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Review

The metabolic basis for developmental disorders due to defective folate transport

Ankuri Desai, Jeffrey M. Sequeira, Edward V. Quadros*

School of Graduate Studies and The Department of Medicine, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA

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ABSTRACT

Folates are essential in the intermediary metabolism of amino acids, synthesis of nucleotides and for maintaining methylation reactions. They are also linked to the production of neurotransmitters through GTP needed for the synthesis of tetrahydrobiopterin. During pregnancy, folate is needed for fetal development. Folate deficiency during this period has been linked to increased risk of neural tube defects. Disturbances of folate metabolism due to genetic abnormalities or the presence of autoantibodies to folate receptor alpha (FRα) can impair physiologic processes dependent on folate, resulting in a variety of developmental disorders including cerebral folate deficiency syndrome and autism spectrum disorders.

Overall, adequate folate status has proven to be important during pregnancy as well as neurological development and functioning in neonates and children. Treatment with pharmacologic doses of folinic acid has led to reversal of some symptoms in many children diagnosed with cerebral folate deficiency syndrome and autism, especially in those positive for autoantibodies to FRα. Thus, as the brain continues to develop throughout fetal and infant life, it can be affected and become dysfunctional due to a defective folate transport contributing to folate deficiency. Treatment and prevention of these disorders can be achieved by identification of those at risk and supplementation with folinic acid.

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1. Introduction

Folic acid (pteroylglutamic acid, PGA) also known as vitamin B 9,

* Corresponding author. MS#20, Department of Medicine, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, USA.
E-mail address: edward.quadros@downstate.edu (E.V. Quadros).

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Abbreviations used

SAM	S-adenosyl methionine
SAH	S-adenosyl homocysteine
GSH	glutathione
GSSH	oxidized glutathione
DHFR	dihydrofolate reductase
THF	tetrahydrofolate
MS	methionine synthase
GTP	guanosine triphosphate
BH4	tetrahydrobiopterin
BH2	dihydrobiopterin
Arg	arginine
Trp	tryptophan
NO	nitric oxide
5-HTP	5-hydroxytryptophan
5-HT	5-hydroxytryptamine (serotonin)
Phe	phenylalanine
Tyr	tyrosine
DA	dopamine
PG-F	polyglutamated folate
GGH	gamma-glutamyl hydroxylase
MG-F	monoglutamated folate
PCFT	proton coupled folate transporter
MRP3	multidrug resistance-associated protein 3
RFC	reduced folate carrier
DHFR	dihydrofolate reductase
DHF	dihydrofolate
THF	tetrahydrofolate
5MTHF	5-methyltetrahydrofolate
FRα	folate receptor alpha
GCS	glycine cleavage system
AMT	aminomethyltransferase
SHMT	serine hydroxymethyltransferase
SDH	sarcosine dehydrogenase
DMGDH	dimethylglycine dehydrogenase
MFT	mitochondrial folate transporter
SAMC	human S-adenosyl methionine (SAM) carrier

is an essential B-complex vitamin required for the transfer of carbon units in the intermediary metabolism of amino acid synthesis, purines, pyrimidines and in the production of S-adenosyl methionine (SAM) for methylation reactions [1]. Because of the role of this vitamin in such fundamental processes, deficiency of the vitamin or disruption in pathways can have a profound effect on cellular replication and metabolism [1]. In humans, folate deficiency can manifest as megaloblastic/macrocytic anemia in all age groups [2]. In the elderly, it could also lead to dementia and cognitive decline [3]. However, its consequences are most evident in pregnancy because folate deficiency during this period could have grave consequences on embryonic and fetal development [4–6]. This review focuses on the consequences of folate deficiency on fetal and neonatal brain development and the resulting functional deficits.

2. Folate dependent pathways

Folate in its many reduced forms is required for synthesis of purines and pyrimidine nucleotides, as well as metabolism of amino acids such as serine and homocysteine [1]. Folate deficiency can result from malnutrition or malabsorption as a result of intestinal diseases, drug interactions, chronic alcohol use [1,7], or disruption of transport due to genetic defects [8,9] or folate receptor autoimmune disorder [10,11].

Inside the cell, this vitamin enters the folate cycle after being reduced by dihydrofolate reductase into its biologically active form, tetrahydrofolate (THF) [1]. THF can then become a donor of single-carbon units as 5-methyltetrahydrofolate (5MTHF), 5,10-methylene, methenyl, and 10-formyl-THF. 5MTHF is also involved in the conversion of homocysteine into methionine, a precursor for the production of S-adenosyl methionine (SAM). As a methyl donor, SAM is critical for control of gene expression and stability through its role in methylation of DNA and histones. Thus, folate plays an integral role in epigenetic regulation and genome stability through its methylation function [12]. Further, folate is an essential component for DNA synthesis and repair. 5,10-methylene-THF provides single-carbon units for *de novo* purine synthesis, and also participates in pyrimidine synthesis at the level of conversion of deoxyuridine 5′-phosphate to deoxythymidine 5′-phosphate [1].

Further, glutathione (GSH) is synthesized in cells to serve as a major anti-oxidant to maintain intracellular redox potential and as a scavenger of free radicals in the cell. GSH metabolism is linked to the folate cycle through regulation of homocysteine and cysteine levels, thus linking the folate pathway to redox homeostasis within the body [13–15]. Oral folic acid supplementation in adults with type 2 diabetes has been shown to increase glutathione levels [16]. Additionally, the folate cycle is linked to the production of GTP needed for the synthesis of tetrahydrobiopterin (BH_4) as a cofactor. BH_4 is a critical cofactor in the production of various neurotransmitters, including serotonin, dopamine, and nitric oxide [17]. These various pathways in which folates are integral are shown in Fig. 1. While all replicating cells are affected by folate deprivation, its effects on the developing brain are most evident and to a large extent may be irreversible. As the brain continues to develop throughout fetal and infant life, it can be affected and become dysfunctional [18].

