels. The levels may rise transiently to the 1200 to 2000 µM range. Excretion of neopterin in urine is increased, the biopterin level is in the subnormal range, and CSF pterin and neurotransmitter metabolite levels are normal [17]. The most striking finding is the occurrence of primapterin (7-biopterin) in the urine of these patients [18]. About 30 to 50 percent of total biopterins are excreted in urine as primapterin (Table 2).
Table 2: Laboratory parameters found in patients with various forms of BH₄ deficiency

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Phe (blood)</th>
<th>Neo (urine)</th>
<th>Bio (urine)</th>
<th>Neo (CSF)</th>
<th>Bio (CSF)</th>
<th>5HIAA (CSF)</th>
<th>HVA (CSF)</th>
<th>5MTHF (CSF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/L</td>
<td>mmol/mol creat.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKU</td>
<td>&gt;1200</td>
<td>1.2–19.8</td>
<td>0.5–7.9</td>
<td>9–118</td>
<td>15–143</td>
<td>14–471</td>
<td>47–1174</td>
<td>n</td>
</tr>
<tr>
<td>Mild PKU</td>
<td>600–1200</td>
<td>1.2–14.5</td>
<td>0.6–5.3</td>
<td>9–118</td>
<td>15–143</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>MHPA</td>
<td>120–600</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>arGTPCH</td>
<td>120–1200</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>0.05–3.0</td>
<td>1.5–7.5</td>
<td>61–183</td>
<td>15–48</td>
<td>n</td>
</tr>
<tr>
<td>PTPS</td>
<td>250–2500</td>
<td>5.0–51.2</td>
<td>&lt;0.5</td>
<td>47–402</td>
<td>1.0–16.0</td>
<td>5–113</td>
<td>5–223</td>
<td>n</td>
</tr>
<tr>
<td>Mild PTPS</td>
<td>240–2200</td>
<td>5.0–51.2</td>
<td>&lt;0.5</td>
<td>25–230</td>
<td>13–56</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>PCD</td>
<td>180–120</td>
<td>4.1–22.5</td>
<td>0.7–1.5*</td>
<td>43–117</td>
<td>16–96</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>DHPR</td>
<td>180–2500</td>
<td>0.5–23.2</td>
<td>3.8–25.6</td>
<td>11–70</td>
<td>43–117</td>
<td>4–75</td>
<td>19–204</td>
<td>↓</td>
</tr>
<tr>
<td>Mild DHPR</td>
<td>280–600</td>
<td>0.5–23.2</td>
<td>3.8–25.6</td>
<td>11–70</td>
<td>43–117</td>
<td>21–66</td>
<td>n</td>
<td>↓-n</td>
</tr>
<tr>
<td>DRD</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>14–51</td>
<td>72–102**</td>
<td>3–15</td>
<td>49–111</td>
<td>n</td>
</tr>
<tr>
<td>Controls</td>
<td>&lt;120</td>
<td>1.0–15.5</td>
<td>0.5–7.6</td>
<td>15–35</td>
<td>20–70</td>
<td>310–1100</td>
<td>150–800</td>
<td>64–182</td>
</tr>
</tbody>
</table>

*Primapterin ↑; **7,8-dihydrobiopterin and sepiapterin ↑
DHPR Deficiency

Quinonoid dihydrobiopterin, formed from BH₄ through a hydroxylation reaction, is an extremely unstable compound and tautomerizes readily to 7,8-dihydrobiopterin [19]. Since 7,8-dihydrobiopterin is not a substrate for DHPR, patients with DHPR deficiency excrete very high amounts of total biopterin (Table 2). In most cases, the percentage of biopterin is greater than 80 percent. In addition, owing to the lack of BH₄ in these patients, there is no feedback inhibition of GTPCH and, therefore, pterin biosynthesis is generally activated. Normal to slightly increased concentrations of neopterin can be found in the urine of these patients, and there is an overlapping with PKU patients in the percentage of biopterin.

Although most DHPR-deficient patients were detected by screening the urinary pterins, some newborns can be missed if the urine is collected during treatment with a low-phenylalanine diet. Some patients might not be detected, even with normal diets.

