

Methylmalonic acidaemia: Examination of genotype and biochemical data in 32 patients belonging to *mut*, *cbIA* or *cbIB* complementation group

B. Merinero · B. Pérez · C. Pérez-Cerdá · A. Rincón ·
L. R. Desviat · M. A. Martínez · P. Ruiz Sala ·
M. J. García · L. Aldamiz-Echevarría · J. Campos ·
V. Cornejo · M. del Toro · A. Mahfoud ·
M. Martínez-Pardo · R. Parini · C. Pedrón ·
L. Peña-Quintana · M. Pérez · M. Pourfarzam ·
M. Ugarte

Received: 25 April 2007 / Submitted in revised form: 7 August 2007 / Accepted: 13 September 2007 / Published online: 22 October 2007
© SSIEM and Springer 2007

Summary Methylmalonic acidaemia (MMA) is a genetic disorder caused by defects in methylmalonyl-CoA mutase or in any of the different proteins involved in

the synthesis of adenosylcobalamin. The aim of this work was to examine the biochemical and clinical phenotype of 32 MMA patients according to their genotype, and to

Communicating editor: Viktor Kozich

Competing interests: None declared

References to electronic databases: OMIM catalogue number: Methylmalonic acidaemia: OMIM 251000; *mut*, OMIM 251000; *cbIA*, OMIM 251100; *cbIB*, OMIM 251110; *cbID*-variant 2, OMIM 277410; *cbIH*, OMIM 606169. Methylmalonyl-CoA mutase: EC 5.4.99.2. ATP:cob(I)alamin adenosyltransferase: EC 2.5.1.17.

The first two authors contributed equally to this work.

B. Merinero · B. Pérez · C. Pérez-Cerdá ·
A. Rincón · L. R. Desviat · M. A. Martínez ·
P. Ruiz Sala · M. J. García · M. Ugarte
Centro de Diagnóstico de Enfermedades Moleculares,
Centro de Biología Molecular, Facultad de Ciencias,
Universidad Autónoma, CIBER de Enfermedades Raras,
Madrid, Spain

L. Aldamiz-Echevarría
Servicio de Pediatría, Hospital Cruces,
Baracaldo, Vizcaya, Spain

J. Campos
Servicio de Pediatría, Hospital Clínico San Carlos,
Madrid, Spain

V. Cornejo
INTA, Universidad de Chile, Santiago, Chile

M. del Toro
Servicio de Pediatría, Hospital Val Hebrón, Barcelona, Spain

A. Mahfoud
IDEA, Unidad de Pesquisa Neonatal, Caracas, Venezuela

M. Martínez-Pardo
Servicio de Pediatría, Hospital Ramón y Cajal,
Madrid, Spain

R. Parini
Clinica Pediatrica, Universita Milano, Monza, Italy

C. Pedrón
Servicio de Pediatría, Hospital Niño Jesús,
Madrid, Spain

L. Peña-Quintana
U. Gastroenterología y Nutrición Pediátrica,
Hospital Universitario Materno-Infantil de Canarias,
Las Palmas de Gran Canaria, Spain

M. Pérez
Servicio de Pediatría, Hospital Virgen del Rocío,
Sevilla, Spain

M. Pourfarzam
Department of Child Health and Clinical Biochemistry,
University of Newcastle, Newcastle upon Tyne, UK

M. Ugarte (✉)
Centro de Biología Molecular ‘Severo Ochoa’ CSIC-UAM,
Facultad de Ciencias, Universidad Autónoma de Madrid,
28049 Madrid, Spain
e-mail: mugarte@cbm.uam.es

study the mutant mRNA stability by real-time PCR analysis. Using cellular and biochemical methods, we classified our patient cohort as having the MMA forms *mut*⁰ ($n=19$), *cblA* ($n=9$) and *cblB* ($n=4$). All the *mut*⁰ and some of the *cblB* patients had the most severe clinical and biochemical manifestations, displaying non-inducible propionate incorporation in the presence of hydroxocobalamin (OHCbl) *in vitro* and high plasma odd-numbered long-chain fatty acid (OLCFA) concentrations under dietary therapy. In contrast, *mut*⁻ and *cblA* patients exhibited a milder phenotype with propionate incorporation enhanced by OHCbl and normal OLCFA levels under dietary therapy. No missense mutations identified in the *MUT* gene, including *mut*⁰ and *mut*⁻ changes, affected mRNA stability. A new sequence variation (c.562G>C) in the *MMAA* gene was identified. Most of the *cblA* patients carried premature termination codons (PTC) in both alleles. Interestingly, the transcripts containing the PTC mutations were insensitive to nonsense-mediated decay (NMD).