3. Folate absorption and cellular uptake

Folate is naturally found in leafy vegetables, nuts, beans, and meats and folic acid is included in fortified foods and vitamin supplements [7]. The minimum daily requirement for adults is ~50 μg of folate per day [19] and is expected to be higher (>400 μg) during pregnancy [20]. Absorption of folate occurs mainly in the upper small intestine [7]. Dietary folate is hydrolyzed from its ingested polyglutamate state to a monoglutamate by gamma-glutamyl hydrolase in the proximal small intestine [7]. This crosses the apical brush border in the proximal small intestine via the proton-coupled folate transporter (PCFT) and in the lower intestine via the reduced folate carrier (RFC) (Fig. 2A).

RFC is a classic transmembrane facilitative carrier, functioning as an anion antiporter system [7,21]. Thus, it is able to transport the negatively-charged folate against a concentration gradient by exchanging with organic phosphates located within the cell [7,21]. RFC functions optimally at a physiologic pH and has higher affinity for reduced folates, including 5-methyltetrahydrofolate (5MTHF) compared to folic acid [22]. RFC is ubiquitously expressed, including localization to the basolateral surface of renal tubular cells, the plasma membrane of hepatocytes, and the apical surface of choroid plexus [21]. Additionally, it is found throughout the intestinal tract, at the brush border surface of the duodenum, jejunum, ileum, and

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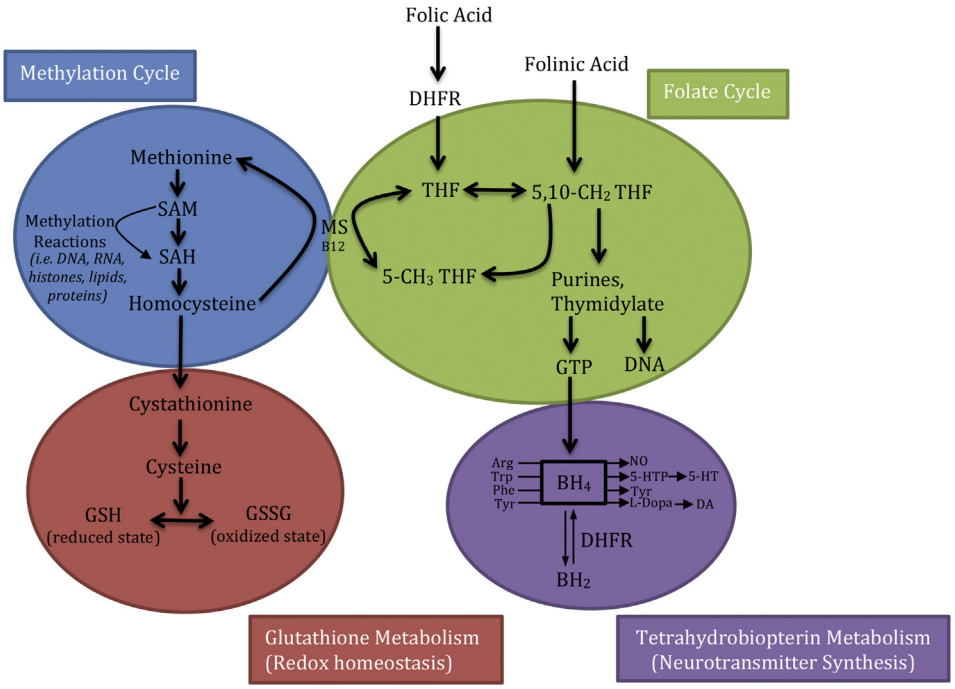

Fig. 1. Folate dependent pathways. Folate cycle is linked to various biological pathways, including DNA synthesis, methylation, redox homeostasis, and production of GTP needed for synthesis of neurotransmitters.

colon [21]. Its contribution to absorption of dietary folate in the gut is not clear.

Recently, the primary role of PCFT in intestinal absorption of folates was ascertained [23]. PCFT has been established as the major transporter of dietary folate in the proximal small intestine. Hereditary folate malabsorption is a disorder of impaired intestinal folate absorption. A loss of function mutation in the gene encoding PCFT has been found to cause this condition [24,25]. PCFT functions optimally at an acidic pH, acting as a symporter of folate with H^+ [7,23]. It is found on the brush border of the duodenum and proximal jejunum where it functions in the surrounding acidic microenvironment to absorb folate [23,26]. PCFT has also been found in other tissue, including kidney [24,26], liver [24,26], placenta [24], and choroid plexus [27]. It is unclear what function this transporter could have in physiologic, non-acidic pH environments. However, it has been suggested that PCFT could play a role in folate receptor-α mediated-endocytosis by exporting folate from the acidified endosomes into the cytoplasm [28,29].

Following absorption into enterocytes via PCFT or RFC, folate is exported across the basolateral surface by multidrug resistance transporter (MRP) 3 into the hepatic portal vein [30]. MRP1 and 5 are also expressed at the basolateral surface of enterocytes, but their contribution is not clear [31]. It is then delivered to the liver, where the mechanism of uptake via hepatocytes is not entirely clear, but may involve RFC, PCFT, or other transporters. The liver highly expresses RFC, only second to the placenta in expression level amongst human tissues [32]. However, the exact functioning of RFC in folate uptake in the liver has not yet been fully elucidated. Additionally, the liver expresses the highest level of PCFT amongst all human tissues, as measured by PCFT transcript present [26]. PCFT appears to localize to the membrane of hepatocytes, but its exact role in folate uptake in the liver is unclear [31,33] Organic anion transporters (OATP1B1 and OATP1B3) present on hepatocytes may also potentially play a role in folate transport [23].

Folate taken up by the liver in the monoglutamate form can be converted to the polyglutamate form via folylpolyglutamate synthetase for storage in the hepatocytes [34]. These can then be hydrolyzed back into the monoglutamate form to be secreted into the bile (enterohepatic folate cycle, where nearly all is reabsorbed), or released into systemic circulation for cellular uptake by tissues [35]. As much as half of all folate that is secreted into systemic circulation first enters the enterohepatic cycle [36]. Draining bile, and so effectively disrupting the enterohepatic cycle, leads to a 30–40% decrease in serum folate levels in 6 h [37].

The liver serves as the main storage organ for absorbed folates. Studies of folate concentration in human liver suggests that levels increase from birth until about the age of 20 (average of 8.8 μg folate per gram liver between ages 11–20 years), following which there is a slow decline (average of 6.9 μg folate per gram liver at age 80 years or older) [38]. No significant difference between males and females in regards to folate levels per gram liver were found [38].

