Cerebral folate deficiency: Analytical tests and differential diagnosis

Simon Pope¹, Rafael Artuch², Simon Heales¹,³,⁴, Shamima Rahman⁴,⁵

¹ Neurometabolic Unit, National Hospital for Neurology, Queen Square, London, UK
² Institut de Recerca Sant Joan de Déu and CIBERER, ISCIII, Barcelona, Spain
³ Department of Chemical Pathology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK
⁴ Mitochondrial Research Group, UCL Great Ormond Street Institute of Child Health, London, UK
⁵ Department of Metabolic Medicine, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

Correspondence to:
Simonpope@nhs.net
Or
Shamima.rahman@ucl.ac.uk

Key words:
Folate, vitamin B9, 5-methyltetrahydrofolate, 5-MTHF, folinic acid, FOLR1, PCFT, DHFR, one carbon metabolism, mitochondrial disease

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jimd.12092

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Abstract

Cerebral folate deficiency is typically defined as a deficiency of the major folate species 5-methyltetrahydrofolate in the cerebrospinal fluid (CSF) in the presence of normal peripheral total folate levels. However, it should be noted that cerebral folate deficiency is also often used to describe conditions where CSF 5-MTHF is low, in the presence of low or undefined peripheral folate levels. Known defects of folate transport are deficiency of the proton coupled folate transporter, associated with systemic as well as cerebral folate deficiency, and deficiency of the folate receptor alpha, leading to an isolated cerebral folate deficiency associated with intractable seizures, developmental delay and/or regression, progressive ataxia and choreoathetoid movement disorders. Inborn errors of folate metabolism include deficiencies of the enzymes methylenetetrahydrofolate reductase, dihydrofolate reductase and 5,10-methylenetetrahydrofolate synthetase. Cerebral folate deficiency is potentially a treatable condition and so prompt recognition of these inborn errors and initiation of appropriate therapy is of paramount importance. Secondary cerebral folate deficiency may be observed in other inherited metabolic diseases, including disorders of the mitochondrial oxidative phosphorylation system, serine deficiency, and pyridoxine dependent epilepsy. Other secondary causes of cerebral folate deficiency include the effects of drugs, immune response activation, toxic insults and oxidative stress. This review describes the absorption, transport and metabolism of folate within the body; analytical methods to measure folate species in blood, plasma and CSF; inherited and acquired causes of cerebral folate deficiency; and possible treatment options in those patients found to have cerebral folate deficiency.
Conflicts of interest
SR is an Editor of the Journal of Inherited Disease and JIMD Reports.

Informed consent
Not applicable since no patients are reported in this manuscript.

Animal Rights
Not applicable since no animal studies were performed in the preparation of this manuscript.
1. Introduction

1.1 History of folate

Folic acid was discovered in 1941 as a factor, isolated from spinach leaves (folium being the Latin for leaves), required for the growth of *Streptococcus lactis* (Mitchell et al 1941). Around this time, in the 1930’s and 40’s, it was also found that yeast and liver extracts containing folic acid or a newly synthesised folic acid supplement could prevent anaemia caused by pregnancy, coeliac disease or malnutrition (see historical reviews of folic acid by Hoffbrand 2001 and Rosenberg 2012).

The term folate refers to a group of compounds containing a pteridine ring, a paraminobenzoic acid ring and glutamic acid (Figure 1). Folic acid, a common form of folate added to fortified foods, contains a fully oxidised pteridine ring and a single glutamic acid residue. Naturally occurring folates can exist in a variety of oxidation states (e.g. dihydro, tetrahydro-) and can also contain more than one glutamate residue (i.e. polyglutamate). Naturally occurring folates with an additional single carbon unit, such as a methyl, formyl, methylene or methenyl group attached to the N5 or N10 nitrogen atom, are frequently observed (Figure 1). These single carbon units are essential to the function of folates in one-carbon metabolism. For example, 5-methyltetrahydrofolate (5-MTHF) is essential for the methylation of homocysteine to methionine (Figure 1) and global deficiency of 5-MTHF results in elevated plasma homocysteine (Freeman et al 1975). S-Adenosyl-methionine (SAM), formed from methionine, is required for more than 100 methylation reactions in cells, including methylation of DNA, RNA, neurotransmitters, lipids, hormones and drug metabolites (Bottiglieri 2002 and 2013).

1.2 Folate absorption and metabolism
Folate absorption and metabolism is shown schematically in Figures 1 and 2. Dietary folate intake is largely from fruits and vegetables as polyglutamates, which are acted on by the enzyme folylpoly-$\alpha$-glutamate carboxypeptidase (Visentin et al, 2014). This enzyme is present at the intestinal brush border and hydrolyses these polyglutamates to their monoglutamates, enabling absorption (Halsted et al 1997; Darcy-Vrillon et al 1988). Two folate transporters are expressed in the intestine - the reduced folate carrier (RFC) (Matherly and Hou 2008) and the proton-coupled folate transporter (PCFT) (Qiu et al 2006; Zhao and Goldman 2013). Defects in the gene encoding the PCFT (SLC46A1) have been described and are associated with systemic folate deficiency, emphasising the key role of PCFT in folate absorption and transport (Qiu et al, 2006; Zhao et al, 2017). Defects in RFC, affecting intestinal transport of folate, have not been reported.

The RFC (encoded by SLC19A1) is an organic anion antiporter that exchanges 5-MTHF for other anions (inorganic or organic). It is ubiquitously expressed and is the main transport mechanism by which cells take-up folates from the systemic circulation (Matherly and Hou 2008). Specialised folate receptors (FRs) are also present on some cell types and represent a higher affinity transport system which operates at the nanomolar range (Chen et al, 2013; Zhao et al, 2011). The precise role and regulation of each of these different folate transport systems is still being elucidated. Folate uptake into the brain is mediated by the high affinity folate receptor α (FRα, encoded by FOLR1), which is highly expressed in the apical membrane of choroid plexus epithelial cells (Grapp et al, 2013) and PCFT (see figure 2). 5-MTHF delivery into the brain parenchyma appears to be mediated via exosomes that transport 5-MTHF-laden FRα molecules, traversing the cytosol of choroid plexus epithelial cells from the basolateral to the apical membranes, before being secreted into the CSF at the apical brush border (Grapp et al, 2013).

