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Prevention of brain disease from severe 5,10-methylenetetrahydrofolate reductase deficiency ☆

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Abstract

Over a four-year period, we collected clinical and biochemical data from five Amish children who were homozygous for missense mutations in 5,10-methylenetetrahydrofolate reductase (*MTHFR* c.1129C>T). The four oldest patients had irreversible brain damage prior to diagnosis. The youngest child, diagnosed and started on betaine therapy as a newborn, is healthy at her present age of three years. We compared biochemical data among four groups: 16 control subjects, eight heterozygous parents, and five affected children (for the latter group, both before and during treatment with betaine anhydrous). Plasma amino acid concentrations were used to estimate changes in cerebral methionine uptake resulting from betaine therapy. In all affected children, treatment with betaine (534 ± 222 mg/kg/day) increased plasma *S*-adenosylmethionine, improved markers of tissue methyltransferase activity, and resulted in a threefold increase of calculated brain methionine uptake. Betaine therapy did not normalize plasma total homocysteine, nor did it correct cerebral 5-methyltetrahydrofolate deficiency. We conclude that when the 5-methyltetrahydrofolate content of brain tissue is low, dietary betaine sufficient to increase brain methionine uptake may compensate for impaired cerebral methionine recycling. To effectively support the metabolic requirements of rapid brain growth, a large dose of betaine should be started early in life.

Keywords: Amish; Betaine; Blood–brain barrier; Homocysteine; Methionine; 5,10-Methylenetetrahydrofolate reductase; Newborn screening; *S*-Adenosylmethionine

Introduction

5-Methyltetrahydrofolate (5MTHF) is used to recycle homocysteine back to methionine in human tissues [1]. Methionine recycling by the brain is absolutely dependent on 5MTHF [2], whereas liver and kidney can recycle homocysteine to methionine by an alternate route, using betaine as the methyl donor for betaine-homocysteine-*S*-methyltransferase (BHMT) [3]. The transmethylation cycle produces *S*-adenosylmethionine, the methyl donor for a large group of methyltransferase enzymes. Collectively, methyltransferases catalyze an array of reactions necessary for brain development, including DNA modification, phospholipid synthesis, polyamine formation, and myelin assembly [4]. Homozygous loss-of-function mutations in 5,10-methylenetetrahydrofolate reductase (MTHFR) result

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in systemic 5MTHF deficiency and are associated with slow brain growth, severe neurological disability, and untimely death [5,6].

In October 2002, a four year-old boy from a small Old Order Amish settlement in Somerset County, Pennsylvania, presented to our clinic with severe psychomotor retardation. Magnetic resonance imaging (MRI) showed generalized cerebral atrophy and hypomyelination (Fig. 1). He had elevated plasma total homocysteine (89 μM; normal 6 ± 2 μM) and low cerebrospinal fluid (CSF) 5MTHF (5 nM; normal 40–150 nM). Direct sequencing of *MTHFR* revealed homozygous c.1129C>T (R377C) mutations [7].[1] Over the ensuing months, we identified three other disabled Amish children who were homozygous for *MTHFR* c.1129C>T, and found 68 *MTHFR* c.1129C>T heterozygotes among 230 healthy Amish residents of Somerset County (estimated population carrier frequency 30%).

All four of the affected children were healthy at birth, but they had slow head growth and arrested development within a few months (Fig. 1). This observation was consistent with other reports, which showed that to prevent brain disease from severe MTHFR deficiency, betaine therapy must be started early in life [8–10].[2] Accordingly, we implemented a method to test Amish newborns for the *MTHFR* c.1129C>T mutation using dried filter paper blood spots [11]. Our observations over a four-year period demonstrate the value of newborn screening for MTHFR deficiency and clarify the therapeutic actions of betaine.

Patients and methods

Patients and clinical methods

The study was approved by the Institutional Review Board of Lancaster General Hospital. Written consents from informed parents were obtained for direct DNA sequencing and molecular testing by real-time polymerase chain reaction (PCR). All data were analyzed by two of the authors (KAS and SHM).

Between October 2002 and September 2003, we identified five Old Order Amish children (present median age 4.5 years, range 3–21 years) who were homozygous for c.1129C>T mutations in *MTHFR*. Four of these children presented with clinical signs of brain disease at ages 7 months, 13 months, 4 years, and 18 years. The fifth child was diagnosed as a healthy newborn using a real-time PCR test (see Laboratory Methods below). For each patient, two of the authors (KAS and DHM) provided all medical care and recorded information about growth, diet, medications, and illnesses.

We treated affected children with betaine anhydrous (Medisca, Plattsburgh, NY), 534 ± 222 mg/kg/day, and aspirin, 40.5 mg per dose, every other day. Other treatments were given inconsistently and over various time intervals, and included creatine (75–100 mg/kg/day), vitamin B12 (25 mg/day), vitamin B6 (25 mg/day), folic acid (4 mg/day), and 5MTHF (2.4–3.2 mg/day). For the youngest child in the group, diagnosed as a newborn, the only medications given during the study period were betaine and aspirin. Children who were hospitalized or undergoing outpatient procedures had an emergency medical letter that outlined the risk for thromboembolism, indicated the use of aspirin, and warned against the use of nitrous oxide [6].

Laboratory methods

The *MTHFR* c.1129C>T mutation was detected either by direct sequencing of DNA isolated from peripheral blood [12] or by real-time PCR LightCycler (Roche Applied Science, Indianapolis, IN) analysis of DNA extracted from dried filter paper blood specimens [11]. The real-time PCR method was developed and validated by Pediatrix Screening Laboratory (Bridgeville, PA). Mutation detection was based on the different melting peaks of the c.1129C>T (58 °C) and wild type (65 °C) *MTHFR* alleles. One standard hole-punch was sufficient for the PCR-based method; this punch was obtained from the same card used for mandatory state newborn screening. To estimate population carrier frequency, we used the PCR method to test for *MTHFR* c.1129C>T in 230 healthy adult volunteers from the Somerset Amish community, all of whom consented in writing to molecular testing. The first 100 of these samples were also tested by direct DNA sequencing to validate the LightCycler method; there was a 100% correlation between the two methods.