In humans, there is a family of folate receptors encoded by three closely located gene loci within the chromosome 11q13.3-q13.5 region consisting of the alpha (FRα), beta (FRβ), and gamma (FRγ) isoforms and are expressed as single-chain glycoproteins [39–41]. The alpha and beta isoforms are glycosyl-phosphatidylinositol (GPI)-anchored to the cell membrane [42,43]. In contrast, the FRγ isoform does not have a GPI anchor for attachment to the plasma membrane, and is thus secreted [44]. Cellular uptake of folate and its reduced form, 5MTHF, which is the predominant form in the blood, occurs mainly via the FR pathway [45].

The expression profile of folate receptor isoforms differs, where FRα is expressed on epithelial cells, including the choroid plexus, thyroid gland, and the cells of the renal proximal tubules [46,47]. FRβ is expressed in the placenta, thymus, and spleen, as well as during the development of myeloid cells [39,48,49]. FRγ is expressed at much lower levels than FRα and FRβ, and is secreted from lymphoid cells found in the thymus, spleen, and bone marrow [39,42,44,50]. The normal biological functions of FRβ and FRγ are not as clear or as well studied as those of FRα. Increased levels of a soluble folate receptor have been reported in pregnancy [51] and in some cancers [52].

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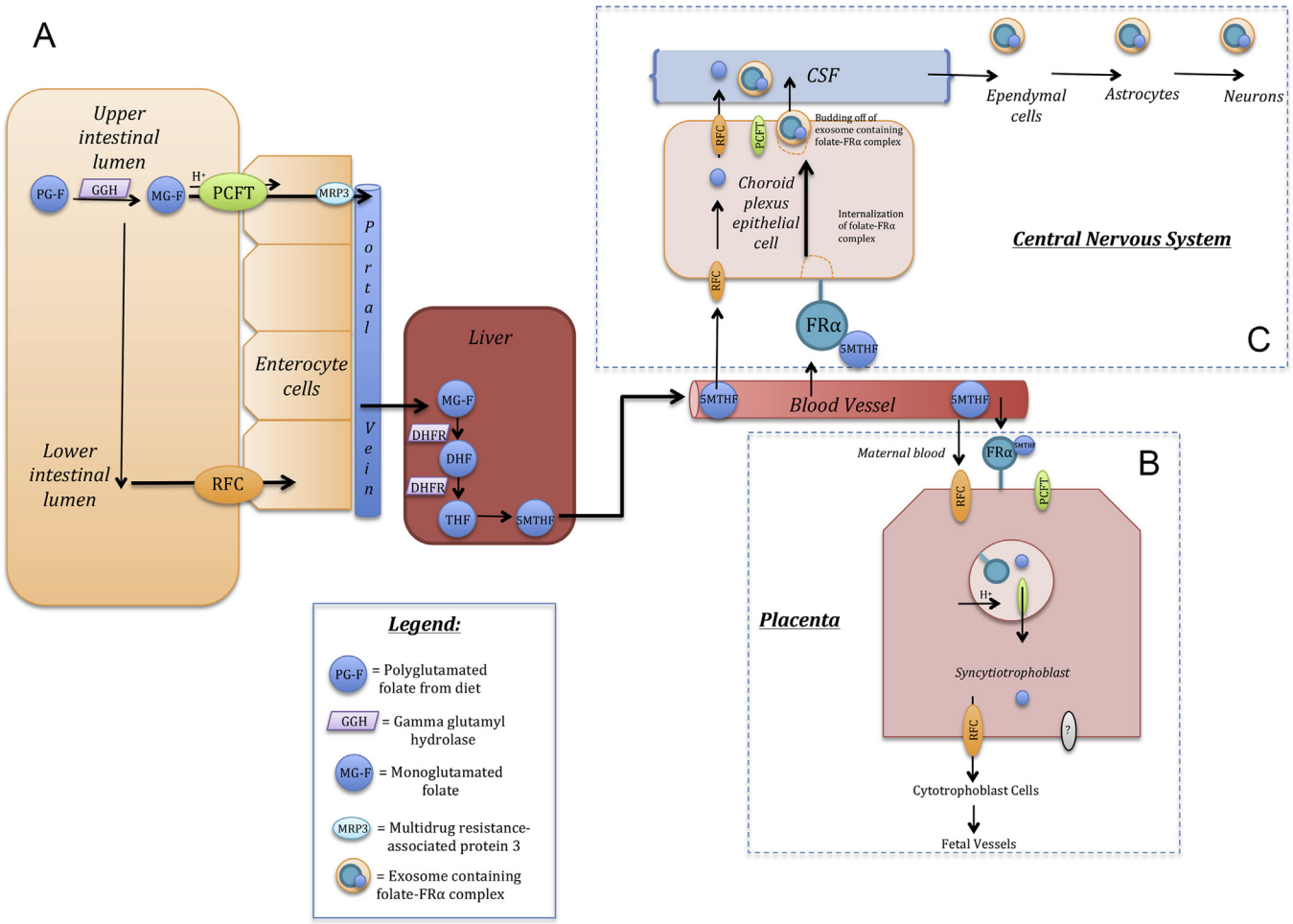

Fig. 2. Mechanisms of folate absorption and cellular uptake. (A) Folate is absorbed mainly in the upper small intestine, following which it is transported to the liver for distribution throughout the body. (B) Folate is transferred to the developing embryo through the placenta via FRα. (C) Folate taken up by choroid plexus cells can be transferred to the CNS via exosomes containing folate bound to FRα.

Folate uptake via FRα begins with folate binding the receptor on the cell surface. Subsequently, there is invagination of the membrane and formation of endosome, which traffics along microtubules to its perinuclear destination [28]. During this process, the endosomes acidify to a pH of ~6.0–6.5, causing release of the ligand from its receptor. The folate is then exported from the intact endosome into the cytoplasm [28]. It has been suggested that PCFT could play a role in this export of folate from the acidified endosome into the cytoplasm [28].