Folate metabolism is complex and has an essential role in nucleic acid biosynthesis, one carbon metabolism and amino acid metabolism, as illustrated in Figures 1 and 2. Dietary forms of folate are absorbed as their monoglutamates. Naturally occurring folates are generally considered to be in their reduced forms. Synthetic and unreduced forms of folate are reduced to dihydrofolate and then tetrahydrofolate by dihydrofolate reductase (DHFR). Tetrahydrofolate is then converted to 5,10-methylene-tetrahydrofolate by transfer of a carbon unit from serine in a reaction catalysed by
serine-hydroxymethyltransferase (SHMT). This reaction also produces glycine. 5,10-methylene-tetrahydrofolate can also be produced via the glycine cleavage system (GCS) (Kikuchi et al 2008; Pai et al 2015). 5,10-methylene-tetrahydrofolate is then converted to 5-methyl tetrahydrofolate (5-MTHF) by methylenetetrahydrofolate reductase (MTHFR). MTHFR is essential for the production of 5-MTHF, which in turn is essential for the remethylation of homocysteine to methionine. This cycle produces S-adenosyl methionine (SAM), which is used in numerous methylation reactions catalysed by methyltransferases, including those of nucleic acids, drug metabolites and neurotransmitters (Blom and Smulders, 2011). Other forms of folate, such as 10-formyl-tetrahydrofolate and 5,10-methylene-tetrahydrofolate, are involved in purine and pyrimidine synthesis, respectively.

1.3 Discovery of cerebral folate deficiency
Neurological disease was first linked to folate deficiency by Pincus and co-workers in 1973 (Reynolds et al, 1973) and low CSF folate levels were first reported in 1981 (Botez and Bachevalier 1981). Cerebral folate deficiency was subsequently defined by Raemakers and Blau (2004) as ‘any neurological syndrome associated with a low CSF 5-MTHF, in the presence of normal folate metabolism outside the nervous system.’ However, it should be noted that cerebral folate deficiency is often used to describe conditions where CSF 5-MTHF is low, regardless of the peripheral folate status. A variety of causes of cerebral folate deficiency have been described, ranging from inborn errors of metabolism including metabolic recycling defects (DHFR and MTHFR deficiencies) and disorders of folate transport (e.g. FOLR1 and PCFT defects, and mitochondrial disorders leading to impaired folate transport) to possible over-utilisation/degradation defects such as L-dopa methylation and neurodegeneration. The primary transport and metabolic defects typically present with extremely low cerebrospinal fluid (CSF) concentrations of 5-MTHF below 10 nmol/L prior to treatment.

2. Analytical methods for assessment of folate status
Folate status is usually initially assessed by total folate in serum or plasma using a folate-binding protein assay (see Sobczynska-Malefora and Harrington 2018 for review of folate assays). The advantages of this assay are that it is easily automated, inexpensive and quick to perform, but it
should be noted that it measures total folate, including folic acid, and therefore does not always accurately assess the levels of active forms of folate such as 5-MTHF. Other peripheral markers of folate status, such as red cell folate and total plasma homocysteine, are more specific and therefore may give a better indication of ‘active’ folate status than total folate in serum.

Methods have been described for the measurement of individual folate species in serum, red cells and CSF, typically using liquid chromatography-mass spectrometry (LC-MS) (Arning 2016; Asante et al., 2018; Kiekens et al., 2015; Meadows 2017; Nandania et al., 2018; Schittmayer et al., 2018). Such methods are not generally available in a diagnostic setting. For the diagnosis of cerebral folate deficiency (CFD), the most frequently used method is high performance liquid chromatography (HPLC) with fluorescence detection or electrochemical detection (ECD) of 5-MTHF in CSF (Akiyama et al. 2015; Aylett et al. 2013; Opladen et al. 2006; Ormazabal et al. 2006; Verbeek et al. 2008). Most rely on the separation of 5-MTHF on a C18 HPLC column followed by ECD or fluorescence detection. Figure 3 shows 5-MTHF HPLC chromatograms, from CSF, performed in our laboratory.

5-MTHF is relatively stable in most CSF samples (Verbeek et al., 2008; Sobczynska-Malefora and Harrington 2018; Aylett et al., 2013) and does not typically degrade on an unrefrigerated autosampler overnight (personal observation). However, we have noted that 5-MTHF may degrade quickly in occasional CSF samples. The reason for this is not clear but could be related to oxidative catabolism due to deficiency of antioxidants such as ascorbic acid and/or increased generation of reactive oxygen species (Aylett et al., 2013). Consequently, we recommend that all CSF samples for 5-MTHF analysis should be snap frozen and stored at -70°C. Repeat analysis of samples with low CSF 5-MTHF should be done on freshly thawed CSF and analysed immediately to distinguish between genuine low CSF 5-MTHF and ex vivo degradation of CSF 5-MTHF due to uncontrollable factors present in a particular sample.

