J Inherit Metab Dis (2015) 38:863–872
DOI 10.1007/s10545-015-9810-3

ORIGINAL ARTICLE

Characterization and review of MTHFD1 deficiency: four new patients, cellular delineation and response to folic and folinic acid treatment

P. Burda · A. Kuster · O. Hjalmarson · T. Suormala · C. Bürer · S. Lutz · G. Roussey · L. Christa · J. Asin-Cayuela · G. Kollberg · B. A. Andersson · D. Watkins · D. S. Rosenblatt · B. Fowler · E. Holme · D. S. Froese · M. R. Baumgartner

Received: 31 October 2014 / Revised: 15 December 2014 / Accepted: 6 January 2015 / Published online: 30 January 2015

Abstract In the folate cycle MTHFD1, encoded by *MTHFD1*, is a trifunctional enzyme containing 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase activity. To date, only one patient with *MTHFD1* deficiency, presenting with hyperhomocysteinemia, megaloblastic anaemia, hemolytic uremic syndrome (HUS) and severe combined immunodeficiency, has been identified (Watkins et al J Med Genet 48:590–2, 2011). We now describe four additional patients from two different families. The second patient presented with hyperhomocysteinemia, megaloblastic anaemia, HUS, microangiopathy and retinopathy; all except the retinopathy resolved after treatment with hydroxocobalamin, betaine and folinic acid. The third patient developed megaloblastic anaemia, infection, autoimmune disease and moderate liver fibrosis but not hyperhomocysteinemia, and was successfully treated with a regime that included and was eventually reduced to folic acid. The other two, elder siblings of the third patient, died at 9 weeks of age with megaloblastic anaemia, infection and severe acidosis and had MTFHD1 deficiency diagnosed retrospectively. We identified a missense mutation (c.806C>T, p.Thr296Ile) and a splice site mutation

Communicated by: Viktor Kožich

P. Burda, A. Kuster and O. Hjalmarson contributed equally to this work.

D. S. Froese and M. R. Baumgartner contributed equally to this work.

P. Burda · T. Suormala · C. Bürer · S. Lutz · B. Fowler · D. S. Froese · M. R. Baumgartner (✉)
Division of Metabolism and Children's Research Center, University Children's Hospital, Steinwiesstrasse 75, 8032 Zurich, Switzerland
e-mail: matthias.baumgartner@kispi.uzh.ch

A. Kuster · G. Roussey
Department of Pediatric Nephrology, University Hospital of Nantes, Nantes, France

O. Hjalmarson
Department of Pediatrics, University of Gothenburg, Gothenburg, Sweden

L. Christa
Metabolic Biochemistry Department, Necker Hospital, AP-HP, University Paris Descartes, Paris, France

J. Asin-Cayuela · G. Kollberg · E. Holme (✉)
Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg SE-413 45, Sweden
e-mail: elisabeth.holme@clinchem.gu.se

B. A. Andersson
Department of Clinical Immunology and Transfusion Medicine, Sahlgrenska University Hospital, Gothenburg, Sweden

D. Watkins · D. S. Rosenblatt
Department of Human Genetics, McGill University, Montreal, QC, Canada

D. S. Froese · M. R. Baumgartner
Radiz – Rare Disease Initiative Zurich, Clinical Research Priority Program for Rare Diseases, University of Zurich, Zurich, Switzerland

M. R. Baumgartner
Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

(c.1674G>A) leading to exon skipping in the second patient, while the other three harboured a missense mutation (c.146C>T, p.Ser49Phe) and a premature stop mutation (c.673G>T, p.Glu225*), all of which were novel. Patient fibroblast studies revealed severely reduced methionine formation from [^{14}C]-formate, which did not increase in cobalamin supplemented culture medium but was responsive to folic and folinic acid. These additional cases increase the clinical spectrum of this intriguing defect, provide in vitro evidence of disturbed methionine synthesis and substantiate the effectiveness of folic or folinic acid treatment.

Introduction

Transfer of single-carbons (1C) is an essential part of many cellular processes, including *de novo* nucleotide synthesis, methylation and production of methionine (Rosenblatt and Fenton 2001). A major carrier of 1C-units is folate, in the form of tetrahydrofolate (THF). THF-mediated 1C metabolism is compartmentalised between the nucleus, mitochondria and cytoplasm. In the mitochondria, the balance of the pathway is towards production of formate, which is then shuttled to the cytoplasm (Tibbetts and Appling 2010). Cytoplasmic formate is reattached to THF for production of purines, or for further processing to 5,10-methylene-THF for dTMP synthesis or to 5-methyl-THF for methionine synthesis (Tibbetts and Appling 2010; Watkins and Rosenblatt 2012; see Fig. 1). In the cytoplasm, the first three reactions of the attachment and reduction of formate on THF are catalysed by the trifunctional enzyme, MTHFD1. These reactions include: the ATP-dependent conversion of formate and THF to 10-formyl-THF (synthetase); the interconversion of 10-formyl-THF and 5,10-methenyl-THF (cyclohydrolase); and the NADP-dependent reduction of 5,10-methenyl-THF to 5,10-methylene-THF (dehydrogenase) (Fox and Stover 2008). In addition to dTMP production via thymidylate synthase, 5,10-methylene-THF is further processed either to 5-methyl-THF by MTHFR to act as a carbon donor in methionine synthesis, or used by serine hydroxymethyltransferase (SHMT) to produce serine from glycine.