4. Folate requirement during pregnancy and fetal development

Fetal development is a time of global and continuous DNA synthesis and cell division, and thus folate requirement is increased to maintain the demand. There is not only rapid tissue growth of the fetus, but an enlargement of the uterus, growth of the placenta, and increased red cell mass during pregnancy [53]. All of these changes are heavily folate-dependent, warranting an increased need for folate during pregnancy [54].

The developing fetus receives folate from the mother through the placenta. It is proposed that folate in the form of 5MTHF, from the maternal circulation binds to FRα present on the microvillous membrane surface of placental syncytiotrophoblasts [29]. The PCFT is also believed to co-localize to this region and so it is proposed that receptor-mediated endocytosis of FRα–folate may also internalize the adjacent PCFT [28,29]. In this endosomal compartment, acidification allows folate to be released from FRα and is transported, coupled to H^+, into the cytoplasm by PCFT [28,29]. Folate is then exported out via RFC and possibly other transporters, into cytotrophoblast cells, and then to the fetal vessels [29] (Fig. 2B).

Randomized controlled trials have confirmed the protection from neural tube defects afforded by folic acid supplementation during the periconceptional period and early pregnancy [4,5]. Low folate status has been linked to increased risk of neural tube defects including spina bifida and anencephaly in humans [55]. The embryonic neural tube and neural crest cells are rapidly undergoing cell division during critical points in early development. Thus folate deficiency during this time can result in defects in the development of the tissues these cells give rise to, resulting in developmental defects [55]. The role of folic acid in the development or prevention of orofacial clefts in humans has been studied but results have often been mixed, and thus inconclusive [56,57]. Defects in folate metabolism have also been associated with other adverse pregnancy outcomes, such as increased risk of placental abruption and preeclampsia [53]. Further, hyperhomocysteinemia, which is indicative of folate deficiency or disruption in the folate cycle, has been associated with increased risk for intrauterine growth restriction and preterm birth [53].

Animal models have further confirmed that defects in folate uptake or transport result in abnormal development arising from

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apoptosis of the embryonic neural tube and neural crest cells [58,59]. Exposure to antibodies to folate receptors during early embryonic development has proven to be teratogenic in a rat model [60]. Further, mice that are nullizygous for *Folbp1* (analogous to human *FOLR1*, encoding FRα) were found to die *in utero* by embryonic day 10. Analysis of these embryos revealed that a majority had neural tubes that had not closed, compared to wild-type embryos that were all fully closed by embryonic day 9.5. Abnormalities in neural crest cell migration were noted in these nullizygous embryos. Folinic acid supplementation of dams rescued the phenotype [61]. Thus, folate uptake during early embryonic development is critical for proper neural development. A disturbance in the uptake of folate at the level of the receptor or transport defect can result in abnormal development. In addition to its direct effect on DNA synthesis and cellular replication, folate appears to play a major role in maintaining the methylation status and gene expression (*vide infra*). These effects may be subtle and yet clinically relevant.

While data on folate requirements and placental transport mechanisms are lacking in pregnancy and fetal development, animal studies have provided some insight into this process. Folate is actively transported across the placenta and made available to the fetus [62]. The expression of both FRα and FRβ in the placenta would suggest a role for both proteins in this process. In human placenta, the ratio of FRα and FRβ is about 3:1 and in the rat, it is about 1:1 [unpublished data]. While the role of FRβ in transplacental transport and fetal uptake of folate is not clear, FRα is likely to play a major role in fetal uptake of folate since administering an antibody to FRα in pregnant rats causes a significant decrease in fetal uptake of folic acid [unpublished data]. Structural development of the fetal brain requires adequate folate as evidenced by neural tube defects in the FRα knockout mouse [61] and decrease in neural tube defect pregnancies following folate supplementation [4].

5. Folate requirement in the CNS, transport, and utilization

In addition to the importance of folate levels for neurodevelopment of the embryo, there are neurological disorders in children with inherited defects of folate absorption and transport, as well as those with autoantibodies to the folate receptor alpha. Neurologic manifestations include cognitive and/or motor impairment, developmental delays, seizures, dyskinesia, and cerebellar ataxia [63,64]. Additionally, folate deficiency is also linked to neurologic and psychiatric disorders in adulthood and in the geriatric population and may include depression, dementia [65], and Alzheimer's disease [3,66]. Increased level of homocysteine, which can be due to folate deficiency, is a risk factor for cardiovascular disease and stroke [67,68]. The mechanism of folate deficiency mediated pathology in the brain is not fully defined. However, folate deficiency in animal models suggests that metabolic disruption of folate dependent pathways could affect synthesis of neurotransmitters, neuronal functions [69], and elevated homocysteine could covalently bind to proteins and affect their function [70,71]. Folate deficiency in combination with homocysteinylation of key neuronal proteins appear to alter vesicular transport along with differentiation and plasticity of hippocampal neurons [72].

The normal level of folate in the cerebrospinal fluid is 44.9 ± 13.2 nmol/L, which is higher than that found in serum, with a ratio of about 2.5–3.5 times greater concentration [73,74]. Similar values are also found in the pediatric population, with younger children having higher levels of 5MTHF in the CSF, implying the importance of folate levels for the developing CNS [75]. This could include folate involvement in synaptogenesis, ongoing development of processes and connections between neurons, as well as in neurotransmitters and signaling [76].

The choroid plexus cells of the central nervous system express high levels of FRα [27,77]. In order to support the folate concentration gradient between the plasma and the CSF, it has been proposed that folate from blood is transported across the basolateral surface of choroid plexus cells via FRα [27]. Once inside the cell, it has been hypothesized that the proton-coupled folate transporter (PCFT) could be responsible for export of folate from the cell into the CSF [27]. A recent study suggests that the PCFT export pathway may only play a minor role in this process. Instead, it is proposed that after folate is taken up at the basolateral surface by FRα, the folate- FRα complex is internalized and processed into microvesicular bodies called exosomes. Eventually, these FRα-containing exosomes bud off from the apical surface of choroid plexus cells and circulate in the CSF until they cross the ependymal layer and are taken up by brain parenchymal cells [78] (Fig. 2C). The precise mechanism of this process is not known and therefore, its role in folate transport to the brain needs further exploration.

