Disorders of Tetrahydrobiopterin Metabolism: Experience from South India

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Abstract

Background Disorders of tetrahydrobiopterin metabolism represent a rare group of inherited neurotransmitter disorders that manifests mainly in infancy or childhood with developmental delay, neuroregression, epilepsy, movement disorders, and autonomic symptoms.

Methodology A retrospective review of genetically confirmed cases of disorders of tetrahydrobiopterin metabolism over a period of three years (Jan 2018 to Jan 2021) was performed across two paediatric neurology centres from South India.

Results A total of nine patients (M:F=4:5) fulfilled the eligibility criteria. The genetic variants detected include homozygous mutations in the QDPR (n=6), GCH1 (n=2), and PTS (n=1) genes. The median age at onset of symptoms was 6-months (range 3-78 months), while that at diagnosis was 15-months (8-120 months), resulting in a median delay in diagnosis of 9-months. The main clinical manifestations included neuroregression (89%), developmental delay (78%), dystonia (78%) and seizures (55%). Management strategies included a phenylalanine restricted diet, levodopa/carbidopa, 5-Hydroxytryptophan, and folic acid. Only, Patient-2 afforded and received BH4 supplementation at a sub-optimal dose later in the disease course. We had a median duration of follow up of 15 months (range 2-48 months). Though the biochemical response has been marked; except for patients with GTPCH deficiency, only mild clinical improvement was noted with regards to developmental milestones, seizures, or dystonia in others.

Conclusion Tetrahydrobiopterin deficiencies represent a rare yet potentially treatable cause for non-phenylketonuria hyperphenylalaninemia with better outcomes when treated early in life. Screening for disorders of biopterin metabolism in patients with hyperphenylalaninemia prevents delayed diagnosis. This study expands the genotype-phenotype spectrum of patients with disorders of tetrahydrobiopterin metabolism from South India.

Key words Tetrahydrobiopterin disorders (BH4 disorders) · Non-PKU Hyperphenylalaninemia · Aromatic amino acid hydroxylases · Sapropterin hydrochloride · Monoamine neurotransmitters

Introduction

Disorders of tetrahydrobiopterin (BH4) metabolism were initially described in the 20th century as ‘atypical’ or ‘malignant Phenylketonuria’ (PKU) in a subset of patients diagnosed to have hyperphenylalaninemia (HPA) with poor response to dietary interventions (Shintaku, 2002; Smith et al., 1975). BH4 is an essential cofactor for the enzymatic hydroxylation of three aromatic amino acids, namely phenylalanine, tryptophan and tyrosine; three isoforms of nitric oxide (NO) synthase and alkylglycerol monooxygenase (Blau et al., 2011; Longo, 2009). In addition to phenylalanine, enzymatic hydroxylation of aromatic amino acids results in the synthesis of monoamine neurotransmitters, particularly serotonin, dopamine, and its metabolites (Blau et al., 2011; Longo, 2009). Biosynthesis of BH4 occurs from guanosine triphosphate (GTP) through a three-step reaction catalyzed by the enzymes guanosine triphosphate cyclohydrolase (GTPCH), 6- pyruvoyl tetrahydropterin synthase.
Disorders of BH4 metabolism are a rare heterogeneous group of inherited neurotransmitter disorders occurring due to disruption in the biosynthesis or regeneration of BH4 (Blau et al., 2011; Longo, 2009). Pathophysiology is two-fold due to – (i) Elevated phenylalanine levels leading to accumulation of toxic metabolites such as phenylacetic acid, phenyl pyruvic acid and phenyl lactic acid resulting in neurological deterioration, and (ii) impaired monoamine neurotransmitter synthesis leading to deficiency of 5-hydroxytryptophan (5-HT), dopamine, norepinephrine and epinephrine (Blau et al., 2011; Longo, 2009). BH4 deficiency usually presents in infancy or childhood with a varied spectrum of manifestations comprising of developmental delay, cognitive impairment, extrapyramidal symptoms, oculogyric crisis, and rarely epilepsy. Diagnosis is primarily by biochemical evaluation of blood phenylalanine levels, cerebrospinal fluid neurotransmitter levels, urinary pterins levels, erythrocyte DHPR activity, and genetic confirmation of the pathogenic variants. Treatment includes management of HPA by dietary restriction, BH4 supplementation, and replacement of monoamine neurotransmitters (Blau et al., 2011; Longo, 2009; Opladen et al., 2012; Ye et al., 2013; Shintaku & Ohwada, 2013).

BH4 synthesis defects were first described by Smith et al. in 1975 (Smith et al., 1975). The incidence of BH4 defects varies geographically between 2 to 10% in patients detected to have HPA (Dhondt, 1984; Han et al., 2015). There is a paucity of literature about BH4 synthesis defects from India. Opladen et al., 2012 reported 626 patients with BH4 deficiencies worldwide, of which 2.1% of patients were from India (Opladen et al., 2012). Vykuntaraju et al., 2018 reported first case of DHPR deficiency from India (Gowda et al., 2018). The aim of this study was to describe in detail the clinical characteristics, laboratory profile, radiological profile, treatment profile, and outcome in genetically confirmed patients with BH4 deficiency from two paediatric neurology centres at south India.

Subjects and methods

Study design

This was a retrospective descriptive study done from two paediatric neurology centres; a quaternary care center for neurological disorders and a tertiary level institute for child health from south India. The case records of the patients who underwent appropriate genetic counseling and were genetically confirmed to have disorders of biopterin metabolism and under the care of the authors from January 2018 and January 2021 were included. A prior informed consent was obtained for all the patients who had undergone genetic testing and were enrolled in the study. Patients were excluded from the analysis if genetic confirmation was unavailable. The institute ethics committee has approved the study (IGICH/ACA/IEC/21/2020-21/155).