There appears to be no rostro-caudal gradient for CSF 5-MTHF but CSF 5-MTHF concentrations are age-related with CSF 5-MTHF typically decreasing with age (Verbeek et al., 2008). Most laboratories have their own age-related reference ranges with broad agreement between different
3. Inborn errors of folate transport and metabolism

Inborn errors of folate metabolism and transport can broadly be divided into those disorders where there is a generalised systemic folate deficiency, and those disorders characterized by cerebral (central) folate deficiency (CFD) with normal peripheral folate levels. The former include defects of intestinal folate absorption and of systemically expressed enzymes. In isolated CFD, defective folate transport across the blood-brain barrier leads to CFD with normal plasma and red cell folate levels. In addition, other inborn errors of metabolism may be associated with what appears to be a secondary CFD, since the underlying pathophysiological mechanisms remain obscure. The largest
group of such disorders are primary mitochondrial disorders, i.e. inherited disorders directly or indirectly affecting the function of the oxidative phosphorylation (OXPHOS) system.

3.1 Disorders of folate transport

3.1.1 Proton-coupled folate transporter (PCFT) deficiency

Hereditary folate malabsorption (OMIM #229050) is an autosomal recessive disorder caused by biallelic mutations in the \textit{SLC46A1} gene encoding PCFT (Qiu et al, 2006; Wolf 2007). This transporter is essential for intestinal folate absorption and transport from blood to the brain. In patients affected by PCFT deficiency, lack of folate availability for the various folate-related pathways leads to decreased levels of all folate species (Qiu et al, 2006). More than 30 affected individuals have been reported to date (Zhao et al 2017). Affected infants typically present a few months after birth with symptoms and signs attributable to both systemic and central folate deficiency. Clinical features include feeding difficulties leading to faltering growth, megaloblastic anemia, neutropaenia, thrombocytopaenia, immune deficiency, recurrent infections (especially 	extit{Pneumocystis} pneumonia), diarrhoea and mouth ulcers (Kronn and Goldman 2017). Neurological features include global developmental delay, seizures and behavioural disturbance. Neuro-imaging may show demyelination and intracranial calcifications. Biochemical investigations reveal both plasma and CSF folate and 5-MHTF deficiencies. Treatment with parenteral or high dose oral folinic acid (5-formyl-tetrahydrofolate, 5-formyl-THF) rapidly corrects blood folate levels in PCFT deficiency but CSF 5-MTHF deficiency may be more difficult to correct. Therefore, it is recommended that CSF 5-MTHF levels are monitored in PCFT deficiency (Manea et al 2018). Early treatment can prevent the neurological complications of the disease (Kronn and Goldman 2017).

3.1.2 Folate receptor alpha deficiency

Mutations in \textit{FOLR1} (OMIM #613068) encoding FR\(\alpha\) were first described in 2009 in 3 children with progressive movement disorder, developmental regression and seizures associated with extremely low CSF 5-MTHF levels and normal peripheral folate levels (Steinfeld et al, 2009). More than 20 affected children have now been reported in the medical literature, and their main clinical features are summarised in Table 2. Symptom onset was between 6 months and 4.5 years in all
reported cases, with a median onset of 2 years. Most affected children appeared normal at birth, although one case had congenital microcephaly. There is usually a symptom-free interval of variable duration prior to symptom onset, which has led to the suggestion that 5-MTHF transport by FRβ may compensate for FRα deficiency in early infancy (Molero-Luis et al, 2015). Typical clinical signs at presentation are tremor, ataxia, hypotonia and developmental delay (especially motor and speech), with subsequent developmental regression and intractable seizures. Seizures are most often myoclonic, but individual patients may have several seizure types including astatic, tonic, tonic-clonic and atypical absences. Recurrent episodes of status epilepticus have been reported in three affected children. Other clinical features include movement disorders (especially chorea and athetosis), acquired microcephaly and polyneuropathy. CSF 5-MTHF levels are extremely low in FRα deficiency, typically < 5 nmol/L. The highest reported value was 11 nmol/L in one of 12 cases for whom absolute values are available (Table 2). Plasma and red cell folate levels are normal. Neuroimaging reveals hypomyelination, particularly affecting subcortical white matter, together with cerebellar and/or cerebral atrophy. Treatment with oral folinic acid supplementation (at doses ranging from 0.5 to 7 mg/kg/dy) has been reported to result in improvement of seizures, neuro-imaging appearances and CSF 5-MTHF levels (Table 2). However, response to treatment is variable and is related to the duration of symptoms at the time of initiation of treatment, emphasizing the importance of early recognition and treatment of this disorder. Because of the poor response to oral folinic acid in many cases, more recent reports have advocated intermittent intravenous folinic acid therapy, in addition to daily oral dosing (Delmelle et al, 2016). Folic acid is contraindicated in this condition, as it competitively binds to FRα, further reducing 5-MTHF transport into the brain.

3.2 Disorders of folate metabolism

3.2.1 Methylene-tetrahydrofolate reductase (MTHFR) deficiency

MTHFR deficiency (OMIM #236250) is the most frequent inborn error of folate metabolism, with >200 patients reported in the literature (Burda et al, 2015). In this autosomal recessive disorder, there is defective synthesis of 5-MTHF, which is the essential methyl group donor for the remethylation of homocysteine to methionine (Watkins et al, 2012). Characteristic biochemical features of severe MTHFR deficiency are hyperhomocysteinemia with low-normal plasma
methionine, and CFD with low-normal plasma total folate levels (Skovby et al. 2003). Since most plasma folate assays measure folate by immunoassay, which does not specifically measure 5-MTHF (Sobczyńska-Malefora and Harrington, 2018), plasma folate is not always low in MTHFR deficiency. Clinical presentations of severe MTHFR deficiency are extremely variable, ranging from early onset in the first months of life with encephalopathy, apnoea, seizures and hydrocephalus, to later onset with neurological decline, ataxia, seizures, cerebral atrophy and delayed myelination. Vascular occlusion, psychiatric manifestations and white matter abnormalities may be observed in older children and adults (Skovby et al. 2003). The limited amounts of 5-MTHF available to be transported to the brain, coupled with diminished formation within the CNS, can explain the profound CFD in MTHFR deficiency. A common genetic variant in MTHFR, c.677 C>T, encodes a thermolabile enzyme with lower activity at higher temperatures. Homozygosity for the c.677 C>T genotype is associated with higher plasma homocysteine and folate levels compared to controls (Watkins et al., 2012), but these individuals do not have CFD (Ormazabal et al. 2011). The c.677 C>T variant has been linked to a large number of disease processes, but as this variant is present very frequently in the general population, in 10-15% of Caucasians and up to 25% of Hispanics in North America (Dean 2012), it is very difficult to prove causality, and much more likely that these are spurious associations. Some authors have also suggested possible association between MTHFR polymorphisms and neural tube defects and cardiovascular disease (Blom et al. 2011; Watkins et al. 2012), but a detailed discussion of this is beyond the scope of this review.