Additionally, repair and regeneration of the CNS following injury is aided by folic acid. In an animal model, CNS axon regeneration and growth following injury was found to improve greatly following folic acid supplementation [79]. This is believed to occur via adequate methyl donors and increased DNA methylation. The effect was shown to be biphasic and reduced at higher doses [80]. While the reason for this effect is not evident, use of a racemic mixture of folic acid at high doses could potentially inhibit folate dependent reactions. However, adequate folate status in the CNS appears to be critical at all stages of life and CNS injury, including during embryonic development, childhood, adulthood, and aging.

The folate content of the fetal brain at various stages of development is not known but is likely to be high based on the observation of higher folate content of the brain on post natal day 10 of ~45 ng/mg protein which decreases to ~11 ng/mg protein by day 23, equal to the level in adult rat brain [81]. What is remarkable about the brain folate status is that even under extreme folate deprivation, the brain retains most of its folate. This has been demonstrated in rats maintained on low folate for 3 weeks [82] and 25 weeks [83]. Despite this, folic acid administered to deficient rats is preferentially shunted to the brain [84]. Our recent study of behavioral deficits in rats made folate deficient only during the pre-weaning period (first 23 days following birth) showed a 70% decrease in liver folate and yet brain folate was unchanged [81]. The brain is unique in that most of the folate is tetrahydrofolate and other reduced derivatives, with 5MTHF accounting for only 18% of the folate [85]. It has been suggested that increased glutamate residues may help retain folate in tissues during deficiency [82]. The predominant form of folate in blood, CSF, and tissues is 5MTHF. In tissues, a small percentage is as monoglutamate (found in small intestine and kidney, since pteroylmonoglutamates are transferred readily across membranes) with penta and hexa glutamates accounting for ~80–100% of the cellular folate [82]. Most of the folate found in the blood is monoglutamate [86]. However, retention of folate with more glutamate residues conjugated is not likely to be the only mechanism in the brain for retention of folate since only a small fraction of the brain folate has more than 6 glutamates [82]. The preferential retention of folate in the brain remains an intriguing observation that raises a number of questions. Is folate critically important to the brain not just during development but also throughout life? Is systemic folate preferentially shunted to the brain at the expense of other tissues? Despite no decrease in brain folate, why does homocysteine increase in the brain? Is this increase more of a reflection of increase in plasma homocysteine? If brain folate does not decrease, why do children with CFD and autism show improvement with high dose folinic acid treatment? Is constant flow of folate to the brain more important than the brain folate content?

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6. Developmental disorders due to defective transport or utilization

As discussed, adequate levels of folate are needed during development of the embryo. Folate deficiency during this period can lead to malformations of the neural tube, as well as cleft lip and palate [5,6]. Genetic disorders of folate metabolism and transport have also been linked to abnormal development; these include mutations of methylenetetrahydrofolate reductase (MTHFR), dihydrofolate reductase (DHFR), PCFT, and FRα that results in decreased activity of the enzyme or protein [87]. MTHFR mutations and polymorphisms are the most common defect in folate metabolism associated with numerous disorders but evidence linking this gene to specific disorders is lacking.

Gene defects of FRα or autoantibodies to FRα could lead to cerebral folate deficiency syndrome (CFD), a neuro-metabolic disorder in which there is decreased levels of 5MTHF in the cerebrospinal fluid [10]. These children suffer from severe neurologic symptoms including seizures, psychomotor retardation, cerebellar ataxia, pyramidal tract signs in the legs, and dyskinesia [10,88]. CFD has a broad clinical presentation; on average, there is normal development during the first four months of life, followed by deceleration of head growth, and marked irritability setting in from 4 to 6 months of age [63]. Psychomotor retardation can become apparent at six months, as developmental milestones are delayed or not attained [63]. Progressive ataxia, pyramidal tract signs in the legs, and dyskinesias may then develop around 1.5–2 years of age and older patients may suffer from spastic tetraplegia [63]. CFD is also found in children with Rett Syndrome and Aicardi-Goutieres Syndrome [89,90]. CFD syndrome responding to folinic acid treatment has been reported in mutations of the FRα gene [77,91,92]. This clinical phenotype raises some important questions. If FRα is the primary transporter of folate to the fetus, why do these children develop normally during gestation and then develop CFD during the first year of life? These children develop low CSF folate and white matter lesions in the brain in the first and second years of life [63]. Is there a normal allele of FRα that is rendered inactive after birth? Could a yet unidentified mechanism operate to meet the fetal needs for folate? The clinical history of these cases implies that another mechanism or transport pathway likely meets the requirement of the fetus *in utero* and needs further study. What is remarkable about CFD either due to mutations in FRα or due to FRα specific autoantibodies is that most children respond well to high dose folinic acid treatment. Pharmacologic doses of folinic acid can be transported to the brain via the reduced folate carrier and could correct some of the metabolic deficits by restoring neurotransmitters and synaptic functions.

7. Role of folates in mitochondrial dysfunction

In eukaryotic cells, one-carbon metabolism is highly compartmentalized with distinct folate-mediated reactions occurring in the cytoplasm, mitochondria and in the nucleus [93]. Fig. 3 shows the compartmentalization and participation of folate in various reactions within the mitochondria. There is an interdependence of the folate-mediated reactions in these different regions of the cell [94]. The majority of folate in the liver is distributed equally between the cytoplasm and mitochondria, with only low levels in the nucleus [95]. Folate uptake into the mitochondria is facilitated by an inner mitochondrial membrane transporter with preference for the reduced monoglutamate forms [96]. Further exchange between the cytoplasm and mitochondria occurs via one-carbon donors including serine [97], glycine [98], and formate [97]. Serine hydroxymethyltransferase (located in both the cytoplasm and mitochondria) catalyzes the conversion of serine to glycine, transforming THF into 5,10 methylenetetrahydrofolate [94]. The glycine and serine can be exported into the mitochondria, where it is involved in identical reactions. Sarcosine can also be formed from glycine via glycine-*N*-methyltransferase [99]. In the mitochondria, THF formed from the production of glycine from serine is eventually converted into formate, which is then transferred back to the cytoplasm [94]. Folate appears to have a global effect in maintaining mitochondrial stability functions since folate deficiency causes increased mutations and a decrease in the content of mitochondrial DNA [100].