Data collection

Data about the clinical features, consanguinity, perinatal events, clinical examination, blood phenylalanine levels, electroencephalography (EEG), neuroimaging findings, genetic analysis, treatment, and outcome were collected and the pertinent data was tabulated in a Microsoft Excel worksheet. Blood phenylalanine levels were measured using electrospray ionization tandem mass spectrometry. Sequential blood phenylalanine levels from baseline, while the patient was on treatment, was also recorded. EEG was performed in the Galileo system by using the standard 10-20 system of electrode placement. Both awake and sleep records were captured. Magnetic Resonance Imaging (MRI) studies of the brain were analyzed by an independent neuroradiologist blinded to the patient’s clinical details. The sequences included T1 (axial), T2 (axial, coronal, sagittal), fluid-attenuated inversion recovery (FLAIR axial), susceptibility-weighted imaging (SWI) and diffusion-weighted imaging (DWI). Targeted exome sequencing was done to detect genetic mutations. In-house bioinformatic pipeline was employed for analysis of raw data, variant calling and annotation.

Results

A total of nine patients (4 males and 5 females) were genetically confirmed to have disorders of biopterin metabolism. Six patients had deficits in the QDPR gene (DHPR deficiency), two had mutations in GCH1 gene (GTPCH deficiency), and Patient-9 had mutation in PTS gene (PTPS deficiency). The demographic details and clinical features have been described in detail in Table 1. The biochemical profile, EEG, and neuroimaging findings are summarized in Table 2.

Clinical features (n=9)

At the onset of symptoms in our cohort, the mean age was 17.3 ± 24.3 months (range - 3 to 78 months). The mean age at diagnosis was 37 ± 42.4 months (range - 8 to 120 months). There was a median delay of 9 months in the diagnosis.
Table 1 Demographic details and clinical features of patients with disorders of tetrahydrobiopterin metabolism

| Details                          | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 | Patient 8 | Patient 9 |
|----------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Age at onset of symptoms         | 2 years   | 3 months  | 3 months  | 6 months  | 11 months | 2 years   | 4 months  | 6 years   | 3 months  |
| Age at diagnosis                 | 4 years   | 9 months  | 18 months | 15 months | 12 months | 10 years  | 8 months  | 8 years   | 8 months  |
| Gender                           | Female    | Female    | Male      | Male      | Female    | Female    | Female    | Male      | Male      |
| Initial Manifestations           | Ataxia    | Delayed milestones | Neuroregression with dystonia and seizures | Neuroregression with seizures | Neuroregression with seizures | Neuroregression with dystonia | Delayed milestones | Abnormal twisting postures of both lower limbs | Delayed milestones |
| Clinical features                | Developmental delay, dystonia and ataxia since 2 years of age | Developmental delay, dystonia at 9-months, epileptic spasms followed by regression | Regression of milestones at 6 months of age, seizures, recurrent respiratory tract infections | Regression of milestones with seizures at 11-months of age, irritability and constipation | Regression of milestones on the background of developmental delay, dystonia since 2-years of age | Developmental delay, irritability, oculogyric crisis, dystonia and choreoathetoid movements since 4-months | Dystonia of lower limb since 6.5 years and upper limbs since 7.5 years of age | Developmental delay at 3-months of age (incidentally detected in newborn period by neonatal screening) |
| Developmental delay              | +         | +         | +         | +         | -         | +         | +         | -         | +         |
| Regression of milestones         | +         | +         | +         | +         | +         | +         | +         | -         | +         |
| Seizures                         | -         | +         | +         | +         | +         | -         | -         | +         | -         |
| Behavioural problems             | -         | -         | -         | -         | -         | +         | +         | -         | -         |
| Oculogyric crisis                | -         | -         | -         | -         | -         | +         | +         | -         | -         |
| Dystonia                         | +         | +         | +         | +         | -         | +         | +         | +         | +         |
| Ataxia                           | +         | -         | -         | -         | -         | -         | -         | -         | -         |
| Consanguinity Examination        | Yes       | Yes       | Yes       | Yes       | No        | Yes       | Yes       | Yes       | Yes       |
| Microcephaly                     | No        | No        | Yes       | No        | No        | No        | Yes       | No        | Yes       |
| Skin and hair findings           | -         | -         | Café – au – lait spots | -         | -         | -         | -         | -         | Hypopigmented skin, hair |
| Ocular findings                  | -         | -         | -         | -         | Oculogyric crisis | -         | Oculogyric crisis | -         | -         |
| Tone                             | Variable  | Variable  | Variable  | Hypotonia | Hypotonia | Variable  | Variable  | Variable  | Variable  |
| Extrapyramidal features          | Dystonia  | Dystonia  | Dystonia  | Hypotonia | Hypotonia | Dystonia  | Dystonia  | Dystonia  | Dystonia  |
**Table 2** Biochemical and radiological pattern in patients with disorders of tetrahydrobiopterin metabolism

| Details                                              | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 | Patient 8 | Patient 9 |
|------------------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Blood Phenylalanine levels before starting treatment (20-150 μmol/l) | 287.2     | 340       | 450       | 676       | 495       | 118       | 300       | 69.2      | 640       |
| Blood Phenylalanine tyrosine ratio (cut off 1.5)     | 21.26     | 9.70      | 7.02      | 6.56      | 14.6      | 3.6       | 18.97     | 1.1       | 18.97     |
| EEG                                                  | Normal    | Multifocal interictal epileptiform discharges | Hypsarrhythmia | Normal | Sleep activated near continuous spike-wave discharges | Normal | Normal | Not done | Normal |
| MRI brain                                            | Normal    | Normal    | Normal    | Delayed myelination | T2 hyperintensity and diffusion restriction of central tegmental tracts | Subcortical T2 hyperintensities with cerebral and cerebellar atrophy | Calcification in bilateral lentiform nuclei, thalami, cerebellar hemispheres and frontal, parietal, temporal and occipital white matter | Normal | Normal | Delayed myelination |
| Mutational analysis                                   | QDPR gene Exon 5 c.488G > T (p.Ser163lle) | QDPR gene Exon 1 c.68G>A (p.Gly23Asp) | QDPR gene Intron 3 c.295+5G>T (5’ splice site) | QDPR gene Exon 7 c.680T>C (p.Leu227Pro) | QDPR gene Intron 3 c.296-1G>T (3’ splice site) | QDPR gene Exon 7 c.635T>C (p.Phe212Ser) | GCH1 gene Exon 6 c.703C>G (p.Arg235Gly) | GCH1 gene Exon 3 c.457C>T (p.His153Tyr) | PTS gene Exon 1 c.65C>G (p.Ala22Gly) |
| Additional variants                                  | -         | -         | -         | -         | -         | -         | -         | -         | -         |
| Diagnosis                                            | DHPR deficiency | DHPR deficiency | DHPR deficiency with Neurofibromatosis-1 | DHPR deficiency with Primary ciliary dyskinesia-22 | DHPR deficiency | DHPR deficiency | GTPCH deficiency | GTPCH deficiency | PTPS deficiency |