The pterin pattern in the CSF is similar to that in the urine, and distribution of the neurotransmitter metabolites is similar to that observed in PTPS deficiency. The decrease of 5HIAA is more pronounced than that of HVA. CSF levels of 7,8-dihydrobiopterin are elevated in DHPR deficiency, and it is likely that it acts as an inhibitor of aromatic amino acid hydroxylases.

Defective folate metabolism is a consequence of DHPR deficiency because the normal enzyme helps to maintain folate in the active tetrahydro form [20].

Dopa-responsive Dystonia (adGTPCH deficiency)

The most informative biochemical investigations in patients with DRD are the measurement of pterins (neopterin and biopterin) and the neurotransmitter metabolites HVA and 5HIAA in CSF (2). Both neopterin and biopterin have been found to be significantly reduced in CSF of patients with DRD (Table 2). Although CSF levels of neopterin and biopterin are higher in DRD than in autosomal recessive GTPCH deficiency, both groups of patients can be clearly differentiated from other forms of BH₄ deficiency and from controls. CSF neopterin may be non-specifcally elevated due to viral infections. A decrease in the levels of 5HIAA is a frequent but not invariable finding, while HVA levels are mostly reduced. Again, reduction of 5HIAA and HVA is less pronounced in DRD patients than in autosomal recessive GTPCH deficiency (Table 2).

An oral loading test with phenylalanine (100 mg/kg) is frequently used when CSF is not available [21]. This test is based on the fact that, due to the partial BH₄ deficiency in the liver, under loading conditions phenylalanine hydroxylase is not able to convert phenylalanine to tyrosine at a normal rate. Profiles of plasma phenylalanine and tyrosine and
phenylalanine/tyrosine ratios are abnormal at 1, 2, 4, and 6 hours after challenge. Although this test can differentiate between asymptomatic and symptomatic gene carriers, false-positive results are possible. In fact, heterozygous carriers for phenylketonuria (PKU) show the same abnormal phenylalanine/tyrosine profiles, whereas a small number of genetically confirmed DRD subjects showed no abnormalities with this test. Additional measurement of plasma total biopterin improves the sensitivity, and blood sampling at 0, 1, 2, and 4 hours seems to be sufficient.

**SR Deficiency**

Diagnosis of SR was missed in the past probably due to the fact that these patients present without hyperphenylalaninemia and with normal urinary pterins excretion. Furthermore, normal neopterin and high biopterin and dihydrobiopterin levels in CSF were rather suggestive of dihydropteridine reductase deficiency (Table 2) [22]. Indeed, all three patients with SR deficiency were initially diagnosed with a “central” form of dihydropteridine reductase deficiency [23]. Diagnosis was misleading because patients with dihydropteridine reductase deficiency present with high biopterin and dihydrobiopterin levels in CSF.

In dihydropteridine reductase deficiency quinonoid-dihydrobiopterin, formed during hydroxylation of aromatic amino acids, can not reduce to BH₄ and the rather unstable quinonoid-dihydrobiopterin is readily isomerized to dihydrobiopterin (7,8-dihydrobiopterin). In patients with SR deficiency one would expect high pterin concentrations due to the non-enzymatic side-chain cleavage of the precursor 6-pyruvoyl-tetrahydropterin. However, due to the fact that aldose reductase (AR) and carbonyl reductase (CR) can reduce 6-pyruvoyl-tetrahydropterin to BH₄ and that these two reductases are differently expressed in different tissues, one may expect different pterin profiles in different body fluids. In CSF of patients with SR deficiency total biopterin, dihydrobiopterin, and oxidized biopterin are increased while neopterin is normal. In contrast to patients with dihydropteridine reductase deficiency, in these patients dihydrobiopterin is formed from sepiapterin in the salvage pathway by the action of CR. Due to the low activity of DHFR in the brain, dihydrobiopterin can not be reduced to BH₄ and accumulates. In contrast to patients with DRD, these patients showed more severe neurotransmitter deficiency with much lower 5HIAA and HVA levels in the CSF.

The phenylalanine loading test was positive in two patients with SR deficiency [2]. Their plasma phenylalanine concentrations remained elevated up to 6 hours after oral administration of phenylalanine (100 mg/kg), indicating impaired hydroxylation in the liver. Simultaneously, in both patients with SR deficiency plasma tyrosine remained almost unchanged during the test.
Acknowledgements

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REFERENCES:


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