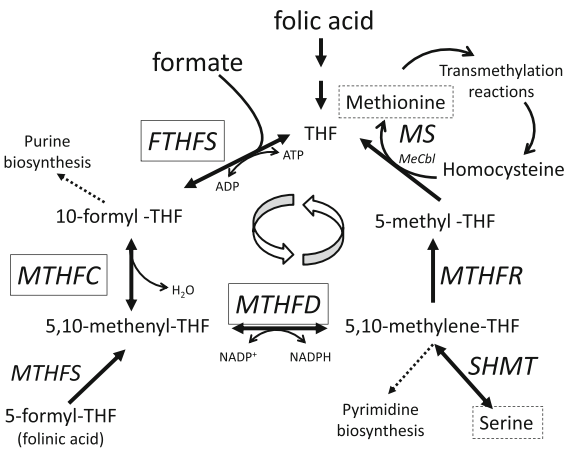

Fig. 1 The cytoplasmic folate cycle. Formate is attached to THF to begin the cycle. The three steps catalysed by MTHFD1 (FTHFS, MTHFC and MTHFD) are boxed in solid lines. Folinic acid (5-formyl-THF) can enter the cycle after conversion to 5,10-methenyl-THF. The endpoints methionine and serine are boxed in dashed lines. *FTHFS*, 10-formyltetrahydrofolate synthetase; *MTHFC*, 5,10-methenyltetrahydrofolate cyclohydrolase; *MTHFD*, 5,10-methylenetetrahydrofolate dehydrogenase; *MTHFS*, 5,10-methenyltetrahydrofolate synthetase; *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *MS*, methionine synthase; *SHMT*, serine hydroxymethyltransferase; THF, tetrahydrofolate

A number of very rare inborn errors of folate uptake and metabolism have been described. Hereditary folate malabsorption and glutamate-formiminotransferase deficiency may cause megaloblastic anaemia and neurological damage without, or with only mild, hyperhomocysteinemia. In contrast, MTHFR deficiency causes severe hyperhomocysteinemia and neurological abnormalities without defects of hematopoiesis. Biochemical changes in cerebral folate deficiency are localised to CSF only with no anaemia (Watkins and Rosenblatt 2012). Mild impairments to one-carbon metabolism, due to insufficient folate or SNPs in genes involved in folate or cobalamin metabolism, are also associated with many pathologies, including neural tube defects (Brody et al 2002; De Marco et al 2002; Parle-McDermott et al 2006), cancers (Stevens et al 2007; Wang et al 2007) and cardiovascular disease (Christensen et al 2009; Shaw et al 2009). It is therefore not surprising that a gene-disruption of *Mthfd1* is embryonically lethal in mice (MacFarlane et al 2009). Deletion of *Mthfd1l* and *Mthfd2*, two mitochondrial homologs of *Mthfd1*, the first of which catalyses only the synthetase reaction and the second both the cyclohydrolase and dehydrogenase in the developing embryo, are also embryonically lethal in murine models (Di Pietro et al 2002; Momb et al 2013), underlining the requirement of this activity for the growing organism.

Thus far, only one patient with MTHFD1 deficiency has been identified (Keller et al 2013; Watkins et al 2011). The 2 months old infant presented with elevated homocysteine, megaloblastic anaemia, atypical hemolytic uremic syndrome (HUS) and severe combined immune deficiency (SCID). The patient was compound heterozygous for two mutations in *MTHFD1*, one affecting a splice acceptor site in intron 8 and the other changing a critical arginine residue in the NADP-binding site. Here, we report four new patients with MTHFD1 deficiency caused by compound heterozygosity of a missense mutation with either a splice site or nonsense mutation and demonstrate a defect in fibroblast methionine formation using synthesis from [^{14}C]-formate.

Materials and methods

Cell culture

Skin fibroblasts, obtained for diagnostic purposes, were used for molecular and biochemical evaluation and were grown in Earle's minimum essential medium supplemented with 10 % FCS with antibiotics (Suormala et al 2004).

Sequencing

For patient 2, RNA and genomic DNA were extracted from cultured skin fibroblasts or blood spots using the QIAamp RNeasy Mini Kit and the QIAamp DNA Mini Kit (Qiagen), respectively. The RT-PCR reaction was performed using the One-Step RT-PCR Kit (Qiagen) following the manufacturer's instructions. Genomic DNA PCR was performed using HOT FIREPol DNA Polymerase (Solis BioDyne) and direct sequencing of all exons and flanking intronic sequences of *MTHFD1* was performed. Primer pairs designed to amplify the complete *MTHFD1* open reading frame in five reactions (fragments) were used. Sequencing was performed using BigDye Terminator v1.1 Cycle Sequencing kit and analysed on a 3500 or 3130xl Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems). Cell treatment for NMD inhibition was performed by incubating cultured fibroblast with 100 μl/ml cycloheximide (Sigma-Aldrich) for 4 h. All primer sequences are available upon request.

For patient 3, whole-exome sequencing (WES) was carried out using Illumina HiSeq 2000 high-throughput sequencing technology. Agilent SureSelect Human All Exon 51 M kit was used for exome capture. Quality assessment of the sequence reads was performed by generating QC statistics with FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Read alignment to the reference human genome (hg19, UCSC assembly, February 2009) was done using BWA (Burrow-Wheeler alignment) with default parameters (Li and Durbin 2009). After removal of PCR duplicates using Picard tools, (http://picard.sourceforge.net) and file conversion using SAMtools (Li et al 2009), quality score recalibration, indel realignment and variant calling were performed with the Genome Analysis Toolkit package (McKenna et al 2010). After excluding all dbSNPs, we focused our search for potential pathogenic mutations on the coding regions and splice sites of genes related to folate metabolism fitting an autosomal recessive mode of inheritance. Mutations identified by WES were verified by Sanger sequencing.

For patients 4 and 5, DNA was extracted from dried blood spots derived from neonatal metabolic screening using the QIAamp DNA Mini Kit (QIAGEN), with confirmation of *MTHFD1* mutations by PCR and Sanger sequencing of exons 3 and 8.

Specific enzyme assays

Cobalamin coenzyme synthesis was determined as described previously (Jusufi et al 2014). Incorporation of [^{14}C]-propionate was assayed by a slight modification of the method already described (Willard et al 1976). 5,10-Methylenetetrahydrofolate reductase (MTHFR) activity was assayed in its physiological forward direction using the natural substrates 5,10-methylenetetrahydrofolate and NADPH and the cofactor FAD as described earlier (Suormala et al 2002). Formation of [^{14}C]-methionine and [^{14}C]-serine from [^{14}C]-formate was determined as described earlier (Fowler et al 1997) with fibroblasts cultured for 72 h in normal medium or supplemented with OHCbl (1 mg/L: ~1 μM; Sigma), 5-formyl-THF (folinic acid, 100 μM; Sigma) or folic acid (100 μM; Sigma). Assays were performed as duplicates in at least two independent experiments.