Abbreviations

AdoCbl	5'-deoxyadenosylcobalamin
ATR	ATP:cob(I)alamin adenosyltransferase
C ₃ -carnitine	propionylcarnitine
C ₄ DC-carnitine	methylmalonylcarnitine
Cbl	cobalamin
GAPDH	glyceraldehyde-phosphate dehydrogenase
GC	gas chromatography
GC-MS	gas chromatography–mass spectrometry
MALDI/TOF/MS	matrix assisted laser desorption ionization/time of flight/mass spectrometry
MCM	methylmalonyl-CoA mutase
MMA	methylmalonic acidaemia
NMD	nonsense-mediated decay
OHCbl	hydroxocobalamin
OLCFA	odd-numbered long-chain fatty acids
PEG	polyethylene glycol
PTC	premature termination codon

Introduction

Methylmalonic acidaemia (MMA; OMIM 251000) is a rare heterogeneous metabolic disorder characterized by an accumulation of methylmalonic acid in body fluids due to the inability to convert L-methylmalonyl-CoA to

succinyl-CoA in the propionate metabolism pathway. This reaction is catalysed by mitochondrial methylmalonyl-CoA mutase (MCM), which requires 5'-deoxyadenosylcobalamin (AdoCbl) as cofactor. MMA is inherited as an autosomal recessive trait, and both mass neonatal screening and selective screening programmes indicate that it is among the most common organic acidaemias worldwide (Leonard et al 2003).

Isolated MMA may be the result of a defect either in the apoenzyme methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) or in the intracellular processing of cobalamin (Cbl), leading to the synthesis of active AdoCbl. The different biochemical forms have been characterized by somatic cell studies which have identified the complementation groups *mut* (OMIM 251000), in which the apomutase itself is affected, and four *cbl* groups showing impaired AdoCbl synthesis: *cblA* (OMIM 251100), *cblB* (OMIM 251110) a deficiency in ATP:cob(I)alamin adenosyltransferase (ATR; EC 2.5.1.17) activity, *cblH* (OMIM 606169), and the recently described *cblD*-variant 2 (OMIM 277410) (Suormala et al 2004). These two last groups may be the same (Moras et al 2007). Patients with the *mut* form of MMA are further divided into the subgroups, *mut*⁰, in which there is no detectable MCM activity in fibroblasts, and *mut*⁻, in which some residual activity can be induced *in vitro* by high concentrations of hydroxocobalamin (OHCbl) in the culture medium.

The mature form of human MCM occurs in the mitochondrial matrix as a homodimer of 750 amino acids. MCM is encoded by the *MUT* gene, which is mapped to chromosome 6p21 and comprises 13 exons spread over 35 kb, expressing a 2.7 kb mRNA transcript. The genes responsible for the *cblA* and *cblB* complementation groups have been identified as *MMAA* and *MMAB* (Dobson et al 2002a, b). The *MMAA* gene maps to chromosome 4q31.1–2, and consists of seven exons that code for a predicted protein of 418 amino acids of as yet unknown function. Two different functions have been proposed based on its homology with prokaryotic proteins: a role in vitamin B₁₂ transport into the mitochondria (Dobson et al 2002b) and the protection or reactivation of MCM (Korotkova and Lidstrom 2004). The *MMAB* gene maps to chromosome 12q24 and consists of nine exons encoding the 250-amino-acid mitochondrial protein ATR (Dobson et al 2002a). The genes that give rise to the groups *cblH* and *cblD*-variant 2 have not yet been identified.