Many of the folate transport and uptake processes in the GI tract as well as the brain are energy dependent and disruption of mitochondrial function is likely to affect these processes and lead to metabolic disruption of folate pathways [101].

In clinical studies, mitochondrial dysfunction has been frequently associated with neurodevelopmental disorders, including autism spectrum disorders (ASD). The prevalence of mitochondrial dysfunction has been much greater in ASD children than in the general population (5% compared to ~0.01%) [102]. Both magnetic resonance spectroscopy (MRS) [103] as well as examination of postmortem brain samples have been used to document mitochondrial dysfunction [104,105] and disruptions have been found in mitochondrial genes and proteins including electron transport chain complexes I–V [105] and aconitase [106].

Further, increased oxidative stress has been reported in brains of those with ASD compared to controls [107]. Studies have reported increased biomarkers of oxidative stress, including decreased reduced glutathione and glutathione redox/antioxidant capacity and increased 3-nitrotyrosine [106,108], Cytoplasmic folate content dictates folate transport into the mitochondria and therefore any disruption of cellular folate uptake is likely to affect folate content of mitochondria. Since mitochondrial folate metabolism is intricately intertwined with mitochondrial functioning, decreased folate transport into the mitochondria could lead to increased oxidative stress or damage and dysfunction, which has been shown to be associated with changes in the brains of those with ASD. Monitoring mitochondrial function before and during treatment with folinic acid in combination with other interventions may be useful in identifying response to therapy.

8. Methyl deficiency and epigenetic effects

As seen in Fig. 1, folate metabolism is closely linked to the methylation cycle. Folate deficiency can lead to impairment of the cell's ability to perform transmethylation reactions including methylation of DNA, histones, RNA, and coregulators of nuclear receptors [12]. The ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) is considered the methylation potential within a cell [109]. The levels of these molecules affects the activity of SAM-dependent methyltransferase enzymes [109]. SAH is known to tightly bind to the active site of these methyltransferases, inhibiting their activity and leading to DNA hypomethylation [110]. Reports have shown that increased levels of SAH in patients with uremia were associated with DNA hypomethylation, leading to altered allelic expression in both imprinted and sex-linked genes [111]. Treatment with oral methyltetrahydrofolate to reduce the homocysteine levels in these patients also corrected the altered DNA methylation patterns and gene expression [111]. These observations provide a link between folate and methylation and connect folate to epigenetic changes and gene expression. Epigenetic influences are most evident in embryonic and fetal development with folate deficiency as well as excessive folate potentially affecting expression profiles of multiple genes (for detailed discussion of fetal programming and epigenetic effects, see Ref. [12]). Among these are the so called “imprinting genes” whose

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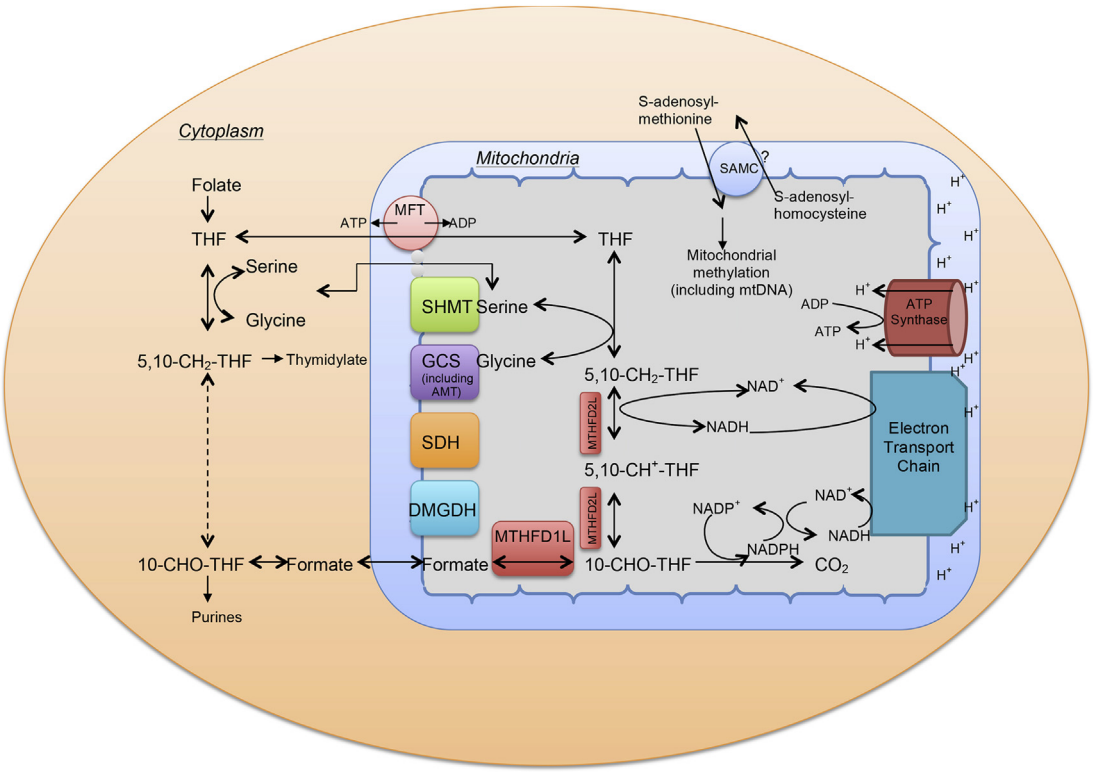

Fig. 3. Mitochondrial transport and utilization of folate. Metabolism of single carbon units in the mitochondria is linked to that of the cytoplasm through THF shuttling through MFT, the serine/glycine shuttle, transport of sarcosine and dimethylglycine, and formate shuttling. Utilization of single carbon units within the mitochondria leads to production of ATP through generation of redox cofactors (electron carriers), linking folate metabolism to both redox status and energy production. SAMC is proposed to transport SAM into the mitochondria where it can participate in methylation reactions, including that of mitochondrial DNA. There is no methionine adenosyltransferase activity inside the mitochondria so it is believed SAMC transport is the only mechanism through which SAM enters the mitochondria for methylation of mitochondrial DNA, RNA, and proteins. Disruption of folate status can thus affect redox status, energy production, and methylation (and thus expression/functioning of genes and proteins) within the mitochondria.

expression is regulated at the germline with "parent-specific" expression of a single allele in the offspring [112]. Such control of imprinting genes could also operate in the brain to dictate behavioral characteristics [113].