Statistics

Statistical significance was determined using an unpaired two-tailed Student's *t*-test with a cut-off of $p<0.05$ unless otherwise indicated. Statistics were performed and graphs generated using GraphPad Prism v6 software.

Results

Patient histories

Previous patient

This patient has been described (Keller et al 2013; Watkins et al 2011).

Patient 2

An 18 month-old girl, the first child of non-consanguineous French parents, presented with asthenia, vomiting and icteric skin. Findings of anaemia, schizocytosis (9/00) and thrombopenia indicated atypical hemolytic uremic syndrome (HUS) with microangiopathy and severe arterial hypertension, although renal function was normal. There was no evidence of post-diarrheic HUS or other known causes of atypical HUS, such as activation of complement factors or alternative pathway proteins. Bone marrow analysis revealed dyserythropoietic and megaloblastic anaemia (Table 1). Leukopenia (2.7×10^9/L) and haptoglobin of less than 0.06 g/L were also noted. Serum cobalamin, transcobalamin and unsaturated cobalamin binding capacity of total serum were normal, as was serum folate. However, free (21 μmol/L; normal: 30–50) and total carnitine (39 μmol/L; normal: 43–65) was

Table 1 Comparison of clinical, biochemical and mutational findings in all MTHFD1 patients reported to date

Reference		Previous patient (Watkins 2011, Keller 2013)	Patient 2 This study	Patient 3 This study	Patients 4 & 5 This study
Clinical characteristics	Haematological	- At 2.5 months: megaloblastic anaemia (Hgb 4.9 g/dL, MCV 95 fL), leukopenia (1.4×10^9/L), thrombocytosis (721×10^9/L)	- At 18 months: megaloblastic anaemia (Hgb 3.5 g/dL, MCV 99.6 fL), dyserythropoesis, neutropenia (0.51×10^9/L), thrombocytopenia (11×10^9/L)	- At 9 weeks: megaloblastic anaemia (Hgb 7.8 g/dL, MCV 108 fL), anisocytosis, thrombocytosis (998×10^9/L)	- Megaloblastic anaemia, anisocytosis, karyorrhexis, leukopenia/granulocytosis, thrombocytosis
	Immunological	- Severe combined immunodeficiency, infections, eczema		- Autoimmune disease	- Moniliasis, skin and mucosa lesions, lethal infections
	Renal	- Atypical HUS	- Atypical HUS with microangiopathy		
	Digestive/Liver		- Vomiting, icteric skin, asthenia	- Moderate liver fibrosis	- Vomiting, severe lactic acidosis
	Other		- Convergent strabism, severe arterial hypertension		
	CNS	- Seizures, mild hearing loss, mild mental retardation	- Normal cognitive development	- Normal cognitive development	
	Present age	- At present 7 years old	- At present 8 years old	- At present 22 years old	- Death at 9 weeks of age (both)
Treatment	Initial	- OHCbl, folate, betaine, IgGs, trimethoprim/sulfamethoxazole	- OHCbl, folinic acid, betaine, antihypertensives	- OHCbl, folate, thiamine	
	Ongoing	- OHCbl, MeCbl, folate, methylfolate, betaine, IgGs	- OHCbl, folinic acid, betaine	- Folate	
Laboratory data	Special	- Plasma methylmalonic acid : 0.5 μmol/L (normal 0.4–1.5)	- Urinary methylmalonic acid: 3 mmol/mol creat. (normal: <7)	- Urinary methylmalonic acid: <10 mmol/mol creat. (normal: 15–29)	
		- Plasma methionine: 9.7 μmol/L (ref range 8–49)	- Plasma methionine: 16 μmol/L (normal: 11–30)	- Plasma methionine: 20–55 μmol/L (normal: 11–30)	
		- Plasma total homocysteine: 29 μmol/L (ref.range: 6–9)#	- Plasma total homocysteine: 50 μmol/L (normal: <12)	- Plasma total homocysteine: 10 μmol/L (normal: <12)	
		- Normal serum cobalamin and folate levels	- Normal serum cobalamin and folate levels	- Normal serum cobalamin and folate levels	
	Biochemical characterisation	- Normal 14C-propionate incorp. 13.8±-4.1 nmol/16 h/mg (normal 10.9±3.4). With OHCbl: 15.4±5.5 (normal 12.6±4.9)†	- Normal 14C-propionate incorp. 11.2±-1.2 nmol/16 h/mg (normal 10.9±3.4). With OHCbl: 12.3±1.4 (normal 12.6±4.9)		
		- Mildly decreased MeCbl (29 % of cellular Cbl; normal: 52–54 %)	- Mildly decreased MeCbl (30 % of cellular Cbl; normal: 48–79 %)	- Mildly decreased MeCbl (46 % of cellular Cbl; normal: 48–79 %)	
		- Normal MTHFR activity. Without FAD: 34.56 nmol/h/mg (control: 13.79–53.06); with FAD: 36.76 nmol/h/mg (control: 14.54–54.65)†	- Normal MTHFR activity. Without FAD: 15.14 nmol/h/mg (control: 13.79–53.06); with FAD: 14.25 nmol/h/mg (control: 14.54–54.65)	- Normal MTHFR activity. Without FAD: 40.31 nmol/h/mg (control: 13.79–53.06); with FAD: 40.66 nmol/h/mg (control: 14.54–54.65)	
MTHFD1 mutational analysis	allele 1	- c.727+1G>A	- c.1674G>A	- c.673G>T (p.Glu225*)	- Older siblings of patient 3
		- Affects intron 8 splice acceptor site	- Leads to skipping of exon 17	- Premature protein termination	
	allele 2	- c.517C>T (p.Arg173Cys)	- c.806C>T (p.Thr269Ile)	- c.146C>T (p.Ser49Phe)	
		- Affects the NADP-binding site	- Affects a highly conserved cyclohydrolase /dehydrogenase active-site residue	- Affects a highly conserved NADP-binding/cyclohydrolase active site residue	

Represents interquartile range (25–75 %)

† This study

slightly decreased, and analysis of amino and organic acids revealed hyperhomocysteinemia and low normal methionine, while methylmalonic acid was normal (Table 1).