In the present study, we revised the biochemical features and clinical course of 32 patients with isolated MMA, representing the entire clinical spectrum of the

disease, according to their genotype. To assess the functional effects of some missense, nonsense and frameshift mutations, we examined mRNA levels by real-time PCR quantification. The results obtained were then correlated with the clinical phenotypes, and possible biochemical phenotype–genotype correlations were explored.

Patients and methods

Subjects

Thirty-two patients with isolated MMA from 30 unrelated families were evaluated over the past 25 years. The disease had already presented in most of the patients, who were from Spain ($n=23$), Latin America ($n=6$), Italy ($n=2$) and the UK ($n=1$). Four patients were siblings: cases 15 and 16 (Table 1A) and cases 29 and 30 (Table 3).

The disease presented in the neonatal period in 16 patients. In a further 10 patients it presented between the ages of 1 and 12 months, and in a further two between 12 and 24 months of age. Three of the 32 patients were investigated because of a previously affected sibling: two were analysed prenatally (patients 11 and 30), and patient 16 was assessed at 12 months of age. The age of onset for patient 22, in whom MMA presented in infancy, was unavailable. The most common presenting clinical symptoms were poor feeding, progressive lethargy, metabolic acidosis, hyperammonaemia and/or pancytopenia. Laboratory diagnostic criteria were persistent elevated urine methylmalonic and methylcitric acids, hypocarnitinaemia and absence of homocystinuria. Plasma acylcarnitines were retrospectively determined in some cases, indicating high levels of propionylcarnitine (C_3 -carnitine) and somewhat elevated methylmalonylcarnitine (C_4 DC-carnitine). Biochemical responsiveness to the oral (5–20 mg/day) or intramuscular (1–5 mg/twice/week) administration of OHCbl was defined as a decrease in urinary MMA concentrations to less than 1000 mmol/mol creatinine.

Biochemical assays

Organic acids were determined as trimethylsilyl derivatives by GC-MS after urease treatment and ethyl acetate liquid–liquid extraction without oxymation. Plasma odd-numbered long-chain fatty acids (OLCFA) were determined by GC and expressed as the percentage of total fatty acids (Coker et al 1996).

Plasma acylcarnitines were quantified by the MALDI-TOF-MS procedure after solid-phase extraction by strong cation-exchange.

Enzyme and metabolic pathway activities

MCM activity in cell homogenates was determined by the permanganate oxidation assay, using 36 $\mu\text{mol/L}$ AdoCbl (Ledley et al 1990). The incorporation of ^{14}C from [$1\text{-}^{14}\text{C}$]propionate into acid-precipitable material was determined in intact cells grown in basal medium and in 1 $\mu\text{g/ml}$ OHCbl-supplemented medium for 72 h, as a measure of propionate metabolism via MCM (Perez-Cerda et al 1989). Control cell lines showed a mild response of propionate incorporation to added OHCbl: [^{14}C]propionate/[^3H]leucine $\times 100$ (+OHCbl/–OHCbl) ratio up to 1.7. A response was considered when the increase was higher than 1.8.

Somatic cell complementation analysis

Complementation studies were performed by determining the uptake of [$1\text{-}^{14}\text{C}$] propionate in fused cells, prepared by exposing the fibroblasts to 50% polyethylene glycol 4000 (PEG; Merck, Hohenbrunn, Germany). Tester cell lines were obtained from the Repository for Mutant Human Cell Strains in Montreal, Canada.

Mutation analysis and mRNA quantification

Molecular studies on cDNA and genomic DNA were performed using the primers and conditions described previously (Martinez et al 2005). In some cases, cDNA studies were conducted in cell lines after treatment with 100 $\mu\text{g/ml}$ emetine or cycloheximide (Skandalis and Uribe 2004).