EEG- electroencephalography, MRI- Magnetic Resonance Imaging, DHPR- dihydropteridine reductase, GTPCH - GTP cyclohydrolase I, PTPS- 6-pyruvoyl-tetrahydropterin synthase
QDPR gene mutation (DHPR deficiency) (n=6) Six patients (2 males and 4 Females) had mutations in the QDPR gene (DHPR deficiency), with the mean age at onset of symptoms being 11.8 ± 9.8 months (range 3 to 24 months). Clinical features in these patients included developmental delay (5/6), regression of milestones (6/6), dystonia (4/6), and ataxia (1/6). Consanguinity was present in (5/6) patients. On examination, microcephaly was seen in (2/6) patients, generalized dystonia in (4/6) patients, generalized hypotonia in two patients and oculogyric crisis in one patient. Patient-3 had multiple café-au-lait spots in the body. Patient 4 had a history of recurrent upper respiratory tract infections.

GCH1 gene mutation (GTPCH deficiency) (n=2) Patients-7 and 8 had mutations in the GCH1 gene. Patient-7 was an eight-month-old girl who presented with developmental delay, oculogyric crisis, intermittent dystonia, and hypotonia from 4-months of age. On examination, she had microcephaly, intermittent oculogyric crisis, and dystonia. Patient-8 was an eight-year-old boy who had presented with toe walking and abnormal posturing of both feet while walking since 6.5 years of age, tremulousness, and posturing of both upper limbs since 7.5 years of age with a diurnal variation. On examination, he predominantly had foot dystonia with preserved reflexes.

PTPS gene mutation (PTPS deficiency) (n=1) Patient-9 was incidentally detected to have increased phenylalanine levels on newborn screening. He was observed to have delayed developmental milestones at around 3-months of age. Later developed dystonia and had myoclonic epilepsy at 6-months. On examination, he had hypopigmented skin and hair, hypotonia, and generalized dystonia. Parents were unable to start on a phenylalanine restricted diet due to financial constraints.

Biochemical findings (n=9)

Before the treatment, in patients with DHPR deficiency, the mean and the median levels of blood phenylalanine was 394.33 ± 191.5 μmol/L and 395 μmol/L respectively (range: 287 - 676 μmol/L). The blood phenylalanine tyrosine ratio (Phe/Tyr ratio) was increased in patients with DHPR deficiency with a mean and median of 10.45 ± 6.45 and 8.36 respectively (range: 3.6 to 21.26). Patient-6 with DHPR deficiency had normal phenylalanine levels when evaluated here at the age of 10 years. However, Phe/Tyr ratio was elevated to 3.5 (cut-off being 1.5) (Eastman et al., 2000) which gave rise to a suspicion of HPA. In patients with GTPCH deficiency (Patients-7,8) and PTPS deficiency (Patient-9) serum phenylalanine levels were 300, 69, and 640 μmol/L, and the P/T ratio was 18.97, 1.1, and 18.97, respectively. Oral BH4 loading test, urinary pterins, CSF neurotransmitter analysis, CSF 5-methyltetrahydrofolate analysis and blood erythrocyte DHPR activity were not performed due to financial constraints, transportation difficulties, and non-availability in India.

Electroencephalography (EEG) findings(n=8)

Eight patients in the study, six with DHPR deficiency, one with PTPS deficiency, and one with GTPCH deficiency had undergone EEG to detect clinical and sub-clinical seizures. EEG was normal in 5/8 patients. EEG was abnormal in patient-2, 3 and 5. Patient-2 had multifocal inter-ictal epileptiform discharges (IEDs), patient-3 had classical hysparhythmia and patient-5 had sleep accentuated, near-continuous, generalized (fronto-central dominant) spike/polyspike wave discharges of 1-2 Hz with a marked reduction in IEDs on awakening consistent with electrical status epilepticus during slow-wave sleep (SESES) (Fig. 1A-C).

Neuroimaging findings (n=9)

MRI brain was normal in 4/9 patients, two patients each with DHPR deficiency (P-1,2) and GTPCH deficiency (P-7,8). MRI findings were consistent with delayed myelination in two patients, one each of DHPR deficiency (P-3) and PTPS deficiency (P-9). Patient-4 with DHPR deficiency had T2 hyperintensity of bilateral central segmental tracts with diffusion restriction (Fig. 2A, B & C). Patient-5 with DHPR deficiency had T2 hyperintensities in the subcortical white matter of both frontal, parietal and occipital lobes with partial inversion on FLAIR (Fig. 2D, E & F). Patient-6 with DHPR deficiency had bilateral symmetrical calcification in the lentiform nuclei, anterolateral thalami, and subcortical white matter of all the lobes with an occipital preponderance (Fig. 2G, H, I). Calcification was also noted in the white matter of both cerebellar hemispheres.