Methyl deficiency during gestation and pre-weaning has been associated with cognitive decline, behavioral changes, and long-term brain alterations in rat models [70,114,115]. Neuronal cell loss was noted and behavioral changes were not correctable following a normal diet after weaning [70,114]. Behavioral changes, including impaired spatial learning and memory, were also reported in mouse models of methyl donor deficiency where mice were fed a diet deficient in B-vitamins [116].

Epigenetic changes have been implicated in various neurodevelopmental disorders, including ASD. Rett Syndrome, which is one of the most common causes of mental retardation in girls, has been attributed to a mutation in the *MECP2* gene [117]. This gene encodes a protein that is part of the methyl CpG-binding domain protein family and consequently is an epigenetic regulator of gene expression [117,118]. MeCP2 protein is also able to regulate transcription as a member of histone remodeling complexes [119]. Altered expression of the *MECP2* gene has been found in ASD as well [120,121]. Unlike Rett Syndrome, where the majority of cases are due to mutations in this gene, coding mutations in *MECP2* are relatively uncommon in ASD, thus favoring the possibility of altered expression due to epigenetic mechanisms as a potential cause [120,121]. Significantly increased *MECP2* promoter methylation in ASD, compared to controls has been correlated with decreased MeCP2 protein expression [121]. While the mechanism of this hypermethylation is not clear, silencing of MECP2 is likely to decrease methylation and activation of other genes. The precise control and regulation of DNA methylation and the role of folate in this process is not clear; both hypomethylation and hypermethylation of certain genes have been reported in folate deficiency as well as folate supplementation [122–125]. Specifically in folate deficiency, global hypomethylation of DNA can be noted, but targeted hypermethylation of specific genes is also concurrently present in the same cell. Therefore, regulation of methylation must reside within the enzymes involved in the process and factors that regulate their expression [122]. Disruption of this critical balance in control of methylation dictates epigenetic influences on expression and function of genes. Thus, methyl deficiency and epigenetic changes, which are linked to folate metabolism, can play a crucial role in neurodevelopment and associated disorders.

9. Folate receptor autoimmune disorders

There are also non-genetic disorders of the folate pathways that can lead to developmental disorders, such as the presence of autoantibodies that bind to FRα and greatly impair its function [126]. The presence of these autoantibodies was first reported in women with a history of neural tube defect pregnancies [127]. Prior to this discovery, the explanation for the benefits attained following folate supplementation was not evident because most women with NTD pregnancy do not have genetic mutations of the folate metabolism pathways or systemic folate deficiency [128]. The discovery of FRα autoantibodies provided a potential mechanism by which fetal folate insufficiency could occur in the presence of normal folate status in the mother. Two types of the FRα autoantibodies have been identified based on their functional property and epitope specificity; blocking autoantibody, which prevents binding of folate

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to FRα (by virtue of directly or sterically interfering with folate binding), and binding autoantibody, which may exert its pathology by triggering an antibody-mediated immune reaction and inflammation [129]. What inflammation markers could potentially be triggered remains an open question and therefore, monitoring cytokines and chemokines in the blood of patients positive for the FRα autoantibody could provide some insight into the pathology.

These antibodies were subsequently identified in children with CFD [10]. The presence of such autoantibodies can elucidate the pathophysiology of decreased folate levels found in the cerebrospinal fluid of affected children as FRα is found on choroid plexus and is needed for folate uptake and transcytosis to the brain. Antibodies that can block this process can effectively reduce folate levels in the CSF [11]. In addition to blocking folate uptake in the brain, antibodies that bind to FRα could be transported into the brain parenchyma as a complex in exosomes Fig. 4.

Folate uptake in the developing brain of a fetus or infant, could thus be decreased in the presence of FRα antibodies, which could consequently result in the pathology of behavior and cognition, as seen in many neurodevelopmental disorders. Treating CFD children with folinic acid has provided clinical improvement, further implicating low folate levels causing these symptoms, and