RNA was subjected to fluorescent real-time PCR quantification. Total mRNA was extracted using TriPure from Ambion and was converted to cDNA using the Archive kit (Applied Biosystems, Foster City, CA, USA) or Superscript III from Invitrogen (Carlsbad, CA, USA). To quantify MUT cDNA, we used the TaqMan fluorogenic probe gene expression assay Hs00604098 in an ABI Prism 7900HT instrument from Applied Biosystems. MMAB cDNA was analysed using Lightcycler FastStart DNA Master Sybr-Green I in a LightCycler instrument, and MMAA cDNA using a target-specific real-time PCR assay designed using probeFinder software from Roche Applied Science (Universal Probe Library) (Mannheim, Germany). The data were analysed using

Table 1A Genotypes, phenotypes and outcomes in *mut* patients (*mut*⁰ phenotype)

Case (Country of origin)	Nucleotide change	Predicted protein change	%OLCFA ^a	MCM activity ^b	[¹⁴ C]Propionate -/+ ^c	Age at onset/ diagnosis	Clinical course
1^d	c.671– 678dup	p.V227fs	NS	ND	0.28/0.27 ^e	4 d/25 d	Hypotonia, tachypnoea, metabolic acidosis. Died 80 d. Previous brother died with acidosis at 9 d
2^d	c.671– 678dup	p.V227fs	NS	ND*	0.56/0.56*	48 h/ 9 mo	Respiratory distress, hypotonia, poor feeding, acidosis. Alive 18 y. Chronic renal insufficiency
3	c.671– 678dup	p.V227fs					
	c.983T>C	p.L328P	3.1–6.3	ND	0.3/0.3	48 h/9 d	Vomiting, progressive lethargy, hypotonia, hyperammonaemia. Torpid clinical course. Died 1 y
	c.983T>C	p.L328P	<i>n</i> = 5				
4^d	c.983T>C	p.L328P	NS	0.1	2.0/3.0	20 d/25 d	Vomiting, lethargy, hepatomegaly, cytopenia, acidosis, hyperammonaemia. Psychomotor delay, failure to thrive. Died <18 mo Twin brother also affected
	c.983T>C	p.L328P					
5^d	c.671– 678dup	p.V227fs	NS	NS	2.6/2.8**	3 d/5 d	Polypnoea, lethargy, acidosis, hyperammonaemia. Alive 12 y. No renal disease, no psychomotor delay
	c.1105C>T	p.R369C					
6^d	c.671– 678dup	p.V227fs	2.7; 2.6	ND	2.1/2.6	1.5 mo/ 1.5 mo	Feeding refusal, hypertonia, polypnoea, hyperammonaemia. Alive 10 y. Growth + height (10th centile), attention problems, no renal disease
	c.1846C>T	p.R616C					
7^d	c.682C>T	p.R228X	2.7–8.45	ND	1.2/0.8	3 d/11 d	Feeding refusal, vomiting, progressive lethargy, coma, metabolic acidosis, hyperammonaemia. Died 12 y
	c.1036– 1038delCTT	p.L346del	<i>n</i> = 3				
8^d	c.682C>T	p.R228X	2.4	ND	0.4/0.3	32 h/10 d	Feeding refusal, metabolic acidosis, polypnoea, hyperammonaemia. Died 9 m
	c.1029delA	p.K343fs					
9^d	c.326A>G	p.Q109R	1.6–6.6	ND	0.9/1.0	2 d/5 d	Respiratory distress, severe metabolic acidosis, anaemia, ketonuria, mild hyperammonaemia. One previous miscarriage. Alive 13 y. Psychomotor delay, slight impaired renal function
	c.572C>A	p.A191E	<i>n</i> = 35				
	c.572C>A	p.A191E					
10^d (Italy)	c.572C>A	p.A191E	NS	ND	3.0/5.0	3 d/3 d	Polypnoea, metabolic acidosis, hyperammonaemia. Alive 18 m. Regular growth at 10th centile for height and weight; slight psychomotor delay
	c.1073T>C	p.L358P					
11	c.1808G>A	p.V583fs	3.7; 5.7	ND	1.0/1.1	4 d/—	Prenatal diagnosis. Previous brother died at 2 m due to MMA. Died 5.5 y
	?	?					
12	c.454C>T	p.R152X	NS	NS	1.1/1.1	3 d/8 d	Feeding refusal, hypotonia, polypnoea, metabolic acidosis, hyperammonaemia. Died 2 y
	c.2159– 2160delAT	p.N720fs					