At six-month intervals over a four year period (2002–2006), we collected blood from five affected children, from eight *MTHFR* c.1129C>T heterozygote parents, and from 16 healthy Amish and non-Amish adult volunteers. Affected patients and their parents provided more than one blood sample over the study period, such that the number of individual data sets (n) used for statistical analyses exceeded the number of patients (N in column headings of Table 1): for eight *MTHFR* c.1129C>T heterozygotes, $n = 20$; for five *MTHFR* c.1129C>T homozygotes prior to treatment, $n = 8$; and for the same five patients on betaine therapy, $n = 13$. We did not measure blood vitamin levels in asymptomatic control subjects; the patient values listed in Table 1 were compared to pediatric reference ranges provided by the laboratories that performed these tests. Cerebrospinal fluid was obtained from one affected child prior to treatment, as well as three affected children on betaine therapy.

Previously described methods were used to measure plasma, serum, and cerebrospinal fluid (CSF) metabolites [13–19]. Plasma concentration ratios (mol:mol) were calculated for methionine to total homocysteine (Met/tHcy), *S*-adenosylmethionine to *S*-adenosylhomocysteine (AdoMet/AdoHcy), and total cysteine to cystathionine. Previously reported CSF data from patients with MTHFR deficiency and control subjects were used to complete Table 2 [20–23]. Published data about organ distribution of enzyme activities and tissue metabolite content (in μmoles per kg wet weight) were used to construct Fig. 4 [1,24–28].

Lymphocyte global DNA methylation was measured for one *MTHFR* c.1129C>T carrier, one affected child prior to treatment, and another affected child on betaine therapy. Lymphocyte DNA was isolated by a standard method (FlexiGene DNA Kit; Qiagen, Valencia, CA) and methylation was determined by measuring methylated cytosine as a percentage of total cytosine [29].

Calculation of brain amino acid uptake

A custom Excel spreadsheet (Microsoft Corporation) was designed to estimate the transport of methionine and nine other amino acids from blood to brain. Briefly, a group of neutral amino acids (glutamine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine), most of which are essential, compete for entry into the brain across a common sodium-independent facilitative

[1] *MTHFR* c.1129C>T (R377C), a severe mutation associated with only 2% activity in vitro, was originally described as *MTHFR* c.1141C>T (R377C) [7]. The nomenclature used in the present report reflects current recommendations (http://www.hgvs.org/mutnomen/): gene numbering begins with the first nucleotide of the initiator codon, always an adenine of the ATG triplet.

[2] A patient diagnosed prenatally with MTHFR deficiency was originally described in 1985 [10]. She was treated from neonatal life to her present age of 21 years with betaine, folic acid and cobalamin. She remains physically and neurologically healthy (Dr. Flemming Skovby, personal communication; information reproduced with his permission).