Treatment was initiated with hydroxocobalamin (OHCbl; 1 mg/day *p.o.* and 1 mg/day i.m.), folinic acid (5 mg/day *p.o.*) and betaine (600 mg x6/day *p.o.*) along with antihypertensive drugs. Red blood cell and platelet transfusions were started and repeated at short intervals. Response to treatment was slow but complete and transfusions and antihypertensive drugs were stopped after 3 months after which plasma homocysteine was <15 μmol/L and methionine 97 μmol/L.

Following treatment, psychomotor development and cerebral MRI were found to be normal at 6 years of age. However, convergent strabism has been noted since the age of 6 months. Despite the continued normalisation of methionine and homocysteine values an electroretinogram showed bilateral alterations due to progressive maculopathy with retinal atrophy which have worsened over the years and are unresponsive to the treatment that has been adapted over time. At 8 years of age, the patient receives OHCbl (1 mg/day *p.o.* and 1 mg/week i.m.), folinic acid (2× 5 mg/day) and betaine (2× 1 g/day). However, CSF 5-methyl-THF measured at 6 and 7 years of age revealed low values (26 and 34, nmol/L, respectively; control values >44 nmol/L), which did not normalise when folinic acid was increased to 2× 5 mg/day (CSF 5-methyl-THF at 8 years: 34 nmol/L).

Patients 3–5

Cases 3, 4 and 5 are from the same non-consanguineous family of Swedish origin. One previous child was healthy. Although born after patients 4 and 5, patient 3 was the index case in whom mutations in *MTHFD1* were identified. This diagnosis was confirmed in patients 4 and 5 retrospectively.

Case 3 A girl, born at 42 weeks gestation, birth weight 2950 g, was monitored closely because of her siblings' medical history. Haematological investigations at 4 days of life showed macroblastic bone marrow with a slight megaloblastic appearance, consistent with cobalamin or folate deficiency, and marked anisocytosis; however, blood haemoglobin (Hgb; 205 g/L), MCV (109 fL) and leukocyte counts were normal over the first week of life. Platelets were high a few hours after delivery ($464*10^9$/L) and increasing.

Intensive metabolic investigation at 12 days of age showed blood and urinary lactate levels mostly within normal limits. Cobalamin and folate levels in blood were normal and high, respectively. Plasma amino acids were normal, with methionine at 20–55 μmol/L; plasma homocysteine (10 μmol/L) and urinary organic acids were normal.

At 9 weeks of age, anaemia (Hgb 78 g/L) and MCV rising to 108 fL was noted. Treatment with cobalamin and folic acid (5 mg/day) was started without obvious results after 2 days. Thiamine responsive megaloblastic anaemia (TRMA) was suspected and parenteral thiamine was added to the existing treatment, with an immediate response in reticulocyte counts, Hgb, and MCV, seeming to confirm this diagnosis. After a further 6 weeks of treatment, at the age of 4 months, Hgb and MCV had normalised and folic acid and cobalamin treatment was terminated. Bone marrow was then almost normalised with normoblastic erythropoiesis and normal myelopoiesis.

At 7 months the girl fell ill with fever, a mononucleosis-like finding in the throat, and obstructive bronchitis. Anaemia (Hgb 78 g/L) but normal MCV was found. A general inflammatory state developed with high C-reactive protein levels, increasing immunoglobulins, high serum acute phase proteins, serum albumin decreasing to 16 g/L (normal 36–50) together with low prothrombin index. Serum lactate dehydrogenase was 19.8 μkat/L (normal <7.5). Auto-antibodies with high titres against DNA and striated muscle appeared and antinuclear antibodies were positive. Serum transaminases were only marginally elevated. Serum titers against several viruses were high but regarded as non-specific. Bone marrow was again macro-megaloblastic with anisocytosis and disturbed myelopoiesis and a liver biopsy showed a low grade lympho-histiocytic inflammation in portal areas and in lobuli. The girl was treated with blood transfusions, gamma globulin infusions, corticosteroids, and cobalamin and folic acid were reintroduced. Reticulocyte counts and Hgb improved rapidly, bone marrow ameliorated and inflammatory markers normalised slowly. The treatment with cobalamin and folic acid was terminated after 2 weeks but thiamine continued. During the following 2–5 months the irregular antibodies disappeared. The girl remained in good health, with normal growth and psychomotor development until at 20 months rising serum transaminases led to a liver biopsy that showed the same mild inflammatory changes as before with the addition of discrete portal fibrosis, a pattern that resolved slowly over the following three years. At 26 months of age Hgb had fallen to 101 g/L and MCV increased to 102 fL. Bone marrow was again megaloblastic and there was disturbed myelopoiesis.

After reintroduction of folic acid, blood and bone marrow normalised and liver fibrosis decreased. The girl developed normally, reached high scores on psychological cognitive tests, and overcame infections, including varicella and influenza, without complications. TRMA was still the presumed diagnosis and folic acid was again terminated at 11 years. Four months later respiratory problems with cough appeared and persisted more than 6 months. At this time sequencing of SLC19A2 to confirm TRMA was performed but failed to show any mutations. Since MCV had increased again, folic acid was reinstituted despite normal Hgb, and thiamine dropped.

At the age of 21 extensive immunological investigations were performed while the patient was on folic acid treatment

demonstrating normal subpopulations of all lymphocytes, and normal responses to pokeweed mitogen and phytohaemagglutin but slightly reduced response to concanavalin A ($53*10^3$, reference $56–110*10^3$ counts per minute) and decreased mitogen stimulated IgG production for Epstein-Barr virus (IgM 500, reference 1000–10,000; IgG 700, reference 1000–10,000; IgA 100, reference 150–2500). At 22 years of age and after continuous supplementation with 5 mg folic acid/day over the last 10 years, the patient has remained in a very good clinical condition. Hgb, MCV, leukocyte and platelet counts have been within normal limits, and bone marrow shows no major disturbances. She is an athlete at national level, and has seldom had infections.