Table 1A Continued

Case (Country of origin)	Nucleotide change	Predicted protein change	%OLCFA ^a	MCM activity ^b	[¹⁴ C]Propionate -/+ ^c	Age at onset/ diagnosis	Clinical course
13	c.1022– 1023dupA ?	p.N341fs ?	NS	ND	0.6/0.5	3 d/<1 mo	Feeding refusal, hypotonia, acidosis. One brother died in the neonatal period. Parents related. Died <2.5 y
14^d	[c.1850T>G; c.205A>G] ?	p.L617R p.I69V ?	1.5–8.9 <i>n</i> = 26	ND	0.7/0.7	36 h/4 d	Vomiting, hypotonia, tachypnoea, severe metabolic acidosis, ketonuria. two previous miscarriages. Severe psychomotor delay, renal and liver failure. Hepatic haemochromatosis (no common mutations in the <i>HFE</i> gene). Died 3.5y.

The genotypes of case numbers in bold type were published in Martínez et al (2005).

h, hour; d, day; y, year; mo, month.

NS, not studied; ND, not detectable.

^aOLCFA control levels: 0.66 ± 0.20 .

^bMCM activity in control fibroblasts (+36 $\mu\text{mol/L}$ AdoCbl) (*n* = 26) 0.94 ± 0.40 nmol/min per mg protein.

^cRatio [¹⁴C]propionate/[³H]leucine ($\times 100$) in control fibroblasts -/+ : grown in basal/OHCbl supplemented medium (*n* = 20): $11.8 \pm 7.5/11.9 \pm 6.9$.

^dParents were tested for identified mutations.

^e[¹⁴C]Propionate incorporation in control cells grown in basal/OHCbl supplemented medium (*n* = 32): $1.90 \pm 1.21/2.25 \pm 1.51$ nmol/10 h per mg protein.

*Results from Dr B. Fowler, Basel; **results from Dr P. Briones, Barcelona (controls 7.1–16.8/6.8–14.3 nmol/17 h/mg protein).

Applied Biosystems SDS software. This program computes threshold cycle (C_T) values for each sample and the endogenous control, glyceraldehyde phosphate dehydrogenase (GAPDH), using various mathematical formulae. To examine the effects of the c.291–1G>A sequence variant identified in the *MMAB* gene, we used a complementary primer to the exon junction sequences generated by exon-skipping (sense primer 5'-TTCAGCTATTGGGTTTGCTCTG G-3'; antisense 5'-ATGGAGGGATCCTCCAAGCT-3').

Data analysis

For statistical analysis of the relative quantification (RQ) values, we subjected the sample data to cluster analysis and classified the MUT mRNA levels in the samples as normal ($\log RQ \geq 0$), moderately reduced ($\log RQ$ between -0.7 and -0.4), or low ($\log RQ \leq -0.7$). The MMAA mRNA levels of the samples were classified as normal ($-0.4 > \log RQ < 0.4$) or slightly higher ($\log RQ > 0.4$). In both cases, Pearson χ^2 distributions were determined.

Results

Genetic classification

Patient cells were typed as *mut* or *cbl* defect according to mutase activity, [¹⁴C]propionate incorporation rate (\pm OHCbl) studies and somatic cell complementation studies performed on cultured fibroblasts. Direct genetic analysis was only undertaken in very few cases for which cell lines were not available. Nineteen patients were classified as mutase deficient, nine as *cblA* and four as *cblB*. The patients are listed in Tables 1A, 1B, 2 and 3, according to their complementation group and genotype.