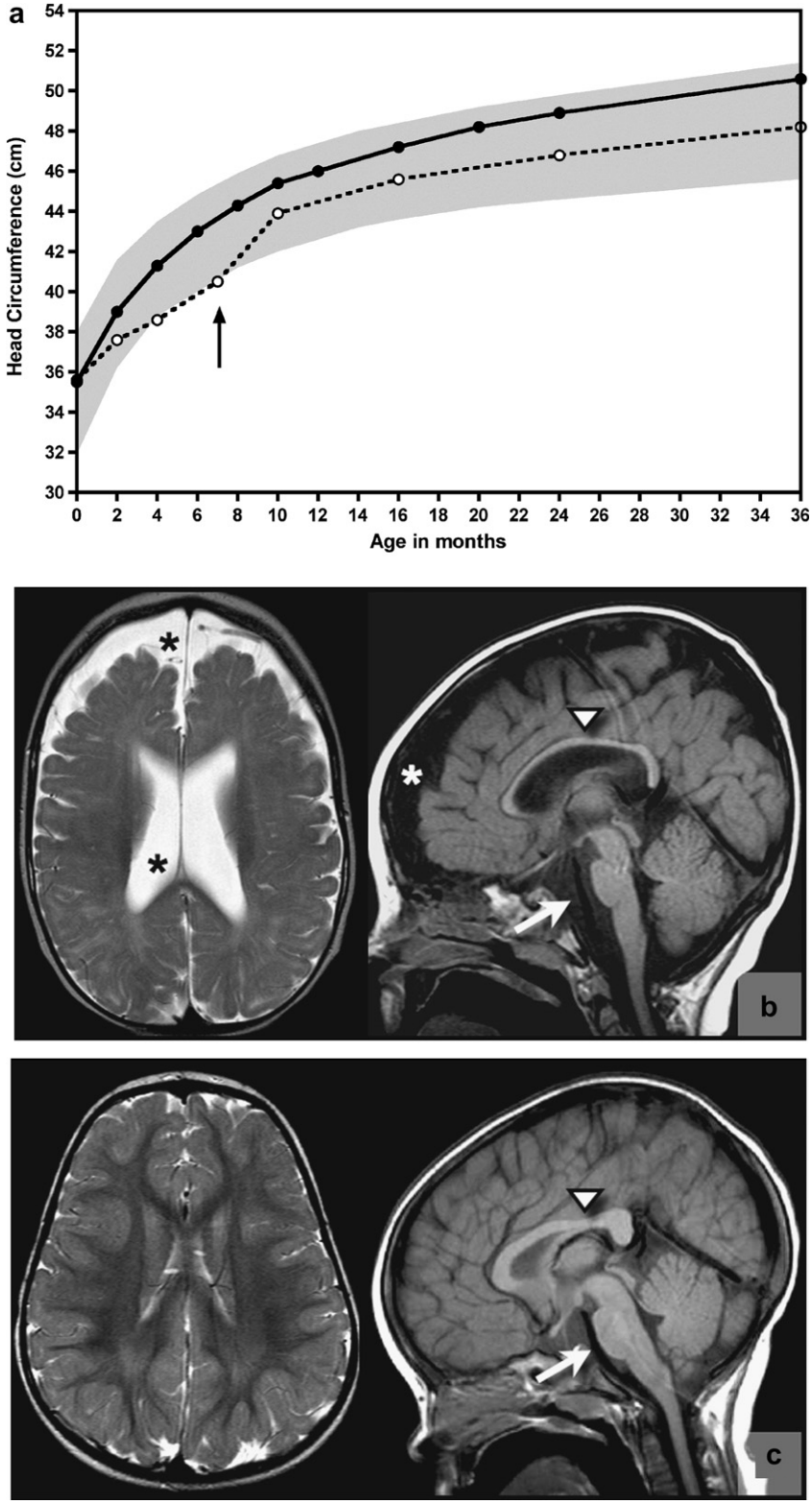

Fig. 1. Patterns of brain growth in Amish children with MTHFR deficiency. (a) Brain growth, as indicated by change in head circumference, for one child who started betaine therapy (arrow) at 7 months of age (dashed line), and one who started betaine therapy at 16 days of age (solid line). Increased head growth on betaine therapy was seen in infants who started betaine at 7 and 13 months of age, but not in those who started treatment at 4 and 18 years of age. The shaded area represents the normal range (+/− 2 standard deviations) for head size from birth to age 36 months. (b) Axial and sagittal T2-weighted MRI of a 14 month-old patient prior to the start of therapy show large ventricular and extra-axial fluid spaces (asterisks) secondary to global brain atrophy. The spinal cord, brainstem (arrow), and corpus callosum (arrowhead) are thin, secondary to poor neuron growth and hypomyelination. (c) Comparable images (obtained at age 18 months) from a child who began taking betaine during the newborn period show a fully developed and appropriately myelinated brain.

Table 1
Blood measurements in MTHFR 1129C>T homozygotes, MTHFR 1129C>T heterozygotes, and control subjects

			MTHFR 1129 C>T Homozygotes		
	Control subjects ($N = 16$)[a]	1129C>T Heterozygotes ($N = 8$)	Untreated ($N = 5$)	Betaine therapy[b] ($N = 5$)	p (one-way ANOVA)
Plasma metabolites					
Betaine (μM)	37 ± 24	41 ± 25	36 ± 25	3318 ± 1818[c,e,g]	<0.001
Methionine (μM)	32 ± 11	29 ± 7	16 ± 7	50± 39[f,h]	0.003
S-Adenosylmethionine (nM)	103 ± 31	81 ± 20	92 ± 44	185 ± 100[d,e,g]	<0.001
S-Adenosylhomocysteine (nM)	29 ± 19	25 ± 12	36 ± 24	21 ± 9	ns
Total homocysteine (μM)	6.2 ± 2.0	8.5 ± 3.2	160.4 ± 60.8[c,e]	54.9 ± 13.5[c,e,g]	<0.001
Cystathionine (nM)	209 ± 82	287 ± 177	1246 ± 946[c,e]	1235 ± 263[c,e]	<0.001
Total cysteine (μM)	255 ± 40	270 ± 38	151 ± 44[c,e]	177 ± 17[c,e]	<0.001
Choline (μM)	9.6 ± 2.6	10.8 ± 3.3	10.1 ± 3.9	13.0 ± 3.3	ns
Phosphatidylcholine (μM)	2009 ± 353	2251 ± 436	1584 ± 470[f]	2136 ± 634	0.046
Dimethylglycine (μM)	5.8 ± 2.0	4.9 ± 1.2	13.7 ± 11.5	190.8 ± 123.3[c,e,g]	<0.001
Homocysteine thiolactone (nM)	0.24 ± 0.11	0.50 ± 0.29	47.3[i]	4.27 ± 2.03[c,e]	<0.001[i]
Metabolite ratios (mol:mol)					
Methionine/total homocysteine	5.77 ± 2.41	3.84 ± 1.58	0.13 ± 0.10[c,e]	1.00 ± 1.11[c,e,g]	<0.001[j]
AdoMet/AdoHcy	4.35 ± 2.02	3.89 ± 2.2	3.11 ± 1.61	9.62 ± 6.62[d,e,g]	<0.001[j]
Total cysteine/cystathionine	1412 ± 589	1211 ± 544	113 ± 34[c,e]	150 ± 40[c,e]	<0.001[j]
Vitamin levels					
Red blood cell folate (ng/mL)	280–791	nd	668 ± 34	nd	
Serum B12 (pg/mL)	200–800	nd	813 ± 443	471 ± 254	
Serum B6 (ng/mL)	3.0–60.0	nd	29 ± 15	nd	
Global DNA methylation					
Lymphocyte percent methylcytosine	[k]	24.1	5.3	17.3	

ns, not significant (one-way ANOVA, $p > 0.05$); nd, not done.

[a] The number of individual data sets (n) used for statistical analyses exceeded the number of patients (N in column headings): for eight MTHFR 1129C>T heterozygotes, $n = 20$; five *MTHFR* 1129C>T homozygotes prior to treatment, $n = 8$; and the same five patients on betaine therapy, $n = 13$.

[b] Betaine anhydrous 534 ± 222 mg/kg/day.

[c] Different from control subjects (Tukey post-test, $p < 0.001$).

[d] Different from control subjects (Tukey post-test, $p < 0.01$).

[e] Different from *MTHFR* 1129C>T heterozygotes (Tukey post-test, $p < 0.001$).

[f] Different from *MTHFR* 1129C>T heterozygotes (Tukey post-test, $p < 0.05$).

[g] Different from untreated *MTHFR* 1129C>T homozygotes (Tukey post-test, $p < 0.001$).