Mutational analysis (n=9)

We detected nine different variations in the three genes related to BH4 deficiency among the cases studied (Table 3). Pathogenic/likely pathogenic (P/LP) variants were identified in all 9 cases according to the American College of Medical Genetics and Genomics (ACMG) criteria (Richards et al., 2015). All the variations identified were in the homozygous state, among which seven were missense, and two were splice site variations. QDPR gene variations were seen in six cases - six of these predicted to be P/LP (c.68G>A; p.Gly23Asp, c.296-1G>T, c.488G>T; p.Ser163Ile, c.635T>C; p.Phe212Ser, c.680T>C; p.Leu227Pro and c.295+5G>T). Three of these variants were novel (c.296-1G>T, c.295+5G>T and c.680T>C) and...
remaining three were known. Among the three known variants, c.68G>A is reported in Clinvar (RCV000000519.2) and Human Gene Mutation Database (HGMD) (CM930632) database, while c.457C>T and c.488G>T have been reported in HGMD. Two different GCH1 gene variations (c.703C>G; p.Arg235Gly and c.457C>T; p.His153Tyr) were observed in 2 cases, both were likely pathogenic and known. Further, both these variants were localized in the GTP cyclohydrolase I domain of the GCH1 protein. PTS gene variation (c.65C>G; p.Ala22Gly) was observed in one case which was classified as likely pathogenic. The observed variation lies in the 6-pyruvoyl tetrahydropterin synthase domain of the PTS protein and has been reported in HGMD database (CM064184). A schematic diagram of the genes - QDPR, GCH1 and PTS, and the mutations found in this study is shown in Fig. 3. Patient-3 with multiple café-au-lait spots had an additional heterozygous LP missense variant c.2288T>C on exon 19 of the NF1 gene (chr17:g.31227254T>C; Depth: 214x) that results in the amino acid substitution of Proline for Leucine at codon 763 (p.Leu763Pro; ENST00000358273.9). Patient-4 with recurrent respiratory tract infections had an additional pathogenic homozygous 5’ splice site variant in intron 11 of the ZMYND10 gene (chr3:g.50379003A>C; Depth: 84x) that affects the invariant GT donor splice site downstream of exon 11 (c.1247+2T>G; ENST00000231749.3).

Treatment and outcome (n=9)

The treatment and follow-up details have been summarized in Table 4. All the patients in the study were treated with levodopa/carbidopa, folinic acid and low phenylalanine diet. Patient-6 and Patient-8 with normal levels of PHA didn’t receive a low phenylalanine diet. Five patients (4 with DHPR deficiency and Patient-7 with GTPCH deficiency) received 5-hydroxytryptophan (5-HTP) at a median dose of 3mg/kg/day. Patient-2 with DHPR deficiency could access BH4 and received it at 2mg/kg/day at a sub-optimal dose later in the disease course. For seizures, patients were treated with levetiracetam, clobazam, valproate, and topiramate. Patient-5 with epileptic encephalopathy showed moderate clinical response to a course of pulse intravenous methylprednisolone with complete electrographic resolution (Fig. 1D). Patients additionally received trihexyphenidyl and...
tetrabenazine for dystonia. The mean and median duration of follow-up of the study cohort was 19.8 ± 15.5 months and 15-months (range 2-months to 4-years). There was variable clinical response in terms of improvement in developmental milestones, reduction in dystonia, and seizures, as described in Table 4. Significant improvement in milestones and complete cessation of seizures was noted in Patient-4 with DHPR deficiency. In Patient-7, administration of levodopa at 1 mg/kg/day resulted in dyskinesias that disappeared with reduction of dose to 0.5 mg/kg/day. This child showed significant motor improvement with low-dose levodopa as well as folinic acid. Folinic acid is given to replete CSF 5-methyltetrahydrofolate levels that might become low as a result of levodopa/carbidopa supplementation (Opladen et al., 2012; Opladen et al., 2011). Patient-8 with GTPCH deficiency had significant alleviation of dystonia with a small dose of levodopa, thus conforming to the criteria of dopa-responsive dystonia. Biochemically, in patients with DHPR deficiency, there was reduction in the phenylalanine levels with mean and median blood levels of 120.6 ± 96.4 μmol/L and 103.3 μmol/L (range 20 to 269.7 μmol/l) respectively at a median follow up of 13.5 months. Patient-2 with DHPR deficiency who was treated with BH4 supplementation, had lower blood phenylalanine levels 20 μmol/l at a follow-up duration of 27 months. In Patient-7 with GTPCH deficiency and Patient-9 with PTPS deficiency the blood phenylalanine levels were 350 and 200 μmol/L at a follow-up duration of 40 and 16-months, respectively. As blood phenylalanine levels were normal at baseline in Patient-6 and 8 repeat analysis wasn’t done. The mean and median development quotient of patients in our study at last follow up was 44.8 ± 28.9 and 30 (range 10 to 100). Repeat neuro-imaging to look for any radiological response was not done in any of our patients.
### Table 3: Detailed description of mutational analysis of patients with disorders of tetrahydrobiopterin metabolism

| Proband | Gene (Location) | Genomic coordinate | Variant | Zygosity | Constraint Scores | Population Frequency (gnomAD, 1kG, 100kGenomeAsia) | Functional prediction | Splicing prediction | Conservation | ACMG classification |
|---------|-----------------|-------------------|---------|----------|-------------------|--------------------------------------------------|----------------------|-------------------|--------------|---------------------|
| Patient 1 QDPR (Exon 5) | chr4:17493912C>G | c.488G>T (p.Ser163Ile) | Homozygous | pLI=0 Z=0.28 | Absent | 11 deleterious (Bayes-Del_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LISTS2, M-CAP, MVP, MutationAssessor, MutationTaster and SIFT) | No impact | Conserved (PhyloP:7.713, GERP++:5.26) | Likely Pathogenic |
| Patient 2 QDPR (Exon 1) | chr4:17513610C>G | c.68G>A (p.Gly23Asp) | Homozygous | pLI=0 Z=0.28 | Absent | 12 deleterious (Bayes-Del_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LISTS2, M-CAP, MVP, MutationAssessor, MutationTaster, PrimateAI and SIFT) | No impact | Conserved (PhyloP: 5.494, GERP++:4.01) | Pathogenic |
| Patient 3 QDPR (Intron 3) | chr4:17505997C>G | c.295+5G>T (5' splice site) | Homozygous | pLI=0 Z=0.28 | Absent | Predicted Splice Donor loss (GeneSplicer, MaxEntScan, NNSplice, PWM) | | | | Likely Pathogenic |
Table 3: (continued)