3.2.2 Dihydrofolate reductase (DHFR) deficiency

DHFR deficiency (OMIM #613839) appears to be an extremely rare autosomal recessive disorder of folate metabolism. DHFR mutations were first reported in 2011 by two research groups, who described a total of 6 individuals from 3 families with CFD associated with megaloblastic anaemia (Banka et al. 2011; Cario et al. 2011). There do not appear to have been any further subsequent reports of this disorder in the literature. The main clinical features were severe megaloblastic anaemia or pancytopenia and a complex neurological picture with acquired microcephaly, refractory seizures, intellectual disability, and white matter lesions and cerebellar and/or cerebral atrophy on neuro-imaging. Biochemically, peripheral biomarkers were normal (folate, cobalamin and homocysteine), but all cases had profound CFD. Improvement of CSF 5-MTHF levels, seizure...
control and other neurological symptoms was observed after oral folinic acid supplementation (Banka et al, 2011; Cario et al 2011).

3.2.3 5,10-Methenyl-tetrahydrofolate synthase (MTHFS) deficiency
MTHFS deficiency is the most recent genetically defined disorder of folate metabolism. MTHFS (OMIM *604197) is an enzyme involved in the conversion of folinic acid (5-formyl-THF) to 5,10-methenyl-tetrahydrofolate (Figure 1). 5,10-methenyl-tetrahydrofolate is in turn converted to 5,10-methylene-tetrahydrofolate, the precursor of 5-MTHF, or 10-formyltetrahydrofolate, which is important for purine metabolism. Recently, Rodan et al. (2018) described two patients with biallelic mutations in MTHFS. These patients presented with microcephaly, global developmental delay, epilepsy and cerebral hypomyelination, at birth in one case and 3 months in the other. CSF 5-MTHF levels were low-normal (~40-50 nmol/L, ref range 40-150). Enzyme analysis revealed impaired MTHFS activity and highly elevated levels of 5-formyl-THF, the substrate of this enzyme, in cultured skin fibroblasts from one patient. The pathophysiology of this disorder is still not fully understood, as the CSF 5-MTHF levels are not as low as typically observed in related folate disorders with a similar clinical phenotype. Rodan et al. hypothesise that although extracellular 5-MTHF levels are not low, intracellular levels may be deficient and suggest that accumulation of intracellular 5-formyl-THF polyglutamates may inhibit synthesis of 5,10-methylene-tetrahydrofolate by SHMT and in turn lead to inhibition of 5-MTHF production. A previous case of suspected MTHFS deficiency was described by Scaglia and Blau (2013) but complete molecular characterisation was not shown. The patients described by Rodan et al. continued to deteriorate with folic acid or folinic acid supplementation, but one patient showed some improvements after oral 5-MTHF and intramuscular methylcobalamin administration. The rationale behind the B12 administration was to decrease methyl group consumption in the endogenous synthesis of methylcobalamin.

3.2.4 Methylenetetrahydrofolate dehydrogenase (MTHFD1) deficiency
MTHFD1 deficiency (OMIM #617780) is a rare inborn error of folate metabolism that has been reported in 7 individuals from 4 families to date (Watkins et al 2011 JMG PMID:21813566, Burda et al 2015 JIMD PMID:25633902, Ramakrishnan et al 2016 J Allergy Clin Immunol Pract
Clinical features include megaloblastic anaemia, atypical hemolytic uraemic syndrome and severe combined immune deficiency (SCID), with seizures, intellectual impairment and abnormal brain imaging in some cases. Biochemical features include elevated blood levels of homocysteine and methylmalonic acid (Watkins et al 2011 JMG), with low CSF 5-MTHF levels documented in some affected individuals (Burda et al 2015). All affected patients had biallelic mutations in MTHFD1 encoding a trifunctional protein involved in cellular folate metabolism. The three reactions catalysed by the MTHFD1 trifunctional protein are 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase, which condense formate with tetrahydrofolate to provide the primary entry point of single carbons into folate-dependent one-carbon metabolism in the cytosol (Field et al 2015 PNAS PMID:25548164, Burda et al 2015). In MTHFD1 deficiency, impaired nuclear de novo dTMP biosynthesis is thought to underlie both the megaloblastic anaemia and the SCID (Field et al 2015 PNAS). Treatment of affected individuals with folinic acid supplementation (in addition to intramuscular hydroxocobalamin, betaine and prophylactic antibiotics) was associated with clinical improvement, including resolution of anaemia and improved lymphocyte function in two cases (Burda et al 2015, Ramakrishnan et al 2016).