[h] Different from untreated *MTHFR* 1129C>T homozygotes (Tukey post-test, $p < 0.05$).

[i] Fewer samples were available for measurement of plasma homocysteine thiolactone: Control = 5; *MTHFR* 1129C>T heterozygote = 6; *MTHFR* 1129C>T homozygote untreated = 1; *MTHFR* 1129C>T homozygote on betaine = 3. One-way ANOVA test did not include the untreated patient with MTHFR deficiency.

[j] Metabolite ratios were log transformed ($Y = \log[y]$) to convert data to Gaussian distributions prior to one-way ANOVA.

[k] There are inadequate control data to establish a reference range for percentage methylcytosine in peripheral lymphoctes using the described HPLC method.

Table 2
Cerebrospinal fluid studies in Amish children with MTHFR deficiency

		MTHFR 1129C>T Homozygotes	
	Reference range	Untreated	Betaine therapy
Calculated brain uptake			
Methionine (nmol/min/gram tissue)	1.02 ± 0.33[a]	0.46 ± 0.24	1.44 ± 1.49[b]
Transmethylation metabolites			
5-Methyltetrahydrofolate (nM)	40–150	5	15 ± 7[d]
Methionine (μM)	2.9 ± 1.0[e]	1.7	2.4 ± 0.6[d]
S-Adenosylmethionine (nM)	260 ± 82[e]	87 ± 30[c]	297[d]
S-Adenosylhomocysteine (nM)	17 ± 5[e]	nf	9
Total homocysteine (μM)	0.19 ± 0.04[e]	2.76 ± 1.01[c]	1.73 ± 0.08[d]
Cystathionine (nM)	24 ± 6[e]	nf	2397
Total cysteine (μM)	0.6 ± 0.2[e]	nf	4.0
Dimethylglycine (μM)	nf	nf	2.5
Metabolite ratios (mol:mol)			
Methionine/total homocysteine	> 8	nf	1.38
S-Adenosylmethionine/*S*-adenosylhomocysteine	16 ± 5[e]	nf	33
Total cysteine/cystathionine	<0.26[e]	nf	1.67
Neurotransmitter metabolites			
Homovanillic acid (nM)	233–928	376	520 ± 146
5-Hydroxyindoleacetic acid (nM)	74–345	145	215 ± 72
3-O-methyldopa (nM)	< 150	83	35 ± 11

nf, test was not done on Amish patients and data were not found in the published literature.
[a] Calculated from the equations of Smith and Stoll (Ref. [30]) based on plasma amino acid profiles from 52 healthy children, eight profiles from untreated MTHFR patients, and 36 profiles from MTHFR patients on betaine therapy.
[b] Different from MTHFR untreated (one-way ANOVA, p <0.0001;Tukey post-test p <0.05).
[c] Previously reported values for seven untreated patients with MTHFR deficiency (Ref. [22]): 5MTHF = 5.1 ± 0.3 nM; methionine = 1.7 ± 1.5 μM; total homocysteine = 2.76 ± 1.01 μM; AdoMet = 87 ± 30 nM.
[d] Previously reported values for seven patients with MTHFR deficiency on betaine therapy (Ref. [22]): 5MTHF = 9.2 ± 7.3 nM; methionine = 7.1 ± 7.8 μM; total homocysteine = 1.06 μM (single value); AdoMet = 248 ± 105 nM.
[e] Reference data from 20 adults (Ref. [21]) and 11 adults (Ref. [20]), and 18 children (Ref. [23]).

transporter (LAT1) [24,30]. The transporter is saturated under physiological conditions, so the cerebral uptake of each amino acid is influenced by the ambient concentrations of competing amino acids [30]. Substrate competition is expressed by an apparent K_m, K_{app} (μM), calculated for each amino acid according to the equation: $K_{app} = K_m[1 + \sum(C_i/K_i)]$, where K_m is the classical Michaelis–Menten affinity constant for the single amino acid of interest, C_i is the plasma concentration of each competitor (μM), and K_i is the classical affinity constant of the competitor (μM).

For a given plasma amino acid profile, K_{app} values were determined for each LAT1 substrate using published Michaelis–Menten parameters for the blood–brain barrier LAT1 amino acid transporter [30]. The K_{app} value was then used to calculate the brain influx (nanomoles per minute per gram of brain tissue) of each amino acid in the competing group, according to the equation: Influx = $(V_{max})(C)/(K_{app}+C)$, where V_{max} and C are the maximal transport velocity (nmol per minute per gram of brain tissue) and plasma concentration (μM), respectively, of each amino acid. Results for estimated brain influx values were compared to calculations from a control population of 52 Amish children not affected by disorders of amino acid, folic acid, or cobalamin metabolism.

Data analyses

Data were depicted as means ± standard deviations, except where indicated otherwise. Statistical calculations were done with Prism 4 software (GraphPad Software, San Diego, CA). Biochemical measurements from three or more experimental groups (i.e. control subjects, *MTHFR* c.1129C>T heterozygotes, untreated *MTHFR* c.1129C>T homozygotes, and *MTHFR* c.1129C>T homozygotes treated with betaine) were analyzed with one-way analysis of variance (ANOVA), followed by the Tukey post-test to compare differences between groups. Tukey *p*-value approximations <0.05 were accepted as significant. Metabolite ratios (i.e. Met/tHcy, AdoMet/AdoHcy, and total cysteine/cystathionine) were log transformed ($Y = \log[y]$) to a Normal distribution for one-way ANOVA analysis, but actual values are given in Table 1. Select pairs of biochemical measurements among treated and untreated patients were tested for correlation using the Spearman rank correlation test. Significant results (*p*-value <0.05) were reported as Spearman correlation coefficients (r_s).