| Proband | Gene (Location) | Genomic coordinate | Variant | Zygosity | Constraint Scores | Population Frequency (gnomAD, 1kG, 100kGenom-eAsia) | Functional prediction Splicing prediction | Conservation | ACMG classification |
|---------|----------------|--------------------|---------|----------|-------------------|------------------------------------------------------|-------------------------------------------------|-------------|---------------------|
| Patient 4 | QDPR (Exon 7) | chr4:17488809A>G | c.680T>C (p.Leu227Pro) | Homozygous | pLI=0 Z=0.28 | Absent | 9 deleterious (Bayes-Del_addAF, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, MVP, MutationAssessor, MutationTaster and SIFT) vs 2 benign (DANN and PrimateAI) | No impact | Conserved (PhyloP:7.713, GERP++:5.26) | Pathogenic |
| Patient 5 | QDPR (Intron 3) | chr4:17503483C>A | c.296-1G>T (3' splice site) | Homozygous | pLI=0 Z=0.28 | Absent | Predicted splice acceptor loss, causes frameshift (GeneSplicer, MaxEntScan) VarSEAK: Loss of function for authentic Splice Site. Use of cryptic site 11 nt downstream of 3' ss | Likely Pathogenic | | |
| Proband | Gene (Location) | Genomic coordinate | Variant | Zygosity | Constraint Scores | Population Frequency (gnomAD, 1kG, 100kGenomeAsia) | Functional prediction | Splicing prediction | Conservation | ACMG classification |
|---------|----------------|-------------------|---------|----------|-----------------|-------------------------------------------------|---------------------|-------------------|--------------|---------------------|
| Patient 6 | QDPR (Exon 7) | chr4:17488854A>G | c.635T>C (p.Phe212Ser) | Homozygous | pLI=0 Z=0.28 | Absent | 10 deleterious (Bayes-Del_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, M-CAP, MVP, Mutation-Assessor, Mutation-Taster and SIFT) vs 2 benign (LIST-S2 and PrimateAI) | No impact | Conserved (PhyloP:7.713, GERP++:5.15) | Likely Pathogenic |
| Patient 7 | GCH1 (Exon 6) | chr14:5531075G>C | c.703C>G (p.Arg235Gly) | Homozygous | pLI=0.9 Z=1.52 | Absent | 11 deleterious (Bayes-Del_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MVP, Mutation-Assessor, Mutation-Taster and SIFT) vs 1 benign (PrimateAI) | No impact | Conserved (PhyloP:4.254, GERP++:4.989) | Likely Pathogenic |
Table 3: (continued)

| Proband | Gene (Location) | Genomic coordinate | Variant | Zygosity | Constraint Scores | Population Frequency (gnomAD, 1kG, 100kGenomeAsia) | Functional prediction | Splicing prediction | Conservation | ACMG classification |
|---------|-----------------|-------------------|---------|----------|------------------|-------------------------------------------|-----------------------|-------------------|--------------|----------------------|
| Patient 8 | GCH1 (Exon 3)   | chr14:55326451G>A | c.457C>T (p.His153Tyr) | Homozygous | pLI=0.9 Z=1.52 | Absent | 12 deleterious (Bayes-Del_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MVP, MutationAssessor, MutationTaster, PrimateAI and SIFT) | No impact | Conserved (PhyloP:9.2, GERP++:5.099) | Pathogenic |
| Patient 9 | PTS (Exon 1)    | chr11:11209723C>G | c.65C>G (p.Ala22Gly) | Homozygous | pLI= 0 Z= 0.13 | Absent | 11 deleterious (Bayes-Del_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MVP, MutationAssessor, MutationTaster, SIFT) vs 1 benign (PrimateAI) | No impact | Conserved (PhyloP:4.480, GERP++:5.059) | Pathogenic |

HGMD- Human Gene Mutation Database
Discussion

In the present study, we have described the clinical features, investigations, treatment, and outcome of 9 genetically confirmed patients with BH4 deficiency from South India. The disorders of BH4 metabolism are broadly classified into six types based on the specific enzyme defects into autosomal recessive (AR) or autosomal dominant (AD) GTPCH deficiency, PTPS deficiency, DHPR deficiency, SR deficiency, and PCD deficiency. PTPS deficiency is the commonest cause of BH4 deficiency (56.7%), followed by DHPR deficiency (34.7%), GTPCH deficiency (4.9%) and PCD deficiency (3.7%) (Opladen et al., 2012; Opladen et al., 2020). SR and PCD deficiencies are mild and rarely described.