3.3 Genetic diseases associated with CFD

3.3.1 Mitochondrial disorders.

The term primary mitochondrial disease is generally accepted to refer to a large group of heterogeneous inborn errors of metabolism that directly or indirectly affect the integrity and/or function of mitochondria, particularly the OXPHOS system (Rahman and Rahman 2018). CFD was first reported in the context of mitochondrial disease in 1983 in a young woman with Kearns-Sayre syndrome (KSS, OMIM #530000) associated with ragged-red fibre myopathy, progressive neurological decline and white matter lesions and basal ganglia calcification on computed tomography of the brain (Allen et al 1983). In the intervening 35 years CFD has been recognized as a relatively frequent occurrence in primary mitochondrial disease, although the exact prevalence remains unknown (Hyland et al 2010). CFD has most frequently been reported in patients with KSS caused by single large-scale mitochondrial DNA deletions (Pineda et al 2006; Garcia-Cazorla et al 2008; Serrano et al 2010; Broomfield et al 2015), but has also been noted in patients with other
mitochondrial disorders including complex I deficiency (not genetically defined) (Ramaekers et al 2007b; Garcia-Cazorla et al, 2008) and Alpers disease caused by biallelic POLG mutations (Hasselmann et al, 2010). Associated clinical features may include seizures but these are not as universal in KSS as in primary CFD disorders such as FOLR1 and DHFR deficiencies. The underlying molecular mechanisms causing CFD in mitochondrial disorders remain obscure, but it is unlikely that mitochondrial CFD is related purely to a bioenergetic failure, since CFD is not a universal feature of mitochondrial disease. In the case of KSS, an attractive hypothesis is that accumulation of deleted mtDNA molecules in the choroid plexus of affected patients leads to breach of the choroidal integrity and thus impaired 5-MTHF transport into the brain (Tanji 2000; Spector and Johanson 2010). More recently CSF FRα and 5-MTHF levels were shown to be correlated in 9 patients with KSS, providing further support for this hypothesis (Grapp et al, 2013). Therapeutic response to folinic acid has been noted in KSS (Pineda et al, 2006, Quijada-Fraile et al, 2014). However, although folinic acid therapy has been reported to correct CSF 5-MTHF levels and cerebral white matter lesions, it does not appear to prevent the relentless multisystem decline associated with this disorder (Broomfield et al, 2015).

3.3.2 Serine deficiency

Three inborn errors of L-serine biosynthesis have been reported, namely deficiencies of 3-phosphoglycerate dehydrogenase (OMIM #601815), phosphoserine aminotransferase (OMIM #610992) and phosphoserine phosphatase (OMIM #614023) (Van der Crabben et al, 2013). In addition, a founder mutation in SLC1A4 (OMIM #616657) leading to defective serine transport to the brain has been reported in the Ashkenazi Jewish population (Damseh et al, 2015). The main clinical features of serine deficiency are congenital microcephaly, postnatal growth retardation, hypertonia (progressive spastic quadriplegia), psychomotor retardation, epilepsy and hypogonadism. The link between serine and folate deficiency is that serine is transformed to glycine by SHMT2 in a reaction that adds a methylene group to THF to form 5,10-methyleneTHF. This is the primary source of one-carbon units in folate metabolism. Thus, serine deficiency leads to mild to moderate CFD and disorders of serine biosynthesis and transport should be considered in the differential diagnosis of CFD (Molero-Luis et al, 2015). Serine supplementation, together with
glycine, usually leads to improved seizure control, but has little impact on psychomotor development.

3.3.3 Dihydropteridine reductase (DHPR) deficiency
DHPR is an enzyme involved in the recycling/reduction of dihydrobiopterin to tetrahydrobiopterin, the active cofactor for aromatic amino acid (phenylalanine, tyrosine and tryptophan) hydroxylases and nitric oxide synthase. DHPR is also able to reduce the structurally related dihydrofolate to tetrahydrofolate. Patients with DHPR deficiency (OMIM #261630) are detected by the presence of hyperphenylalanenaemia on newborn screening, with normal pterins and reduced DHPR activity in dried blood spots. If treated with a low phenylalanine diet alone, neurological symptoms (coarse tremor, rigidity, drooling and progressive cognitive and motor decline) develop because of neurotransmitter deficiency. Treatment therefore consists of dietary phenylalanine restriction together with L-dopa, carbidopa (a peripheral decarboxylase inhibitor) and 5-hydroxytryptophan supplementation. Progressive cerebral calcification affecting the basal ganglia and white matter has been reported to result from secondary CFD in patients with DHPR deficiency and can be prevented by folinic acid supplementation (Irons et al, 1987; Woody et al, 1989). A putative mechanisms to explain the CFD in these patients is that MTHFR enzyme activity might be redirected in the face of DHPR deficiency from 5-MTHF synthesis to the production of BH4 from qBH2, since MTHFR has similar Km values for its natural substrate 5,10-methylenetetrahydrofolate and for qBH2 (Irons et al, 1987).

3.3.4 Aromatic L-amino acid decarboxylase (AADC) deficiency
Patients with AADC deficiency (OMIM #608643) may also develop secondary CFD (Wassenberg et al, 2017). In AADC deficiency, affected patients have a severe combined deficiency of serotonin, dopamine, noradrenaline and adrenaline. More than 100 patients have been reported in the literature and clinical presentation is typically in infancy with hypotonia, global developmental delay, movement disorders (oculogyric crisis, dystonia and hypokinesia) and autonomic symptoms (Wassenberg et al, 2017). The enzyme deficiency affects the conversion of L-dopa to dopamine, leading to a build-up of L-dopa and consequently an increased reliance on folate for L-dopa
methylation. Folinic acid therapy is therefore recommended for patients with AADC deficiency if CSF 5-MTHF is low (Wassenberg et al, 2017).