Results

Clinical patterns

All five Amish children with MTFHR deficiency were identical by descent for the *MTHFR* c.1129C>T allele. Analyses of all *MTHFR* exons and splice sites revealed no other sequence variations. Amish newborns with homozygous *MTHFR* c.1129C>T mutations were born healthy. If untreated, slow brain growth and psychomotor arrest were apparent within a few months of life (Fig. 1). By late childhood, the oldest patient had microcephaly, dystonia, spastic quadriplegia, intractable epilepsy, and severe mental retardation. When first examined at age 18 years, he was immobile, non-communicative, and fully dependent on others for daily care.

Betaine was the only treatment that influenced biochemical indices and improved neurological outcome. The efficacy of betaine was inversely related to the age at which

it was started: diagnosis during the newborn period, followed by sustained treatment, was associated with normal brain growth and development assessed at age 3 years (Fig. 1); two patients diagnosed during infancy (ages 7 and 13 months) gained weight, had catch-up brain growth, and showed marked neurological recovery after starting betaine (Fig. 1), but remained delayed in all developmental streams; the two oldest patients, diagnosed at ages 4 and 18 years, gained weight and motor skills on betaine therapy, but these children had no surge of head growth and they experienced only minimal cognitive improvement.

No vaso-occlusive or thromboembolic events occurred in *MTHFR* c.1129C>T homozygotes or heterozygotes. Treatment was well tolerated. We observed no adverse effects of betaine or aspirin therapy over the study period.

Biochemical changes in blood

Laboratory results are summarized in Table 1, Figs. 2, and 3. Physiological relationships among key metabolites are depicted in Fig. 4c.

There were no biochemical differences between control subjects and *MTHFR* c.1129C>T heterozygotes. Amish children homozygous for *MTHFR* c.1129C>T had low plasma Met/tHcy, total cysteine, and phosphatidylcholine, accompanied by elevations of total homocysteine and cystathionine. The cysteine/cystathionine ratio was decreased approximately 10-fold, and did not correct with therapy. For the one untreated *MTHFR* c.1129C>T homozygote in whom they were measured, Hcy-thiolactone was very high and lymphocyte DNA was hypomethylated.

Treatment with betaine increased plasma betaine levels approximately 100-fold, and led to predictable elevations of dimethylglycine, Met/tHcy, AdoMet, and AdoMet/AdoHcy. These changes indicated remethylation of homocysteine to methionine through BHMT [3] as well as a parallel increase of AdoMet biosynthesis (Table 1) [31]. There was no correlation between the weight-based dose of betaine and its plasma concentration, consistent with the short plasma half-life (1 h) of this compound [32]. Plasma total homocysteine decreased an average of 66% on therapy, but remained elevated (54.9 ± 13.5 μM; control subjects 6.2 ± 2.0 μM) in all treated patients. In the four oldest patients, we observed a large but transient increase of plasma methionine (sometimes to levels greater than 300 μM) during the first 6–12 months of treatment with a relatively constant weight-based dose of betaine (Fig. 2). This surge of plasma methionine presumably reflected adaptive changes of tissue enzyme expression and regulation, and was not observed when betaine therapy was started during the newborn period.

For *MTHFR* c.1129C>T homozygotes (but not heterozygotes or control subjects), plasma betaine concentrations before and during therapy correlated with plasma Met/tHcy ($r_s = 0.92$, $p < 0.001$; Fig. 3), methionine ($r_s = 0.80$, $p = 0.002$), AdoMet/AdoHcy ($r_s = 0.82$, $p = 0.001$), and AdoMet ($r_s = 0.85$, $p = 0.001$). There were also significant correlations between plasma methionine and AdoMet ($r_s = 0.78$, $p < 0.001$; Fig. 2), and between Met/tHcy and AdoMet/AdoHcy ($r_s = 0.92$, $p < 0.001$; Fig. 3). Plasma Hcy-thiolactone was inversely proportional to Met/tHcy ($r_s = -0.71$, $p = 0.003$; Fig. 3) across all experimental

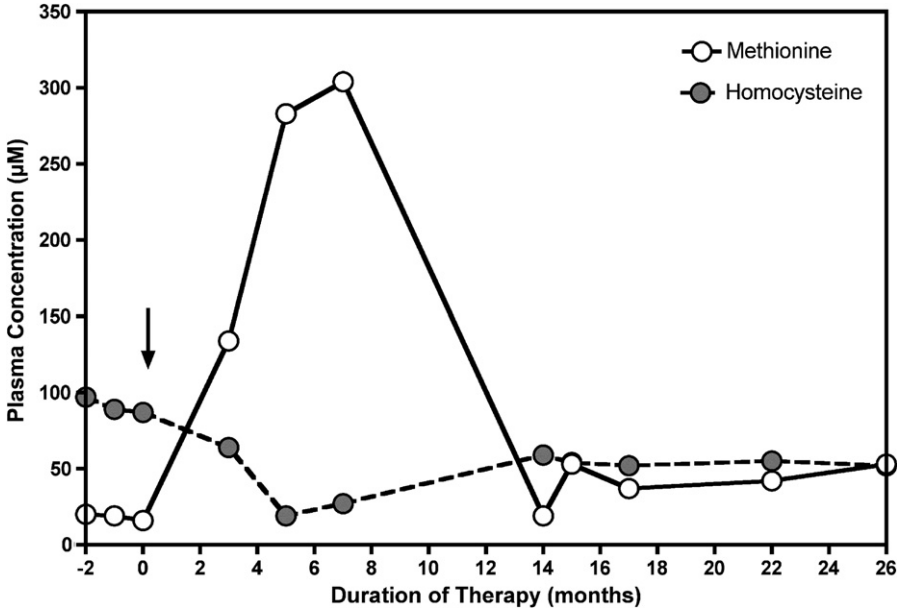

Fig. 2. Initial biochemical response to betaine therapy. The index child was started on betaine (500 mg/kg/day) at 4 years of age (arrow). Plasma methionine (white circles, solid line) increased transiently to more than 300 μM over the first 12 months of betaine therapy, and then stabilized at values of 40–50 μM. Plasma total homocysteine (gray circles, dashed line) initially decreased to 20 μM, but thereafter stabilized at 50–60 μM. The weight-adjusted dose of betaine remained relatively constant over the depicted time interval.