Case 4 A girl, born at 37 weeks gestation, birth weight 2550 g, was breast-fed and thrived up to 6 weeks of age when weight gain diminished. Moniliasis appeared in her mouth. On routine check-up at 9 weeks of age anaemia (Hgb 80 g/L) was found, iron deficiency was suspected and medication started. She was hospitalised the day after due to vomiting, laboured breathing and unconsciousness. Severe anaemia (Hgb 56 g/L), granulocytosis ($20.5*10^9$/L) with 7 % immature forms, and extreme metabolic acidosis were found. She died after a few hours of intensive care. No cultures were obtained. The only pathological finding on autopsy was macro-megaloblastic bone marrow with marked anisocytosis and karyorrhexis. Myelopoesis was judged to be normal.

Case 5 A boy, born at 37 weeks of gestation, birth weight 2560 g, was breast-fed. At 10 days of age, urine organic acids were normal. At 6 weeks of age, diminished weight gain, tiredness and 7 days of fever up to 39.5 °C led to hospitalisation. Subsequently, moniliasis in the mouth was found but no other pathological signs were noted. However, Hgb decreased from 97 to 87 g/L over 1 week, granulocytes decreased to $0.3*10^9$/L, mean corpuscular volume (MCV) was 98 fL and the platelet count was high ($761*10^9$/L). The bone marrow was megaloblastic with anisocytosis and karyorrhexis and the myelopoesis was affected with prominent giant cells and aberrant nuclei.

Soon after discharge, skin and mucosal lesions appeared that led to a presumed diagnosis of hand-foot-and-mouth disease. After a few days of apparent recovery he suddenly started vomiting, breathed intensively and became unconscious. In hospital, anaemia, leukopenia, thrombocytosis and extreme metabolic acidosis were noted. Despite intensive care, he died at 9 weeks of age. At autopsy, general oedema was found with ascites, hydrothorax, hydropericardium and lung oedema. The myocardium was diffusely infiltrated by inflammatory cells, there was interstitial oedema, inflammatory cells also around coronary arteries but no true arteritis, and foci of pronounced myocardial degeneration and early necrosis was found. Inflammatory foci were also seen in lungs, liver, pancreas and in perivascular areas in the brain. Organic acids in urine close to death showed high levels of lactate, fumarate and malate. No etiologic agent was found.

Mutation analysis

Following the identification of the first patient with an *MTHFD1* defect (Watkins et al 2011), the *MTHFD1* gene was screened for coding mutations in patient 2, while patient 3 was screened by whole exome sequencing, which by searching for folate-related genes rendered only one candidate gene, *MTHFD1*. A similar search focused on genes related to thiamine metabolism gave no hits. In total, four novel mutations were found. Patient 2 harbours a missense (c.806C>T; p.Thr269Ile) and splicing (c.1674G>A) mutation, while patient 3 harbours a missense (c.146C>T, p.Ser49Phe), of which the father was a carrier, and nonsense (c.673G>T, p.Glu225*) mutation, of which the mother was a carrier. Both missense mutations change highly conserved amino acids in the active-site of the cyclohydrolase/dehydrogenase domain (Fig. 2) (Schmidt et al 2000) and are predicted by SIFT (both 3.21, damaging) (Kumar et al 2009) and Polyphen-2 (both 1.000, probably damaging) (Adzhubei et al 2010) to be damaging. Neither mutation was found in the 1000 genomes genetic variation database, while only c.146C>T was found in the exome variant server, in 1 allele of 13,005. The splicing variant in patient 2 codes for a silent mutation (p.Thr558=) but is located at the last nucleotide of exon 17 and is predicted to affect the splice donor site of exon 17. This splice site resulted in almost completely absent mRNA from that allele, since on RT-PCR experiments the c.806C>T mutation appeared to be homozygous. Confirmation of the splicing defect was performed by RT-PCR of patient RNA using primers encompassing exons 15–19 (Fig. 3a). In the presence of cycloheximide, which suppresses nonsense mediated decay (NMD), a band was visualised corresponding to a shorter transcript lacking exon 17, which was confirmed by sequencing. This shorter band was very faint in the absence of cylcoheximide and not seen in the control. Sequencing of patients 4 and 5 confirmed the same mutations as their sibling patient 3.

Biochemical determination of MTHFD1 deficiency

Results of biochemical assays performed in fibroblasts of our patients (patient 2 and 3) as well as in the previous patient reported by Watkins et al were as follows. MTHFR activity in all three patients was within the normal range without and with FAD supplementation (Table 1). Cobalamin synthesis assay demonstrated slightly decreased methylcobalamin synthesis for patients 2 and 3 (Table 1), consistent with reported results for the previous patient (Watkins et al 2011). Analysis of [^{14}C]-propionate incorporation showed normal levels for the previous patient and patient 2, neither of which was responsive to OHCbl (Table 1). However, analysis of formation