Biochemical and molecular studies

Mutase-deficient patients

Nineteen patients in our cohort were mutase deficient. Mutase activity could not be determined in patients 5 and 12 (Table 1A). All the cell lines showed

Table 1B Genotypes, phenotypes and outcomes in *mut* patients (*mut*⁻ phenotype)

Case (Country of origin)	Nucleotide change	Predicted protein change	%OLCFA ^a	MCM activity ^b	[¹⁴ C]Propionate -/+ ^c	Age at onset/ diagnosis	Clinical course
15^d	c.970G>A c.1482– 1501del20	p.A324T p.E495fs	0.7–0.95 <i>n</i> = 7	ND	2.2/4.3	9 mo/ 20 mo	Reye syndrome, metabolic acidosis, hypotonia, pancytopenia, altered EEG. Alive 19 y. Motor handicap. Normal CT and renal function
16 ^d	c.970G>A c.1482– 1501del20	p.A324T p.E495fs	0.4–0.9 <i>n</i> = 7	ND	0.8/1.6	–/12 mo	Mild acidosis crises after infections. Younger sister of case 15. Alive 16 y. Good clinical condition. Mild renal insufficiency
17^d	c.655A>T c.970G>A	p.N219Y p.A324T	0.5–0.8 <i>n</i> = 5	ND	2.8/7.6	15 mo/ 15 mo	Vomiting, gastrointestinal infections, severe metabolic acidosis, basal ganglia affected. Alive 9 y. Slight motor disorder. Normal renal function
18 ^d (Venezuela)	c.607G>A c.2080C>T	p.G203R p.R694W	NS	ND	3.0/17.0	24 mo/ 24 mo	Vomiting, somnolence, metabolic acidosis, two previous miscarriages; one healthy twin brother. Alive 4 y. Normal physical and psychomotor development
19 ^d (Italy)	c.19C>T c.691T>A	p.Q7X p.Y231N	NS	0.17	3.0/40	4 d/–	Metabolic acidosis, hyperammonaemia. Alive 18 m. Regular growth at 10th centile for weight and height. Normal psychomotor development

The genotypes of case numbers in bold type were published in Martínez et al (2005).

d, day; mo, month.

NS, not studied; ND, not detectable.

^aOLCFA control levels: 0.66 ± 0.20 .

^bMCM activity in control fibroblasts (+36 $\mu\text{mol/L}$ AdoCbl) (*n* = 26) 0.94 ± 0.40 nmol/min per mg protein.

^cRatio [¹⁴C]propionate/[³H]leucine ($\times 100$) in control fibroblasts –/+ : grown in basal/OHCbl supplemented medium (*n* = 20): $11.8 \pm 7.5/11.9 \pm 6.9$.

^dParents were tested for identified mutations.

diminished propionate incorporation in the absence of OHCbl compared to controls. Fourteen were unresponsive to OHCbl supplementation *in vitro* (phenotype *mut*⁰, Table 1A), and five displayed increases in propionate utilization (1.95–13.3 times) and were classified as phenotype *mut*⁻ cell lines (Table 1B). Three of these *mut*⁻ cell lines showed no detectable mutase activity even when assayed in the presence of up to 130 $\mu\text{mol/L}$ AdoCbl (over 360-fold the normal K_m) (Ledley et al 1990).

All *mut*⁰ patients except one presented clinical symptoms during the neonatal period and a severe course of the disease. All five *mut*⁻ patients show a milder clinical and biochemical phenotype. No biochemical response to vitamin B₁₂ administration could

be detected in the mutase-deficient patients. Ten patients (seven *mut*⁰ and three *mut*⁻ patients) under conventional therapy were monitored (Leonard 1995). Despite good compliance with diet, the *mut*⁰ patients showed persistent significantly increased plasma %OLCFA levels, while levels were always within the control range for the three *mut*⁻ patients. However, no significant concentration differences in plasma C₃-carnitine levels (11–85 $\mu\text{mol/L}$; normal < 0.4) between *mut*⁰ and *mut*⁻ patients were detected in five subjects examined.