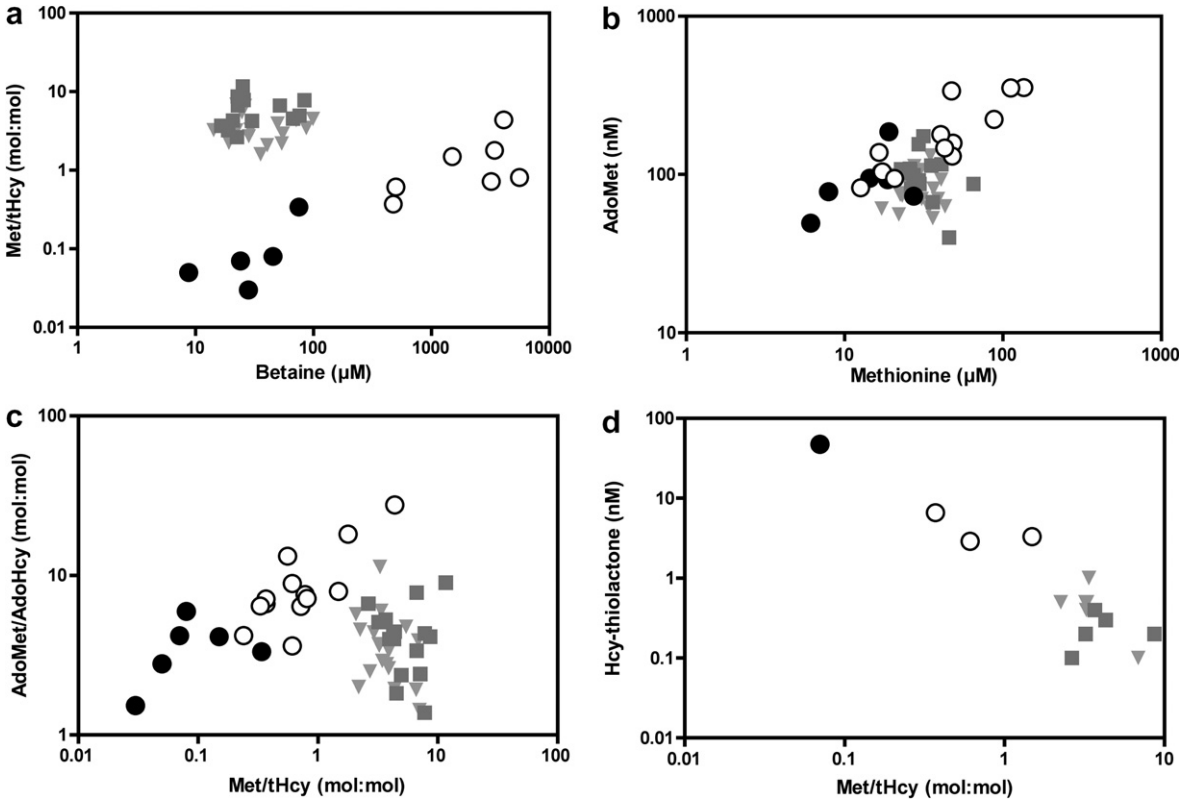

Fig. 3. Biochemical responses to betaine therapy. All axes are log10 scale. Four panels depict representative relationships among various biochemical measurements in plasma of control subjects (gray squares), *MTHFR* c.1129C>T heterozygotes (gray triangles), and *MTHFR* c.1129C>T homozygotes before (black circles) and during (open circles) treatment with betaine. (a) In *MTHFR* c.1129C>T homozygotes, but not controls or heterozygotes, there were strong correlations ($r_s \geqslant 0.80$; $p < 0.002$) between plasma betaine and the methionine to homocysteine concentration ratio; (b) between plasma *S*-adenosylmethionine and methionine; and (c) between Met/tHcy and AdoMet/AdoHcy. (d) There was a correlation between Met/tHcy and homocysteine thiolactone across all subjects.

groups. In the one child in whom it was assayed, lymphocyte DNA methylation during betaine therapy (17.3%) was higher than the value in a different untreated child (5.3%). During betaine treatment plasma phosphatidylcholine corrected to normal (Table 1), but plasma cystathionine remained elevated.

Calculated brain methionine uptake and biochemical findings in cerebrospinal fluid

Cerebrospinal fluid data and calculated brain methionine uptake are summarized in Table 2 and Fig. 4, respectively. Among ten LAT1 amino acid substrates, plasma methionine was selectively reduced prior to treatment and was closely related to calculated brain methionine uptake ($r_s = 0.86$, $p < 0.001$). Treatment with betaine increased plasma methionine from 16 ± 7 μM to 50 ± 39 μM (one-way ANOVA $p = 0.003$, post-test $p < 0.01$) and led to an average threefold increase of calculated brain methionine uptake, from 0.46 to 1.44 nanomoles per minute per gram brain tissue (one-way ANOVA $p < 0.001$; post-test $p < 0.05$).

Affected children on betaine therapy had normal CSF values for glucose (52 ± 8 mg/dL), lactate (1.4 ± 0.3 mM), and protein (17 ± 5 mg/dL), as well as normal CSF methionine, AdoMet, AdoHcy, AdoMet/AdoHcy, homovanillic acid, and 5-hydroxyindoleacetic acid. The 5MTHF of CSF remained low during therapy (15 ± 7 nM; normal 40–150 nM), whereas CSF total homocysteine, total cysteine, and cystathionine levels remained elevated.