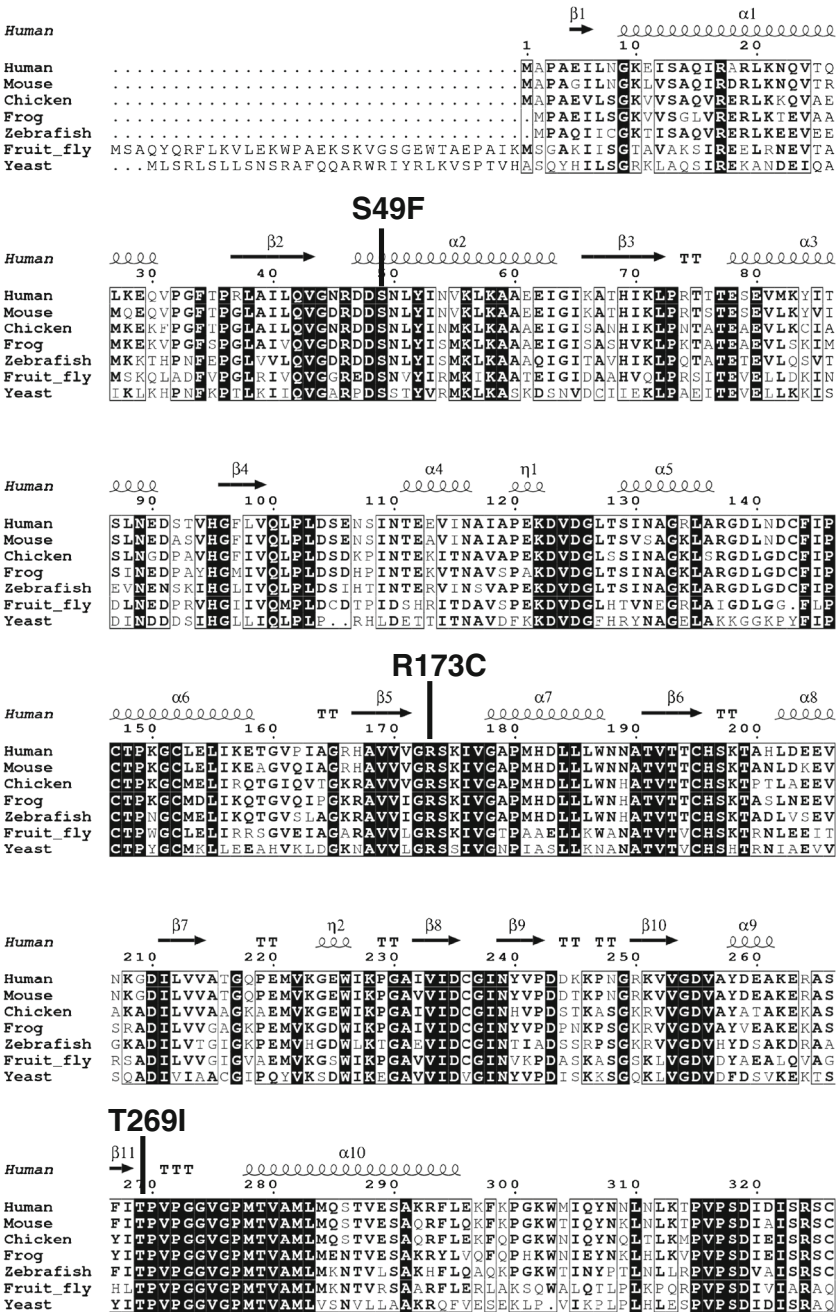

Fig. 2 Multiple sequence alignment of the MTHFC and MTHFD domains of MTHFD1 across evolution. For clarity only the first 326 of 935 residues are shown. Amino acids that are identical in all organisms listed are highlighted in black; residues conserved in most organisms are in black font. Missense mutations from the previous patient (R173C), and patients 2 (T269I) and 3 (S49F) are pointed out. Secondary structure elements are shown above, with α-helices (coils) and β-sheets (arrows) derived from the structure of the MTHFS/MTHFD domains of human MTHFD1 (PDB ID: 1A4I). Sequences are shown for human (Homo sapiens: NP_005947), mouse (Mus musculus: NP_620084), chicken (Gallus gallus: NP_001034392), frog (Xenopus laevis: NP_001080574), zebrafish (Danio rerio: NP_955823), fruit fly (Drosophila melanogastor: NP_731489) and yeast (Saccharomyces cerevisiae: NP_009640). Alignment was created by multalin (Corpet 1988) and visualised by ESPript (Gouet et al 2003)

of methionine from [^{14}C]-formate demonstrated markedly reduced methionine in unsupplemented medium in all three patient fibroblasts compared to those of wild-type controls (Fig. 3b). Supplementation of medium with OHCbl (1 mg/L) had no effect, but addition of folic or folinic acid (each 100 μM) increased methionine formation in all patients' fibroblasts, but not in control cells (Fig. 3b). Formation of serine was also slightly but significantly decreased in fibroblasts from the previous patient and patient 2, but not in patient 3 compared to control, and did not significantly increase upon addition of OHCbl, folinic acid or folic acid for any patient cell line tested (Fig. 3c). An MTHFR deficient cell line showed similar defects in methionine formation but was not responsive to OHCbl, folinic acid or folic acid supplementation (Fig. 3b). However, it had much higher serine formation, even compared to controls (Fig. 3c).

Discussion

Here we describe four new patients, two still living, with MTHFD1 deficiency. These patients showed important similarities and differences from each other and the previous