Most of the reports on disorders of BH4 metabolism are from western literature, Japan, and China, where universal newborn screening for PKU is practiced (Opladen et al., 1984; Han et al., 2015). Opladen et al., 2012 have described the largest cohort of 626 patients with BH4 deficiency from the BIODEF database (Opladen et al., 2012). Unlike previous studies, most of the patients in our cohort belonged to DHPR deficiency (66.6%), followed by AR GTPCH deficiency (22.2%) and PTPS deficiency (11.1%). This discrepancy could be because of the referral bias as ours was a tertiary/quaternary health care centre. All our patients were diagnosed when symptomatic except one who was detected on newborn screening. Our patients had a median age at onset of symptoms of 6 months and were diagnosed around 15 months (8 months – 120 months) which is delayed as compared to previous studies (Ye et al., 2013). Though there is a glaring biochemical difference among the various BH4 deficiencies clinical features overlap in most and can mimic cerebral palsy, extrapyramidal disorder or genetic epilepsies. The cardinal clinical features described include hypotonia with developmental delay, cognitive impairment, seizures,
autonomic disturbances, and movement disorders, mainly dystonia, oculogyric crisis, dyskinesias, and early-onset Parkinsonism (Blau et al., 2011; Longo, 2009; Opladen et al., 2012). Other less-described symptoms are swallowing difficulties, hypersalivation, sleep disturbances, psychological issues, prematurity, low birth weight, and central hypothyroidism (Werner et al., 2011). Hypopigmented skin and hair, characteristic musty body odor, and eczema are few systemic findings (Lee et al., 2006). In our cohort, neuroregression (89%) was the most common presentation, followed by developmental delay (77.7%), dystonia (77.7%), seizures (55.5%), and behavioral problems (22.2%). Extrapyramidal symptoms dominated in patients with GTPCH deficiency, seizures in DHPR and PTPS deficiency, whereas regression and developmental delay was present across the cohort. Ataxia was observed in Patient-1. Consanguinity was high and noted in 89%. Microcephaly (33%), oculogyric crisis (22%), hypotonia (22%), and dystonia (77.7%) were remarkable examination findings. Microcephaly is commonly reported in patients with PTPS and DHPR deficiency, while

| Table 4 | Treatment and follow up of patients with disorders of tetrahydrobiopterin metabolism |
|---|---|---|---|---|---|---|---|---|---|
| Details | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 | Patient 8 | Patient 9 |
| Treatment | Low phenylalanine diet | Yes | Yes | Yes | Yes | Yes | No | Yes | No | Yes |
| BH4 (mg/kg/day) | No | Yes, 2 mg/kg/day | No | No | No | No | No | No | No |
| Levodopa-carbidopa (mg/kg/day) | 3 mg/kg | 3 mg/kg | 3 mg/kg | 5 mg/kg | 3 mg/kg | 10 mg/kg | 1 mg/kg | 6 mg/kg | 3 mg/kg |
| 5-HTP (mg/kg/day) | No | 3 mg/kg | No | 5 mg/kg | 2 mg/kg | 1 mg/kg | 3 mg/kg | No | No |
| Folinic acid (mg/day) | 15 mg | 15 mg | 15 mg | 10 mg | 10 mg | 15 mg | 10 mg | 15 mg |
| Follow up | | | | | | | | | |
| Follow up duration in months | 48 | 27 | 6 | 2 | 15 | 12 | 40 | 12 | 16 |
| Improvement of the delay in developmental milestones | Complete | Minimal | Minimal | Significant | Minimal | No | Significant | Baseline milestones were normal | Minimal |
| Improvement in dystonia | Yes, mild improvement | Yes, mild improvement | Yes, mild improvement | N/A | N/A | Yes, mild improvement | Yes, significant improvement | Yes, significant improvement | Yes, mild improvement |
| Improvement in seizures | N/A | No, seizures remained drug refractory | No, seizures remained drug refractory | Yes, complete cessation of seizures | N/A | N/A | N/A | N/A | Yes, mild reduction in seizure frequency |
| Serum phenylalanine levels in last follow up (μmol/l) | 150 | 20 | 60 | 269.7 | 103.3 | Not repeated as baseline was normal | 350 | Not repeated as baseline was normal | 200 |
| Development quotient (DQ) at last follow up | 70 | 30 | 30 | 41.1 | 22.2 | 10 | 70 | 100 | 30 |

BH4- tetrahydrobiopterin, 5-HTP- 5 hydroxytryptophan, N/A- Not applicable
it is uncommon in GTPCH deficiency (Blau et al., 1996a). In our study, the patients with PTPS and GTPCH deficiency as well as one patient with DHPR deficiency, reportedly had microcephaly. Patients with PKU or BH4 deficiencies can have decreased skin and hair pigmentation due to reduced levels of tyrosine and competitive inhibition of tyrosine uptake by phenylalanine (Farishian & Whittaker, 1980). But skin and hair changes were not that prominent in our cohort (11%), probably as blood phenylalanine levels were not markedly elevated. Prematurity, low birth weight, musty odor and autonomic nervous system symptoms was not present in any of the patients in our cohort.

Hyperphenylalaninemia (HPA) is classically defined as plasma phenylalanine levels greater than 120 μmol/l (2mg/dl) (Regier & Greene, 2000). HPA can result either from a deficiency of the enzyme phenylalanine hydroxylase or its cofactor BH4. Disorders of BH4 metabolism are rare and contribute to around 2% of the cases detected to have HPA (Regier & Greene, 2000). HPA is seen in almost all the subtypes of BH4 deficiency except in AD-GTPCH and SR deficiency. Only mild HPA was detected in our cohort, with highest blood phenylalanine levels pre-treatment being 676 μmol/l. Patient-6 with DHPR deficiency had an increased Phe/tyr of 3.5 with borderline baseline phenylalanine levels suggesting HPA. Surprisingly, Patient-8 with AR-GTPCH deficiency didn’t have HPA (Horvath et al., 2008). Ideal investigation after detecting HPA is to assess blood or urinary pterins (bioppterin, neopterin, sepiapterin), CSF analysis of neurotransmitters [5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA)], pterins and 5-MTHFR and erythrocyte DHPR activity which will help in diagnostic confirmation and also in differentiating among the various subtypes of BH4 deficiencies (Opladen et al., 2020). The presence of HPA with normal BH4 cofactor metabolites suggests PKU and excludes BH4 disorders. Unfortunately, as ours is a resource-limited setting, the above-mentioned biochemical tests could not be performed in our study.