Discussion

We used a fast and accurate molecular test to diagnose severe MTHFR deficiency within a regional Amish population. Treatment of affected infants with betaine was affordable, well tolerated, and effective. Our results, along with those of others [9,10,33][2] suggest that neonatal diagnosis of MTHFR deficiency, based on either the Met/tHcy ratio from dried blood spots [34] or microarray strategies [35], could prevent death and disability in other populations. Current analyte screening tests for "homocystinuria" are inadequate for this purpose. They are based on measurements of blood methionine [36], and do not detect defects of homocysteine remethylation that result in hyperhomocysteinemia and methionine deficiency.

Large doses of betaine were used to maximize remethylation of homocysteine to methionine (Fig. 2), but the resulting high plasma betaine concentrations did not reduce plasma or CSF homocysteine levels to normal

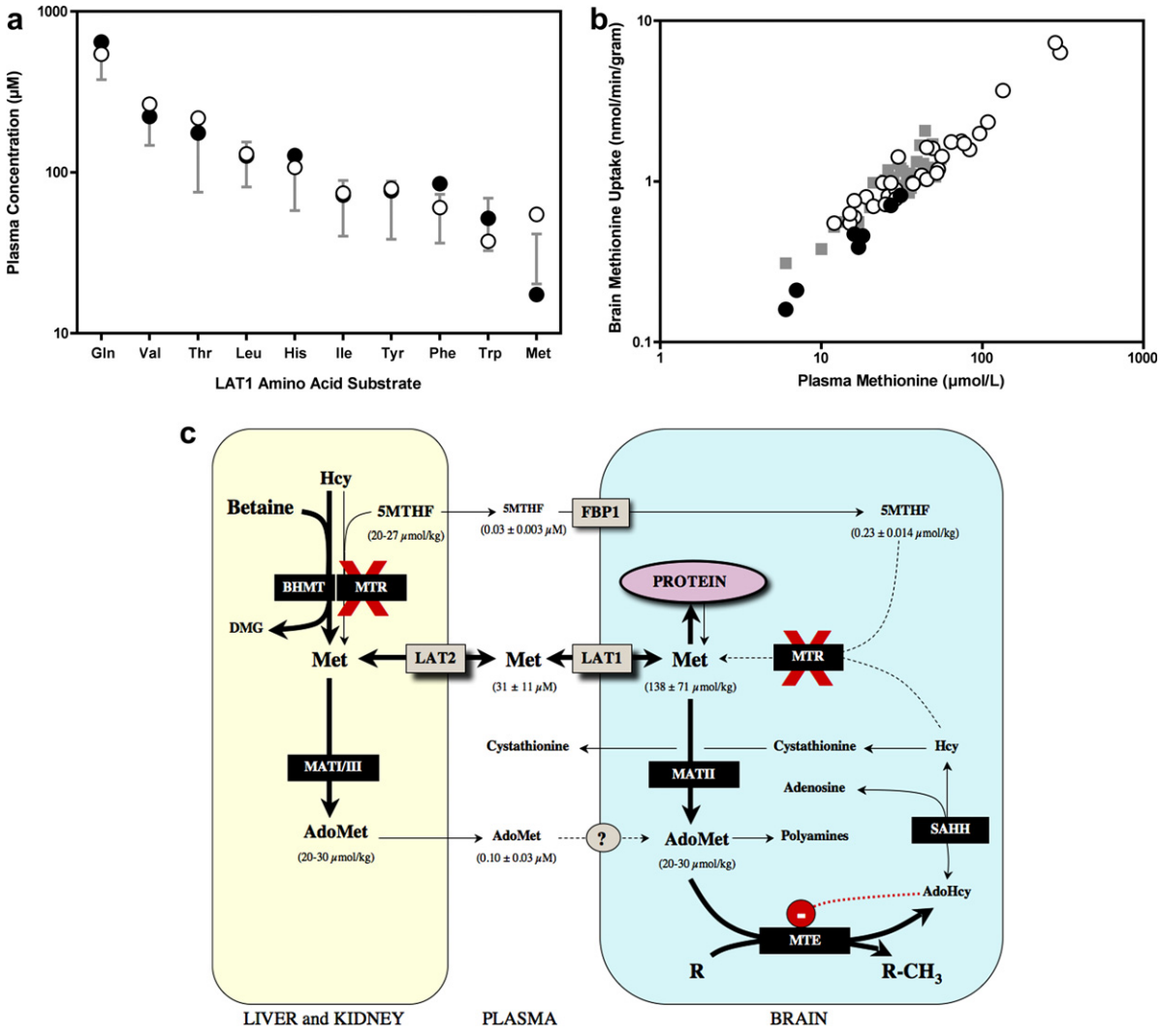

Fig. 4. Proposed action of betaine on brain nutrition. Panel a and b axes are log 10 scale. (a) Plasma values (in μM) for ten amino acids that compete for entry into the brain via a common transporter (LAT1). Gray bars represent the range of values (±2 standard deviations) for a reference population of 52 Amish children without disorders of folate, cobalamin, or amino acid metabolism. Mean values are shown for *MTHFR* c.1129C>T homozygotes before (black circles) and during (open circles) treatment with betaine. Methionine selectively increased on therapy (one-way ANOVA, $p = 0.003$; Tukey post-test, $p < 0.01$). Panel b shows how this affected calculated brain uptake of methionine relative to control subjects (gray squares). We hypothesize that an average threefold increase (one-way ANOVA, $p < 0.001$; Tukey post-test $p < 0.05$) of cerebral methionine uptake on betaine therapy is sufficient to support normal rates of cerebral protein accretion, lipid synthesis, polyamine formation, and methyl-group transfer when brain 5MTHF content is low. (c) A model of nutrient interactions between liver and brain is shown. A proportion of methyl groups derived from 5MTHF or betaine enter the brain as methionine [44–46]. Cerebral 5MTHF deficiency results in a functional block of cerebral methionine recycling (indicated with an "X"), and brain tissue becomes critically dependent on methionine influx to meet its material requirements for normal growth and one-carbon transfer. The proposed paths of inter-organ and intra-organ nutrient flow during betaine therapy are shown with heavy arrows. Metabolic flows that are absent, minimal, or poorly characterized are shown with dashed lines. The red dotted line indicates the inhibitory effect of high AdoHcy or low AdoMet/AdoHcy ratio on tissue methyltransferase activity. *Abbreviations*: BHMT, betaine:homocysteine methyltransferase; FBP1, folate binding protein; LAT1 and LAT2, high- and low-affinity, respectively, large neutral amino acid transporters; MATI/III, hepatic and renal isoform of methionine adenosyltransferase; MATII, the only isoform of methionine adenosyltransferase expressed in brain; MTR, 5-methyltetrahydrofolate:homocysteine methyltransferase; MTE, various cerebral methyltransferase enzymes; R, substrate for methyltransferase enzyme; R-CH_3, methylated derivative; SAHH, reversible *S*-adenosylhomocysteine hydrolase. See text for additional abbreviations. Biochemical data are from Refs. [1,24–28].