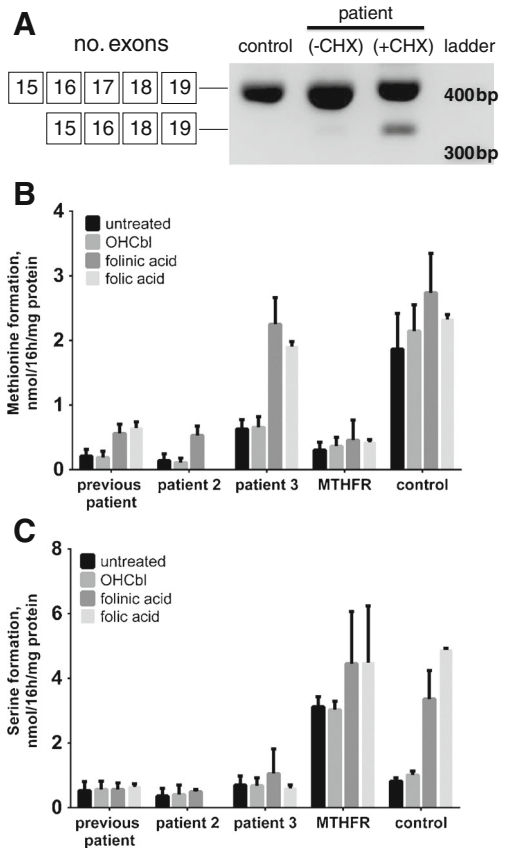

Fig. 3 RT-PCR demonstrating skipping of exon 17. **a**. RT-PCR was performed using primers spanning exons 15–19 on RNA extracted from either wild-type fibroblasts (control) or MTHD1 patient 2 fibroblasts without (−CHX) or with (+CHX) pre-incubation of cycloheximide. Bands were resolved using agarose gel electrophoresis. Expected size with exon 17: 423 bp; without: 346 bp. Formation of serine and methionine from formate. **b**. [^{14}C]-methionine formation from [^{14}C]-formate in fibroblasts from MTHD1 patients, an MTHFR patient and wild-type control. Methionine formation of cells from all three MTHFD1 and the MTHFR patient was decreased compared to control (at least $p<0.01$). Folinic acid and folic acid treatment increased MTHFD1 patient fibroblast methionine formation (each $p<0.001$), but not control or MTHFR (*n.s.*). **c**. [^{14}C]-serine formation from [^{14}C]-formate in fibroblasts from MTHD1 patients, an MTHFR patient and wild-type (control). Serine formation was decreased in fibroblasts from the previous MTHFD1 patient and patient 2 compared to control ($p<0.05$) but not patient 3 (*n.s.*). MTHFD1 patient fibroblasts did not increase serine production upon incubation of OHCbl, folinic acid or folic acid (*n.s.*), however, in control fibroblasts serine production was slightly increased with treatment of OHCbl ($p<0.05$) and greatly increased with folinic acid and folic acid (each $p<0.001$). Untreated MTHFR cells had increased serine formation compared to controls ($p<0.001$), which did not significantly increase upon incubation with OHCbl, folinic acid or folic acid (all *n.s.*). For (**b**) and (**c**): Control results shown were performed in parallel with mutants. In all control cell lines tested so far ($n=18$), methionine synthesis: 2.73±0.80 nmol/16 h/mg protein (no OHCbl) and 2.97±1.02 nmol/16 h/mg protein (OHCbl); serine synthesis: 1.38±0.76 nmol/16 h/mg protein (no OHCbl) and 1.93±1.12 nmol/16 h/mg protein (OHCbl). *n.s.*, not significant

patient (Table 1). All patients had severe megaloblastic anaemia, which seems to be a consistent diagnostic hallmark. Patient 3 showed pathologic appearance in the bone marrow at as early as 4 days of age, indicating that this may be an important early marker of the disease, if tested. While the previous patient was originally reported to have methylmalonic aciduria (Watkins et al 2011), a later publication mentioned normal plasma methylmalonic acid levels (Keller et al 2013), and methylmalonic aciduria was not seen in any of our patients. Plasma homocysteine varied from normal to moderately increased in all living patients and thus may not be a good biomarker for the disease. Patient 2 showed atypical HUS and severe arterial hypertension along with microangiopathy and retinopathy. It is of interest that despite treatment with folinic acid, 5-methyl-THF in the CSF remained low; however, this did not result in abnormal cognitive development and we currently do not understand the mechanism behind it. Patients 4 and 5, in addition to progressive anaemia, at the same early age very suddenly developed signs of intensive inflammation, fatal circulatory shock with severe acidosis, and, in patient 5, widespread inflammatory lesions in internal organs. The most likely cause is overwhelming infection, although no agents were found. The almost identical and rare clinical course is suggestive of an immunological weakness at that phase of life. Patient 3, their sister, had received folic acid at the age when her sibs died. She escaped an early infection but had an episode of autoinflammation at 7 months of age when folic acid therapy was withdrawn. Together with the SCID seen in the previous patient, this is suggests that immunological defects may be an important characteristic of the disease, although these did not play a role in the history of patient 2. Atypical HUS was found in the previous patient and patient 2, but not in patients 3, 4 and 5, although the marked anisocytosis in case 3 might have been a sign of microangiopathy, suggesting HUS is also a potential symptom of MTHFD1 deficiency. Thus, even with these few patients there is varied clinical presentation; however, haematological, immunological and renal symptoms may be prevalent.

MTHFD1 catalyses the 3-step interchange between 5,10-methylene-THF and formate+THF (Fig. 1). While possible to function in either direction, stable isotope and whole-body flux studies suggest that the flow is preferentially in the direction of incorporation of formate into THF (Tibbetts and Appling 2010), although recent quantitative flux analysis of NADPH production suggests it may also flow the other way (Fan et al 2014). Importantly, the single carbon moeity in 5, 10-methylene-THF is a precursor for both the production of serine, via transfer onto a glycine molecule by serine hydroxymethyltransferase, and methionine, via reduction to 5-methyl-THF by MTHFR followed by incorporation into homocysteine by methionine synthase (Fig. 1). Using [^{14}C]-formate we measured the synthesis of both [^{14}C]-serine and [^{14}C]-methionine in our patient cells. We found methionine to be decreased in each MTHFD1 patient cell line, and serine to be decreased in two of three cell lines compared to control

fibroblasts. This is in contrast to MTHFR deficient cells, which show defects in methionine synthesis only and actually have increased serine production compared to controls, as observed here (Fig. 3c) and previously (Fowler et al 1997), and is consistent with the location of the defect in the pathway (Fig. 1). It must be noted that the measure of MTHFD1 activity used here is indirect and not a classical direct assay with saturating substrate and cofactor concentrations. Nevertheless this assay has been clearly shown to reliably detect other remethylation defects (Fowler et al 1997) and in fact may represent a more appropriate reflection of physiological conditions.

Our data, therefore, is compatible with production of 5,10-methylene-THF from formate and THF. Together, these data provide evidence for disturbance of methionine synthesis in this folate disorder and suggest that this assay may be used as a diagnostic tool for the delineation of MTHFD1 deficiency.