3.3.5 Pyridoxine dependent epilepsy
Pyridoxine dependent epilepsy (PDE, OMIM #266100) is most commonly caused by mutations in ALDH7A1 encoding antiquitin (α-aminoacidipic semialdehyde dehydrogenase), an enzyme of the lysine degradation pathway (reviewed by Wilson et al 2019 in this issue of the Journal of Inherited Metabolic Disease). Antiquitin deficiency results in accumulation of ±-aminoacidipic semialdehyde (AASA) and piperideine-6-carboxylate (P6C). P6C condenses with and inactivates pyridoxal phosphate, the active form of vitamin B6 (Mills et al, 2006). Most patients achieve complete seizure control on monotherapy with pyridoxine, the first dose being given in a setting where respiratory support can be provided, since apnoea may develop in B6 responsive patients. The presence of cognitive impairment in a significant majority of patients with PDE despite adequate seizure control led to the hypothesis that this was mediated by accumulating toxic intermediates in the lysine degradation pathway (Van Karnebeek et al, 2016). Current treatment protocols recommend dietary lysine restriction, together with arginine supplementation as this competes with lysine for cellular uptake, and B6 at a maximum dose of 300mg/day (to avoid peripheral neuropathy and other features of B6 toxicity). A disorder initially reported as folinic acid responsive epilepsy was subsequently shown to be allelic with PDE – both are caused by biallelic mutations in ALDH7A1 (Gallagher et al, 2009). The precise mechanism for folinic acid responsiveness of the seizures in these patients remains obscure. Typically these patients have been treated with both pyridoxine and folinic acid and baseline levels of CSF 5-MTHF are rarely measured (Stockler et al, 2011). However, we have noticed in some older patients with PDE, that CSF 5-MTHF can fall below the reference range (unpublished observation from our laboratory - two teenage siblings with confirmed PDE had CSF 5-MTHF levels around half of the lower end of the reference range), so consideration should be given to monitoring 5-MTHF levels and supplementing with folinic acid if indicated.

4 Acquired cerebral folate deficiency
4.1 Environmental and unknown factors
To date, delineation of all neurological conditions leading to CFD is probably incomplete, as evidenced by some patients with remarkably low CSF 5-MTHF values with no genetic diagnosis. The presence of folate receptor autoantibodies in the CSF has been claimed as a cause of CFD in a wide range of conditions, ranging from an infantile onset CFD syndrome (Ramaekers et al, 2005) to schizophrenia, autistic spectrum disorder and Rett syndrome (Ramaekers et a. 2014; Frye 2013; Moretti 2008; Ramaekers et al, 2007c and 2003). These results have not been replicated in other laboratories and it is possible that in some neurological conditions, the observed mildly low CSF 5-MTHF values are an epiphenomenon with no pathophysiological implications. In the authors’ experience, a relatively frequent cause of mild to moderate CFD is a suboptimal blood folate status due to inadequate intake (Perez-Dueñas et al, 2011). This common condition should be ruled out as a first step in the diagnostic investigations for CFD. For example, inappropriate folate nutrition has been associated with many neuropsychiatric disorders (Morris et al, 2002) and folate deficiency and hyperhomocysteinemia are commonly observed in psychiatric geriatric patients.

4.2 Drugs and CFD

In some cases, CFD may be secondary to medication effects. Many drugs are methylated during the normal detoxification process (Jancova et al, 2010) and this may contribute to a secondary loss of folate. 5-MTHF is required for SAM production, which is used by methyltransferases to methylate a wide range of substrates including L-dopa, a drug used to treat dopamine deficiency. Long term L-dopa/carbidopa therapy can result in secondary folate deficiency so folinic acid supplementation is recommended (Surtees and Hyland 1990). CSF 5-MTHF levels should be monitored in such patients if folate deficiency is suspected. Some antiepileptic drugs, such as phenytoin, carbamazepine, barbiturates and valproate, may reduce folate levels (Wegner et al, 1992; Morrell 2002; Opladen et al, 2010), and this should be taken into consideration when prescribing AEDs for patients with CFD and epilepsy.

Other drugs known to deplete folate levels include anti-folate cancer drugs such as methotrexate (inhibits DHFR) (Lambie and Johnson 1985; Vezmar et al, 2009) and some antibiotics, such as trimethoprim, whose mechanism of action is via inhibition of bacterial DHFR (Lambie and Johnson 1985). Nitrous oxide (laughing gas) is used as an anaesthetic and may be abused as a recreational
drug. Long term or excessive use can result in inhibition of methionine synthase via deactivation of its cobalamin cofactor, resulting in cellular folate becoming trapped as 5-MTHF - ‘the folate trap hypothesis’ (Lumb et al, 1980). 5-MTHF levels may be normal or elevated, but this folate would be trapped due to the inactivity of methionine synthase and inhibition of other downstream pathways requiring different folate species.

4.3 Oxidative stress and toxic insult resulting in CFD
Aylett et al (2013) showed a positive correlation between levels of CSF 5-MTHF and those of the antioxidant ascorbic acid (vitamin C). It was hypothesised that ascorbic acid and other antioxidants may protect 5-MTHF in the CSF and some cases of CFD may be due to a deficiency of these antioxidant defences and/or an increase in oxidative stress/toxic insult (see figure 2B). Chronic immune response activation is one possible cause of increased oxidative stress and interestingly there have been some reports of CFD associated with elevated levels of the immune response activation marker, neopterin (Hasselmann et al, 2010; Papandreou et al, 2018; Blau et al, 2003). In our laboratory, we have noticed that in some CSF samples 5-MTHF degrades rapidly on the HPLC autosampler (unpublished findings) even though 5-MTHF is typically thought to be relatively stable in biological samples (Verbeek et al, 2008). This may suggest that in some samples, antioxidant defences are compromised and/or oxidising species are present in the CSF. Such findings may have clinical relevance.

5 Therapeutic aspects: different folate forms and routes of administration
5.1 Folate, folinic acid and 5-methyltetrahydrofolate
Folate is an umbrella term for several compounds including 5-MTHF, 5-formyl-THF (folinic acid), 10-formyl-THF, 5,10-methylene-THF and THF (Scaglione et al, 2014). It should be noted that the currently available formulations (folic acid, folinic acid and 5-MTHF) have different properties (Scaglione et al, 2014). In most studies reporting treatment of CFD, folinic acid has been used, with good results in many cases, under the rationale that the brain has a limited capacity to reduce folic acid. Folic acid is not an active drug since it needs to be converted into metabolically active forms by several metabolic reactions, including reduction by DHFR (Forssen et al, 2000; Scaglione et al,
Genetic or acquired disturbances in folate availability may decrease synthesis of the active vitamers, and so therapy with metabolically active forms of folate is preferred.