(Tables 1 and 2). Enduring hyperhomocysteinemia in treated children may in part reflect disparate fates of methionine in tissues [1]. When the liver and kidneys are deprived of 5MTHF, they still use betaine to remethylate a proportion of homocysteine back to methionine; this recycling rate can be increased by pharmacological doses of betaine (Table 1, Fig. 4) [1,3]. In contrast, 5MTHF-deficient organs that do not express BHMT have impaired recycling of homocysteine back to methionine, and will accumulate homocysteine when methionine uptake exceeds the requirement for growth. Thus, the homocysteine-lowering effect of betaine therapy is self-limiting; as fluxes through hepatic and renal BHMT increase, methionine flows into the circulation and drives homocysteine produc-

tion in other organs (Fig. 4). Under these circumstances, adipose and brain tissue, which have low or absent cystathionase activity [1,37], may also accumulate cystathionine (Table 2).

Our results suggest that brain disease associated with MTHFR deficiency results predominately from cerebral methionine and AdoMet deficiency, rather than from neurotoxic actions of homocysteine. The youngest affected child grew and developed normally on treatment, despite persistent and marked elevations of homocysteine and cystathionine in blood and CSF (Fig. 1, Tables 1 and 2). Prior to treatment, the other four affected children grew poorly and had signs of abnormal methyltransferase activity in tissues, including low plasma Met/tHcy, plasma phosphatidylcholine [38], and DNA methylcytosine [39]. Betaine therapy resulted in a concerted increase of these markers, a reduction of cellular Hcy-thiolactone formation (Fig. 3) [40], and increased growth of the brain (Fig. 1) and other tissues.

Normally, the total flow of methionine into the labile cerebral amino acid pool is the sum of methionine uptake from plasma and in situ remethylation of homocysteine to methionine [27]. The latter is functionally impaired in children with severe cerebral 5MTHF deficiency [2,41]. Consequently, higher rates of brain methionine uptake may be required to maintain normal cerebral protein accretion, myelin assembly [38,42], polyamine formation [1], and methyl group transfer (Fig. 4) [43,44]. For the latter, AdoMet is the principal methyl donor [1,42], and although some AdoMet may cross the blood–brain barrier in vivo [45], methionine transported into the brain appears to be an important source of cerebral AdoMet (Fig. 4) [1,44,46]. Thus, for individuals with severe systemic 5MTHF deficiency, cerebral methionine influx may place an absolute limit not only on brain protein synthesis, but also on the rate of cerebral methyl group transfer (Fig. 4).

The human brain can apparently develop normally when deprived of 5MTHF (Fig. 1, Table 2) [2]. This suggests that the major therapeutic action of betaine is to increase brain methionine uptake to a level sufficient to compensate for the loss of brain methionine recycling (Fig. 4) [27,43,44]. Because the blood–brain barrier transport K_m value for methionine is high (40 μM) relative to several competing substrates (e.g. phenylalanine 11 μM, tryptophan 15 μM, leucine 29 μM), its uptake by the brain can be influenced by disturbances in concentration ratios among circulating amino acids [30]. Such was the case in affected children: among ten LAT1 amino acid substrates, plasma methionine was selectively reduced prior to treatment, and betaine therapy was associated with a threefold increase in both the methionine concentration of plasma and its calculated transport from blood to brain (Table 2, Fig. 4).

Our observations have important implications beyond the Amish community. First, the study of brain disease in folate and cobalamin deficiency syndromes must account for the material requirements of the brain during its rapid phase of growth; these requirements apparently include a threshold inflow of methionine and its derivative, AdoMet, but not 5MTHF per se [41]. Smith and colleagues [47] reached a similar conclusion about the pathophysiology of subacute combined degeneration of the spinal cord in a patient with a hereditary defect of cobalamin metabolism. Second, therapeutic timing is critical: high-dose betaine must be started early in life to be maximally effective (Fig. 1). This is consistent with observations in both humans [9,10][2] and experimental animals [43]. Third, we show that molar concentration ratios among key metabolites (Met/tHcy, AdoMet/AdoHcy) and in vivo markers of methyltransferase activity (e.g. global DNA methylation, plasma phosphatidylcholine) may provide more information about tissue methylation status and growth potential than measurements of homocysteine alone [31]. Such indices could be applied in patients with cobalamin dysmetabolism [47] and in larger studies of homocysteine-related vascular and cerebral diseases in outbred populations [48,49]. Fourth, our experience with the youngest child in this group suggests that betaine alone, if given at an adequate dose, may be sufficient nutritional therapy for severe MTHFR deficiency.

Finally, it is widely recognized that specific pathological mutations are often concentrated within certain ethnic or cultural groups; the Amish are not unique in this way. Indeed, as knowledge of population genetics grows, defining a "general population" with regard to genetic disease risks becomes increasingly difficult. We show that resources can be directed effectively, and local health improved, by targeting specific disorders or mutations within specific populations.

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