After sequencing *MUT* cDNA or the 13 exons and their flanking intron sequences, 22 different sequence variations were identified, all of which have been described previously (Acquaviva et al 2005; Martinez

Table 2 Genotypes, phenotypes and outcomes in *cb1A* patients

Case (Country of origin)	Nucleotide change	Predicted protein change	%OLCFA ^a	MCM activity ^b	[¹⁴ C]Propionate -/+ ^c	Age at onset/ diagnosis	Clinical course
20^d	c.397C>T c.397C>T	p.Q133X p.Q133X	0.6; 0.9	0.74	0.9/3.9	8 mo/ 19 mo	Anorexia, vomiting, failure to thrive, severe metabolic acidosis. Parents related. Alive 18 y. Slight psychomotor delay. Normal renal function
21	c.433C>T c.433C>T	p.R145X p.R145X	NS	NS	0.39/1.15 ^e	2 mo/8 y	Vomiting, metabolic acidosis, altered EEG, motor delay. Parents related. Lost from 17 y
22^d	c.433C>T c.433C>T	p.R145X p.R145X	NS	1.06	1.9/12.9	?/2 y	No data. Lost; alive 21 y
23^d (Ecuador)	c.433C>T c.433C>T	p.R145X p.R145X	0.9; 0.7	0.85	0.8/4.3	8 mo/ 15 mo	Vomiting, metabolic acidosis, respiratory arrest, neurological regression, dystonic posturing, microcephaly. Cortical atrophy and bilateral basal ganglia lesions on MRI. Alive 5 y. Growth and psychomotor delay
24 ^d (Chile)	c.562G>C c.562G>C	p.G188R p.G188R	NS	0.58	NS	7 mo/6 y	Measles, metabolic acidosis, stupor. Parents related. Alive 8 y. Psychomotor delay
25^d (Chile)	c.594dupT c.594dupT	p.E199fs p.E199fs	NS	1.45	1.9/7.3	4 mo/ 7 mo	Vomiting, failure to thrive, metabolic acidosis. Alive 11 y. Slight hypotonia, psychomotor delay, attention problems, no renal disease
26 ^d	c.64C>T c.433C>T	p.R22X p.R145X	NS	0.49	1.3/4.8	5 mo/ 20 mo	Vomiting, metabolic acidosis, refusal of protein intake. Alive 21 y. Normal psychomotor development and renal function
27^d (Chile)	c.358C>T c.812– 813dupAG	p.Q120X p.L272fs	NS	2.07	1.9/6.8	2 mo/ 9 mo	Vomiting, failure to thrive, metabolic acidosis. Alive 13 y. Slight hypotonia, attention problems, no renal disease
28 (Chile)	c.450dupG c.733G>A	p.P151fs p.S189fs	NS	1.08	1.9/4.1	7 mo/ 8 mo	Vomiting since neonate, failure to thrive, metabolic acidosis, hyperammonaemia. Lost to follow-up from age 3 y

The genotypes of case numbers in bold type were published in Martínez et al (2005).

mo, month; y, year.

NS, not studied; ND, not detectable.

^aOLCFA control levels: 0.66 ± 0.20 .

^bMCM activity in control fibroblasts (+36 $\mu\text{mol/L}$ AdoCbl) ($n=26$) 0.94 ± 0.40 nmol/min per mg protein.

^cRatio [¹⁴C]propionate/[³H]leucine ($\times 100$) in control fibroblasts -/+ : grown in basal/OHCbl supplemented medium ($n=20$): $11.8 \pm 7.5/11.9 \pm 6.9$.

^dParents were tested for identified mutations.

^e[¹⁴C]Propionate incorporation in control cells grown in basal/OHCbl supplemented medium: $1.92 \pm 1.31/2.28 \pm 1.60$ nmol/10 h per mg protein.

et al 2005; Worgan et al 2006). In all the patients except three, at least one mutant allele was identified, the mutation detection rate being close to 90%. In these cases the unidentified mutation could be located in gene regions not sequenced by standard mutation

detection techniques such as deep in introns. In these three patients, the identified mutation was detected in cDNA in homozygosity. Four *mut*⁰ unrelated families were homozygous for the mutations p.V227fs and p.L328P.

The four *mut*⁻ families were compound heterozygous for two different missense mutations or hemizygous for one missense and one null mutation. Two of them displayed the change p.A324T thought to confer the *mut*⁻ phenotype (Martinez et al 2005; Worgan et al 2006) affecting the substrate-binding domain, in combination either with a null mutation (p.E495fs) or with the previously established *mut*⁰ change p.N219Y. The third family showed the genotype p.G203R/p.R694W, in which p.R694W, located in the cofactor binding domain, is the already described *mut*⁻ mutation (Janata et al 1997). The fourth family's genotype was p.Q7X/p.Y231N, in which p.Y231N located in the substrate domain has already been established as a *mut*⁻ mutation (Janata et al 1997).