EEG changes previously described in the literature are non-specific (Opladen et al., 2012; Ye et al., 2013). Three patients in our cohort with DHPR deficiency had abnormal EEG findings, Patient-2 had multifocal epileptiform discharges, Patient-3 had classical hypersarrhythmia, and Patient-5 had an EEG consistent with ESES. Drug refractory epilepsy was observed in all three cases with abnormal EEG. Neuroimaging findings in BH4 deficiencies are indeterminate and not routinely recommended for diagnosis (Opladen et al., 2020). In the cohort by Ye et al., 2012 cerebral MRI abnormalities were seen in nearly 43% and mainly showed cerebral white matter changes (Ye et al., 2013). Imaging changes are commonly described in patients with DHPR and PTPS deficiency. Similar to our cohort, the imaging findings described include delayed myelination, signal changes in parieto-occipital white matter, cerebral atrophy, and central tegmental tract (CTT) hyperintensities (Kuseyri Hübschmann et al., 2021; Chien et al., 2001). Intracranial calcifications like in our Patient-6 with DHPR deficiency, mainly in basal ganglia and subcortical regions have been previously described in patients with DHPR deficiency and in only one patient with PTPS deficiency (Wang et al., 2006). Intracranial calcifications have been hypothesized to be secondary to cerebral folate depletion in DHPR deficiency. Other imaging findings in BH4 deficiency include subcortical cyst-like lesions on T1 weighted images (Chien et al., 2001). However, no correlation has been observed between the imaging features and degree of neurological impairment (Wang et al., 2006). A total of 11 genetic variants were identified in our cohort with 9 P/LP variants related to BH4 disorders: 6 in the QDPR gene, 2 in the GCH gene, and 1 in the PTPS gene. Most of the variants have been previously reported in ClinVar or HGMD except three novel variants of QDPR gene c.296-1G>T, c.295+5G>T, and c.680T>C.

The earlier the initiation of treatment in BH4 disorders, the better is the neurodevelopmental outcome (Opladen et al., 2012; Ye et al., 2013). The main target of treatment is to lower the plasma phenylalanine levels by low phenylalanine diet in conjunction with BH4 supplementation and replacement of monoamine neurotransmitters, namely L-Dopamine and 5-HT. Sapropterin treatment can be an alternative to phenylalanine-reduced diet in BH4 deficient patients except for DHPR deficiency (Opladen et al., 2020). FDA first approved sapropterin hydrochloride, synthetic analogue of BH4 in 2007 for the treatment of BH4 responsive HPA (Shintaku & Ohwada, 2013). Oral BH4 at therapeutic dose doesn’t cross blood-brain barrier and is mainly involved in lowering peripheral blood phenylalanine levels. However, oral BH4 is expensive, and most of our patients didn’t receive it due to non-affordability and non-availability in India. Only one of our patients could access BH4 and received it at a suboptimal dose of 2mg/kg/day later in the disease course. The actual recommended dose of BH4 is around 5-10mg/kg/day. Sometimes a higher dose of BH4 (up to 20 mg/kg/day) is required to maintain optimal CSF BH4 levels (Opladen et al., 2020; Winn et al., 2016). Among patients with DHPR deficiency, traditionally, treatment with only a low phenylalanine diet and neurotransmitter replacement has been advocated with the controversial role of BH4 as the defect is mainly in BH4 recycling. This is because a very high dose of BH4 (>20mg/kg) will be required if BH4 is solely used for the treatment of HPA in DHPR deficiency. This leads to an excess of BH2, which leads to inhibition of aromatic acid hydroxylases, NO uncoupling resulting in oxidative stress and neurotoxicity (Crabtree et al., 2009). Nonetheless, Coughlin et al., 2013 have reported clinical improvement in a case of DHPR deficiency with oral supplementation of BH4 (up to 40mg/kg) along with levodopa.
and 5-HT (Coughlin 2nd et al., 2013). Patient-2 with DHPR deficiency who received BH4 at 2mg/kg/day had no adverse effects, and the dose was inadequate for any significant clinical improvement.

Levodopa in combination with peripheral decarboxylase inhibitor (carbidopa/benserazide) is recommended at a dose of 3-7 mg/kg/day (GTPCH deficiency) and up to 10 mg/kg/day in rest of the BH4 disorders starting at a low dose of 0.5-1mg/kg/day (Opladen et al., 2020). All the patients in our cohort received levodopa/carbidopa at a median dose of 3mg/kg/day (range 1-10mg/kg). 5-HT starting at a dose of 1-2 mg/kg/day (target dose 5mg/kg/day) is recommended in all BH4 disorders except AD-GTPCH and PCD deficiency (Opladen et al., 2020). Due to affordability issues, only five patients in our cohort received levodopa at a median dose of 3 mg/kg/day (range 1-5mg/kg). Folinic acid (10-20mg/day) is beneficial in patients with DHPR deficiency as it leads to secondary cerebral folate depletion and in cases with low CSF 5-MTHFR (Opladen et al., 2020). Owing to the lack of facility for monitoring CSF 5-MTHFR, all the patients in our study received folinic acid at a dose of 10-15mg/day. Additionally, children received symptomatic management for seizures and dystonia. Medication-related adverse events were not observed in our patients, except Patient-7 with GTPCH had mild dyskinesias with L-Dopa. Ideally, monitoring of treatment by blood/plasma phenylalanine levels, CSF HVA, 5-HIAA, and 5-MTHFR, and titration of dosage of BH4, levodopa and 5-HT is essential to ensure optimal level of neurotransmitters required for brain development (Opladen et al., 2020). Prolactin is another indirect marker used for assessing the effectiveness of levodopa in order to avoid invasive lumbar puncture (Opladen et al., 2020; Ng et al., 2015). Selective monoamine oxidase inhibitors, dopamine agonists, catechol-o-methyltransferase inhibitors, selective serotonin reuptake inhibitors are the next-line agents suggested for the treatment of BH4 disorders (Opladen et al., 2020).