Stimulation of methionine synthesis was achieved using folinic acid in the MTHFD1 cell lines. Folinic acid, also known as 5-formyl-THF, enters the 1C-THF pathway as 5, 10-methenyl-THF after conversion by 5,10-methenyl-THF synthetase (Fig. 1), but still requires MTHFD1 dehydrogenase activity to form the serine/methionine precursor 5,10-methylene-THF. Therefore, stimulation of methionine production by folinic acid suggests some activity remains in the patients' enzymes, and increasing substrate can drive the reaction towards increased product synthesis. Addition of folinic acid caused increased methionine synthesis in patient cells and increased serine synthesis in the control, perhaps reflecting the feedback regulation of cellular methionine synthesis. Interestingly, patient fibroblasts also responded to folic acid treatment, suggesting increasing substrate concentration can push the reaction forward. Patient 2 responded clinically to therapy which included folinic acid treatment as well as hydroxocobalamin and betaine, while patient 3 responded to therapy which contained, and was eventually limited to, folic acid. Therefore, folic or folinic acid should be considered as a possible treatment for MTHFD1 deficiency, provided that residual activity is present.

Acknowledgments This work was supported by the Swiss National Science Foundation [grant number 31003A_138521 to M.R.B. and B.F.] and the Rare Disease Initiative Zurich (radiz), a clinical research priority program for rare diseases of the University of Zurich, Switzerland.

Compliance with Ethics Guidelines

Conflict of interest None.

Human and animal rights and informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

References

Adzhubei IA, Schmidt S, Peshkin L et al (2010) A method and server for predicting damaging missense mutations. Nat Methods 7:248–9

Brody LC, Conley M, Cox C et al (2002) A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group. Am J Hum Genet 71: 1207–15

Christensen KE, Rohlicek CV, Andelfinger GU et al (2009) The MTHF D1 p.Arg653Gln variant alters enzyme function and increases risk for congenital heart defects. Hum Mutat 30:212–20

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881–90

de Marco P, Calevo MG, Moroni A et al (2002) Study of MTHFR and MS polymorphisms as risk factors for NTD in the Italian population. J Hum Genet 47:319–24

di Pietro E, Sirois J, Tremblay ML, MacKenzie RE (2002) Mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase is essential for embryonic development. Mol Cell Biol 22:4158–66

Fan J, Ye J, Kamphorst JJ, Shlomi T, Thompson CB, Rabinowitz JD (2014) Quantitative flux analysis reveals folate-dependent NADP H production. Nature 510:298–302

Fowler B, Whitehouse C, Wenzel F, Wraith JE (1997) Methionine and serine formation in control and mutant human cultured fibroblasts: evidence for methyl trapping and characterization of remethylation defects. Pediatr Res 41:145–51

Fox JT, Stover PJ (2008) Folate-mediated one-carbon metabolism. Vitam Horm 79:1–44

Gouet P, Robert X, Courcelle E (2003) ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res 31:3320–3

Jusufi J, Suormala T, Burda P, Fowler B, Froese DS, Baumgartner MR (2014) Characterization of functional domains of the cblD (MMAD HC) gene product. J Inherit Metab Dis

Keller MD, Ganesh J, Heltzer M et al (2013) Severe combined immunodeficiency resulting from mutations in MTHFD1. Pediatrics 131:e629–34

Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 4:1073–81

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–60

Li H, Handsaker B, Wysoker A et al (2009) The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–9

MacFarlane AJ, Perry CA, Girnary HH et al (2009) Mthfd1 is an essential gene in mice and alters biomarkers of impaired one-carbon metabolism. J Biol Chem 284:1533–9

McKenna A, Hanna M, Banks E et al (2010) The genome analysis toolkit: a mapreduce framework for analyzing next-generation DNA sequencing data. Genome Res 20:1297–303

Momb J, Lewandowski JP, Bryant JD, Fitch R, Surman DR, Vokes SA, Appling DR (2013) Deletion of Mthfd1l causes embryonic lethality and neural tube and craniofacial defects in mice. Proc Natl Acad Sci U S A 110:549–54

Parle-McDermott A, Kirke PN, Mills JL et al (2006) Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population. Eur J Hum Genet 14:768–72

Rosenblatt DS, Fenton WA (2001) Inherited disorders of folate and cobalamin transport and metabolism. In: Scriver CR, Beaudet AL, Valle D, Sly WS, Childs B, Kinzler KW, Vogelstein B (eds) The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp 3897–3933

Schmidt A, Wu H, MacKenzie RE et al (2000) Structures of three inhibitor complexes provide insight into the reaction mechanism of the human methylenetetrahydrofolate dehydrogenase/cyclohydrolase. Biochemistry 39:6325–35

Shaw GM, Lu W, Zhu H et al (2009) 118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects. BMC Med Genet 10:49

Stevens VL, McCullough ML, Pavluck AL, Talbot JT, Feigelson HS, Thun MJ, Calle EE (2007) Association of polymorphisms in one-carbon metabolism genes and postmenopausal breast cancer incidence. Cancer Epidemiol Biomarkers Prev 16:1140–7

Suormala T, Gamse G, Fowler B (2002) 5,10-Methylenetetrahydrofolate reductase (MTHFR) assay in the forward direction: residual activity in MTHFR deficiency. Clin Chem 48:835–43

Suormala T, Baumgartner MR, Coelho D et al (2004) The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. J Biol Chem 279:42742–9

Tibbetts AS, Appling DR (2010) Compartmentalization of Mammalian folate-mediated one-carbon metabolism. Annu Rev Nutr 30:57–81

Wang L, Ke Q, Chen W et al (2007) Polymorphisms of MTHFD, plasma homocysteine levels, and risk of gastric cancer in a high-risk Chinese population. Clin Cancer Res 13:2526–32

Watkins D, Rosenblatt DS (2012) Update and new concepts in vitamin responsive disorders of folate transport and metabolism. J Inherit Metab Dis 35:665–70

Watkins D, Schwartzentruber JA, Ganesh J et al (2011) Novel inborn error of folate metabolism: identification by exome capture and sequencing of mutations in the MTHFD1 gene in a single proband. J Med Genet 48:590–2

Willard HF, Ambani LM, Hart AC, Mahoney MJ, Rosenberg LE (1976) Rapid prenatal and postnatal detection of inborn errors of propionate, methylmalonate, and cobalamin metabolism: a sensitive assay using cultured cells. Hum Genet 34:277–83