Folinic acid is converted to THF without the need for DHFR enzymatic activity (Rajagopalan et al, 2002). 5-MTHF, which represents >90% of total plasma folate (Scaglione et al, 2014) has important advantages over other folate forms, since it is well absorbed in the intestine and its bioavailability is not affected by DHFR and MTFHR deficiencies. The use of 5-MTHF could also prevent the potential negative effects of unconverted folic acid in the brain, and it is thought to be the most efficient way to restore CSF 5-MTHF concentrations (Knowles et al, 2016).

### 5.2 Routes of administration

Specific therapeutic approaches have been described for each of the individual disorders of folate transport and metabolism in the relevant sections above. As documented above, oral folate therapy has been used extensively in CFD, with variable results. Because neurodevelopmental outcomes have not been normal, unless therapy is started extremely early (the best results have been observed in siblings treated at a pre- or oligosymptomatic stage), recently it has been suggested that parenteral (either intramuscular or intravenous) folinic acid or 5-MTHF administration may further improve the clinical outcome of patients with different forms of CFD. Although current experience is limited owing to the rarity of the genetic conditions leading to CFD, promising preliminary results highlight the importance of designing clinical trials to assess alternative routes of folate administration (Delmelle et al, 2016; Manea et al, 2018; Torres et al, 2015).

### 5.3 Adverse effects

To our knowledge, there are no publications of adverse effects of folinic acid or 5-MTHF in patients with primary CFD, other than some anecdotal observations in small cohorts of patients (Frye et al, 2013). Nevertheless, it is important to consider the possibility of adverse effects of folate therapy in the pathological conditions described above, and to perform appropriate monitoring for potential toxicity. For example, excess folic acid may act as a competitive inhibitor of DHFR activity (Reynolds et al, 2006). Another consideration is that administration of high doses of folate may lead to significant excitatory effects in neuronal cells (Morrell et al, 2002).
6. Conclusion

As can be seen from this review, folate species are vital in many metabolic pathways. Concentrations of 5-MTHF are maintained at a higher concentration in the CSF than plasma, indicating the importance of this folate species in the brain. Primary deficiencies in folate metabolism or transport can result in CFD. However, it is becoming increasingly acknowledged that CFD is also a common secondary finding in many other metabolic defects as well as being observed after toxic insults/drug therapy. Although prolonged CFD can result in a range of clinical symptoms such as white matter changes on MRI due to impaired myelination, developmental delay and seizures, it is also a potentially treatable condition. Therefore, it is important to highlight that measurement of CSF 5-MTHF is useful in a wide range of conditions, as a biomarker to monitor the condition, to understand the underlying aetiology (oxidative stress, transport impairment, metabolic defects in one carbon metabolism, increased utilization of 5-MTHF) and also to monitor treatment with folate species such as folinic acid and 5-MTHF.

Acknowledgements

The authors acknowledge research grant funding from Great Ormond Street Hospital Children's Charity, the NIHR Great Ormond Street Hospital Biomedical Research Centre and the Lily Foundation.
References


Scaglione F, Panzavolta G (2014) Folate, folic acid and 5-methyltetrahydrofolate are not the same thing *Xenobiotica* 44(5), 480–488.


Wilson et al (2019) in this issue of the Journal of Inherited Metabolic Disease


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Figure 1. Folate structures and Folate/One-carbon metabolism.

See text for description.

Key: DHF - dihydrofolate; DHFR - dihydrofolate reductase; DHPR - dihydropteridine reductase; THF - tetrahydrofolate; MS - methionine synthase; MTHFR - methylenetetrahydrofolate reductase; 5-MTHF - 5-methyl-tetrahydrofolate; SAM - S-adenosyl methionine; SAH - S-adenosyl homocysteine; CBS - cystathionine beta-synthase; B6 - pyridoxal phosphate dependent enzyme; B12 - cobalamin dependent reaction.

Figure 2A and B. Folate transport, mitochondrial metabolism and oxidative stress

A. Folate species are absorbed in the gut via PCFT and are then converted to the main transport form, 5-MTHF. 5-MTHF crosses the blood brain barrier via the choroid plexus. Both vesicle-mediated folate receptor (FR) transport and PCFT are required for this process to work optimally. The main dietary and supplementary forms of folate are shown, as are drugs such as methotrexate and trimethoprim, which inhibit folate synthesis. It is important to note that there is a concentration gradient between the blood and CSF, meaning 5-MTHF transport into the brain is energy dependent. See text for more details.

B. Oxidative stress has been proposed as a mechanism that could cause cerebral folate deficiency (see Aylett et al 2013). Oxidative phosphorylation (OXPHOS) within the mitochondria can result in electron leakage and production of oxidising species such as hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (OH$^-$$^-$). Antioxidants, such as ascorbate (ASC) and glutathione (GSH), scavenge these oxidants and protect the cellular components from oxidation. Oxidative stress can be caused as a result of increased oxidants (for example from defective oxidative phosphorylation or free
transition metal ions (Fe^{2+}) or impaired antioxidant defences, In conditions of oxidative stress, labile folate species could be oxidized, resulting in CFD (Aylett et al. 2013). It should be noted that the mitochondria have their own enzyme isoforms for some steps of folate metabolism and NAD(P)H produced during folate metabolism can be used to recycle ascorbate and glutathione.