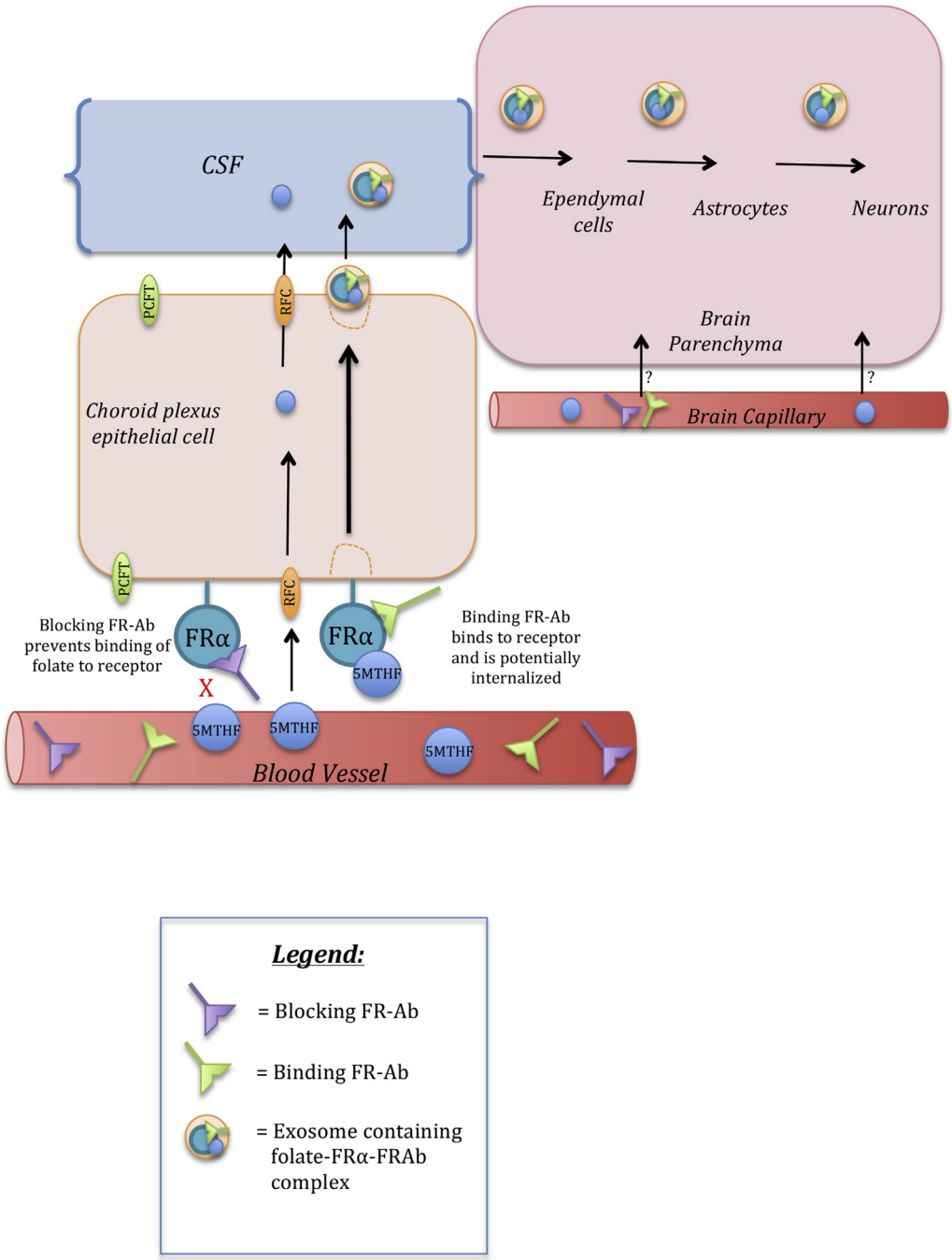

Fig. 4. Potential effect of exposure to FRα antibody in the brain. FRα antibody could block uptake of folate at the basal surface of choroid plexus epithelial cells, thus producing folate deficiency in the CNS. Additionally, FRα antibody bound to holo-FRα on the basal surface of choroid plexus cells could be transported into the brain parenchyma as a complex in exosomes.

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demonstrating the importance of recognizing and treating FRα autoimmunity [11,126].

In addition to CFD, FRα autoimmunity has been implicated in numerous developmental disorders in children including Rett syndrome [130] and low functioning autism with neurological deficits [131]. Maternal antibodies reactive against fetal brain proteins have been reported in a small number of mothers of autistic children [132]. They identified two proteins, a 37-kDa and a 73-kDa but did not report the identity of these proteins. Incidentally, the 37-kDa protein is very similar in size to the FRα.

Immunohistochemical analysis of postmortem brains from patients with autism indicated glial activation and inflammation in most regions, including the cerebellum [133]. The extensive inflammation and the presence of proinflammatory cytokines in the brain and the CSF point to an active immune response. Whether this is secondary to an autoimmune response to FRα and folate deficiency remains to be determined. Studies in the rat have further demonstrated that antibodies to folate receptors produce developmental abnormalities in the embryos [60].

FRα autoantibodies are prevalent in ASD with more than 70% of these children positive for serum FRα autoantibodies [134]. Many of the parents of autistic children are also positive for these autoantibodies [135]. Thus, the presence of autoantibodies against FRα, whether transferred to the fetus from the mother during pregnancy or developed postnatally in the infant, can disrupt the transfer of folate to the brain, depriving the brain of this essential nutrient and consequently leading to potential changes in the brain that cause the behavioral deficits seen in ASD. Thus, genetic disturbances of folate metabolism or the presence of autoantibodies to FRα can impair and cause disruption in physiologic processes dependent on folate, resulting in a variety of developmental disorders.

10. Prevention and treatment of these disorders

Maintaining adequate folate status has proven to be important during pregnancy for the development of the embryo as well as neurologic development and functioning in children. Folate supplementation during pregnancy and the periconceptional period can prevent neural tube defect births [4,5]. Rescue of embryos with folinic acid administration has also been demonstrated in animal models, including a model of FR-antiserum exposure during gestation [60] as well as in a *folbp1*-knockout model [61].

Folate supplementation in children with CFD has also shown to be efficacious [77,91,92]. Treatment with pharmacologic doses of folinic acid has led to reversal of symptoms in many patients diagnosed with CFD, including those with genetic mutations [77,91,92]. For example, a loss of function mutation for the *FOLR1* gene encoding FRα due to a nonsense mutation led to brain-specific folate deficiency [77]. For this inherited disorder, folinic acid therapy was shown to reverse symptoms and improve functioning in these patients [77]. Additionally, children with CFD due to presence of autoantibodies to FRα have also greatly benefited from treatment with folinic acid [10].

More recently, treatment with high-dose folinic acid in a subset of children with autism, in whom the neurologic presentation is not complicated by other genetic or metabolic disorders, has resulted in remarkable improvement in functional deficits [134,135]. Thus, the benefit of folinic acid supplementation has also been observed in ASD children positive for serum FRα autoantibodies. A folinic acid dose of 0.5–2.0 mg/kg body weight has been used in the treatment of these patients. The advantage of using folinic acid is that it can be transported via the RFC and can readily participate in folate dependent reactions whereas folic acid and MTHF will need to be further processed prior to use in folate dependent reactions. While the racemic mixture of folinic acid is currently used, the availability of levofolinic acid in the future would make this the preferred form to use since the D-isomer is not physiologically active and at the high doses used could potentially interfere with folate dependent pathways.

11. Epilogue

Prevention of most of these developmental disorders should be the primary goal. Prenatal testing of women and men for mutations of folate dependent pathways and for FRα autoantibodies could pave the way for pre-pregnancy treatment of both parents and the mother throughout pregnancy. Treating the newborn with folinic acid at an early age may prevent many of the neurologic deficits. While the debate on excessive use of folic acid continues, for the most part, pharmacologic use of folinic acid has been deemed relatively safe and in this case, the immediate benefits may outweigh the potential risks of long-term use of folinic acid.

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Conflict of interest statement

Two of the authors (JMS and EVQ) are inventors on a US patent for the detection of FRalpha autoantibodies issued to the Research Foundation of the State University of New York, USA.

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