We had a median duration of follow-up of 15 months in our study, with a median reduction of blood phenylalanine levels by 190 μmol/l. Though the biochemical response has been marked, except for patients with GTPCH deficiency, only mild clinical improvement was noted with regards to developmental milestones, seizures, or dystonia. Developmental quotient at follow-up in 8/9 patients was < 70 suggesting severe developmental delay. This finding is in line with other studies wherein plasma phenylalanine levels did not correlate with the developmental quotient. Instead, developmental quotient inversely correlated with the age of initiation of treatment (Ye et al., 2013; Wang et al., 2006). Irreversible neuronal damage (basal ganglia calcification, neuronal loss) in DHPR deficiency occurs at a very early age that fails to amend with therapy. Hence, detection of the disorder within the first month of life will ensure a good neurological prognosis (Jäggi et al., 2008). Patients who were initiated on treatment at an early age; had a higher incidence of better neurological outcomes with fewer children having mental retardation, hypotonia, or seizures (Opladen et al., 2012; Jäggi et al., 2008). However, few patients develop severe phenotypes despite initiation of treatment at an early age (Ponzone et al., 2004). Children with later age of onset of therapy displayed mental retardation, aggressive behavior, irritability, and autistic features, despite improvement in IQ (Wang et al., 2006; Manzoni et al., 2020). Moreover, patients with earlier age of onset of treatment had achieved normal IQ. Although our study comprised only nine patients, the mean development quotient of 44.8 ± 28.9 must be interpreted with caution. Yet, it is much lower than the development quotient observed after three years of treatment in another study (78±15) (Wang et al., 2006).

Our data indicate that patients in our follow-up have much worse neurological outcomes than reported in previous studies (Manzoni et al., 2020). There are two factors leading to this wide discrepancy. Firstly, these studies were done in countries where newborn screening for these disorders has been implemented with good coverage, resulting in detection in the neonatal period, leading to early initiation of treatment. Secondly, most of the patients were treated with BH4 supplements, unlike our patients, hence affirming the fact that BH4 is indispensable in the treatment for BH4 deficiency. However, these factors may not hold true for all patients. Patient-1 diagnosed and started on treatment at the age of 4 years improved tremendously, while Patient-2 and Patient-5 diagnosed and started on earlier treatment has poor developmental outcome, with drug refractory seizures (Jäggi et al., 2008). The overall outcome of these patients with BH4 deficiency is tremendously conditioned by the age of initiation of treatment and the adequacy of the dose of the medication. Folinic acid administration mainly plays a very significant role in the developmental outcome (Ponzone et al., 2004). Patients who have developed severe developmental delay and low CSF HVA and HIAA values before starting treatment are unlikely to make complete recovery even with the best medical management (Jäggi et al., 2008). The lack of wide accessibility to pterin and CSF neurotransmitter analysis makes monitoring treatment highly dependent on clinical assessment, which may not give sufficient evidence for dosage optimization, thereby leading to subtle progressive brain damage (Lee et al., 2006; Wang et al., 2006). In addition to serum phenylalanine, regular monitoring of serum prolactin may remove impediments to a certain extent in this regard.

Management of BH4 deficiency in India poses a significant challenge. The non-availability of diagnostics and lack of national neonatal screening programs for the detection of these disorders is compounded by the lack of BH4
supplements for the diagnosed patients (Verma et al., 2015). Universal newborn screening for HPA in conjunction with BH4 cofactor metabolites is essential since 25% of children with PTPS and GTPCH deficiency and 40% of children with DHPR deficiency are asymptomatic in the newborn period and remain asymptomatic until 3 months of age (Opladen et al., 2012; Blau et al., 1996b; Opladen et al., 2016). The newborn screening may, in fact, be normal on day one of life, and hence a repeat testing is essential later after day-3 of life (Manzoni et al., 2020). The first step towards inclusion into the newborn screening programme in India, as well as the availability of BH4 supplements, will pave the way for earlier identification and better neurodevelopmental outcome for Indian children with this rare yet treatable neurotransmitter disorder.

To the best of our knowledge, this is the first Indian study described on a cohort of genetically confirmed patients with BH4 deficiency. Strengths of the study include strict inclusion of only genetically confirmed cases of disorders of biotinidase metabolism, detailed description of clinical features, investigations, treatment, and outcome. Limitations include the retrospective nature with non-uniform data and follow-up, lack of facility to perform BH4 cofactor analysis, segregation analysis, parental analysis, and functional studies. Although the study suffers from its inherent limitations, this is a novel endeavor to highlight the challenges faced during the management of patients with BH4 deficiency in India. This study also highlights the need for initiation of nationwide newborn screening which is an unmet need of BH4 cofactor metabolites is essential since 25% of children with PTPS and GTPCH deficiency and 40% of children with DHPR deficiency are asymptomatic in the newborn period and remain asymptomatic until 3 months of age (Opladen et al., 2012; Blau et al., 1996b; Opladen et al., 2016). The newborn screening may, in fact, be normal on day one of life, and hence a repeat testing is essential later after day-3 of life (Manzoni et al., 2020). The first step towards inclusion into the newborn screening programme in India, as well as the availability of BH4 supplements, will pave the way for earlier identification and better neurodevelopmental outcome for Indian children with this rare yet treatable neurotransmitter disorder.

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**Conclusion**

Tetrahydrobiopterin deficiencies represent a rare yet potentially treatable cause for non-phenylketonuria hyperphenylalaninemia when diagnosed and treated early in life. Developing a policy of newborn screening and implementing at the national level can help prevent devastating neurological deterioration. This study expands the genotype-phenotype spectrum of patients with disorders of tetrahydrobiopterin metabolism from South India.

**Availability of data and material**  Everything has been presented in the manuscript. Any additional data required shall be provided

**Code availability**  Not applicable

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**Declarations**

**Conflicts of Interest**  None

**Ethics approval**  As it’s a retrospective study of only 9 cases ethics approval is not mandatory

**Consent to participate**  Yes

**Consent for publication**  Yes

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