Key: DHFR – dihydrofolate reductase; SHMT1 – cytosolic serine hydroxymethyltransferase; SHMT2 – mitochondrial serine-hydroxymethyltransferase; MTHFR – methylenetetrahydrofolate reductase; MTHFS – methenyl-tetrahydrofolate synthase; MTHFD1 – cytosolic methylenetetrahydrofolate dehydrogenase; MTHFD1L – mitochondrial methylenetetrahydrofolate dehydrogenase 1-like; mitochondrial methylenetetrahydrofolate dehydrogenase 2; FR – folate receptor; PCFT – proton-coupled folate transporter; RFC – reduced folate carrier;

Figure 3 5-MTHF chromatograms

HPLC chromatograms from our laboratory showing a prominent and well resolved 5-MTHF peak in ‘control’ CSF and no peak in a deficient control CSF from a patient with MTHFR deficiency. This highlights the importance of running ‘normal’ and disease quality controls in analytical methods.

Table 1
Overview of conditions that can lead to cerebral folate deficiency

Table 2
Reported cases with FOLR1 mutations
Table 1. Overview of conditions that can lead to cerebral folate deficiency.

<table>
<thead>
<tr>
<th>Defect</th>
<th>Clinical signs</th>
<th>Typical CSF 5-MTHF levels and other key biochemical changes</th>
<th>Treatment</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary causes of CFD</strong></td>
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<tr>
<td><strong>MTHFR</strong></td>
<td>Meomaloblastic anaemia or pancytoapatnena, seizures, hydrocephalus, ataxia</td>
<td>Generally very low (&lt;10nmol/L) Elevated plasma total homocysteine and low methionine</td>
<td>Betaine, folinic acid or 5-methyltetrahydrofle</td>
<td>Skovby 2003</td>
</tr>
<tr>
<td><strong>DHFR</strong></td>
<td>Meomaloblastic anaemia or pancytoapatnena, microcephaly,</td>
<td>Generally very low (&lt;10nmol/L)</td>
<td>Folinic acid</td>
<td>Banka et al 2011; Cario et al 2011</td>
</tr>
<tr>
<td>MTHFS</td>
<td>White matter changes, seizures, microcephaly</td>
<td>Low normal</td>
<td>5-methyltetrahydrofolate and intramuscular methylcobalamin</td>
<td>Rodan et al 2018</td>
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<tr>
<td>FOLR1</td>
<td>White matter changes, developmental delay</td>
<td>Generally very low (&lt;10nmol/L)</td>
<td>Folinic acid</td>
<td>Steinfeld et al 2009; Perez-Duenas et al 2010</td>
</tr>
</tbody>
</table>

**Secondary causes of CFD**

| Mitochondrial disorders | Pigmentary retinopathy, seizures, white matter changes, ataxia | Variable - very low to normal | Folinic acid | Garcia-Cazorla et al 2008; Batllori et al |

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<table>
<thead>
<tr>
<th>Condition</th>
<th>Symptoms</th>
<th>CFD Severity</th>
<th>Treatment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serine deficiency</strong></td>
<td>Microcephaly, growth retardation, hypertonia, epilepsy, hypogonadism</td>
<td>Mild to moderate CFD</td>
<td>Oral l-serine</td>
<td>Van der Crebban et al 2013; Damseh et al 2015</td>
</tr>
<tr>
<td><strong>DHPR deficiency</strong></td>
<td>Features of dopamine and serotonin deficiencies - dystonia, truncal hypotonia, temperature instability, disturbed intestinal mobility</td>
<td>Low-Normal</td>
<td>Folinic acid</td>
<td>Irons et al 1987; Smith et al 1985;</td>
</tr>
<tr>
<td><strong>AADC deficiency</strong></td>
<td>Features of dopamine and serotonin deficiencies - dystonia, truncal hypotonia, temperature instability, disturbed intestinal mobility</td>
<td>Low-Normal</td>
<td>Folinic acid</td>
<td>Wassenberg et al 2017</td>
</tr>
<tr>
<td><strong>Antibodies to folate receptor</strong></td>
<td>Schizophrenia, autism spectrum disorder.</td>
<td>Variable - low to normal</td>
<td>Folinic acid</td>
<td>Raemakers et al. 2005</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Disorders with low CSF 5-MTHF and low peripheral total folate</th>
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<tbody>
<tr>
<td><strong>PCFT1 deficiency</strong></td>
</tr>
<tr>
<td><strong>Nutritional deficiency</strong></td>
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<tr>
<td><strong>Environmental/Drugs such as Methorexate, L-dopa, antifolates</strong></td>
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<tr>
<td>Patient *</td>
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<td>-----------</td>
</tr>
<tr>
<td>1 (P9 in Grapp et al)</td>
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<tr>
<td>2 (sib of 1, P10 in Grapp et al)</td>
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<td>3 (P8 in Grapp et al)</td>
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<td>Case</td>
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<td>4 (P11 in Grapp et al)</td>
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<td>5</td>
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<td>6 (P1)</td>
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<td>7 (P2)</td>
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<td>8 (P3)</td>
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<td>9 (P4)</td>
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<td>10 (P5)</td>
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<td>11 (P6)</td>
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<td>12 (P7)</td>
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<td>Case No.</td>
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<td>21 (sib of 20)</td>
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<td>23</td>
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Key: * number in brackets refers to identification number in original publication; † age and laboratory-specific reference ranges given in brackets after patient’s value; ‡ precise value not stated; AEDs antiepileptic drugs; BG basal ganglia; CT computed tomography; DD developmental delay; dy day; GDD global developmental delay; ID intellectual disability; LAMM congenital deafness syndrome with labyrinthine aplasia, microtia and microdontia; m months; ND not determined; PV periventricular; SC subcortical; Sz seizures; T2W T2-weighted; wk week; WM white matter; y years
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Retention time in minutes

Control CSF

Deficient control CSF

No 5-MTHF peak in deficient control CSF

Fluorescence absorbance AU