The analysis of mRNA levels in patients affected by the *mut* form of MMA identified three groups of cell lines using real-time PCR and TaqMan technology (Fig. 1): those (2, 8 and 1) with intensely reduced mRNA levels (72–175 times lower than those of controls ($n=2$); $p<0.05$), all bearing PTC mutations in both mutant alleles; cell lines 14, 13, 7, 6 and 11

displaying a moderate decrease (3– to 11-fold) in MUT mRNA levels ($p<0.05$) carrying hemizygoty for one missense mutation and one frameshift mutation or in-frame deletion; and finally cell lines 19, 4, 10, 18, 3, 9 and 17 with normal mRNA levels ($p<0.05$), bearing *mut*⁰ or *mut*⁻ missense mutations in both alleles (Tables 1A and 1B).

cblA and *cblB* forms of MMA

Nine patients in our cohort had the *cblA* form of MMA (Table 2). Propionate incorporation was stimulated by OHCbl in the corresponding cell lines, reaching control values in one. Eight cell lines displayed normal mutase activity.

Onset of the disease occurred in infancy in eight of the original nine *cblA* patients, who underwent less life-threatening episodes than the mutase-deficient patients despite lack of dietary compliance in some. A biochemical response to oral or intramuscular administration of OHCbl was recorded in four patients (21, 25, 26, and 27). Patients 20 and 23, monitored regularly in the laboratory, presented plasma %OLCFA

Table 3 Genotypes, phenotypes and outcomes in *cblB* patients

Case (Country of origin)	Nucleotide change	Predicted protein change	%OLCFA ^a	MCM activity ^b	[¹⁴ C]Propionate -/+ ^c	Age at onset/diagnosis	Clinical course
29	c.291-1G>A c.291-1G>A	p.G97fs p.G97fs	5.2	NS	NS	5 d/8 d	Feeding refusal, hypotonia, metabolic acidosis, pancytopenia, hyperammonaemia. Parents related. Died 48 d with scalded skin syndrome
30^d	c.291-1G>A c.291-1G>A	p.G97fs p.G97fs	NS	1.33*	0.04/0.03*	-/-	Prenatal diagnosis. Index case 29. Miscarriage
31^d (UK)	c.556C>T c.556C>T	p.R186W p.R186W	NS	1.52	1.9/1.5	24 h/4 d	Hyperammonaemia, ketosis, hypoglycaemia. Died <10 d
32^d	c.287T>C c.571C>T	p.I96T p.R191W	1.8–2.9 $n=4$	0.99	6.0/62.5	6 d/<1 mo	Feeding refusal, progressive neurological impairment, coma, metabolic acidosis, pancytopenia, hyperammonaemia. Alive 3 y. Encephalopathy, leukopathy on CT, psychomotor and growth delay

The genotypes of case numbers in bold type were published in Martínez et al (2005).

h, hour; d, day; mo, month.

NS, not studied.

^aOLCFA control levels: 0.66 ± 0.20 .

^bMCM activity in control fibroblasts (+36 $\mu\text{mol/L}$ AdoCbl) ($n=26$) 0.94 ± 0.40 nmol/min per mg protein. MCM activity in control amniocytes (+36 $\mu\text{mol/L}$ AdoCbl) ($n=6$): 1.14 ± 0.54 nmol/min per mg protein.

^cRatio [¹⁴C]propionate/[³H]leucine ($\times 100$) in control fibroblasts -/+; grown in basal/OHCbl supplemented medium ($n=20$): $11.8 \pm 7.5/11.9 \pm 6.9$. Ratio [¹⁴C]propionate/[³H]leucine ($\times 100$) in control amniocytes -/+; grown in basal/OHCbl supplemented medium ($n=16$): $24 \pm 18/21 \pm 12$.

^dParents were tested for identified mutations.

*Determined in amniocytes.

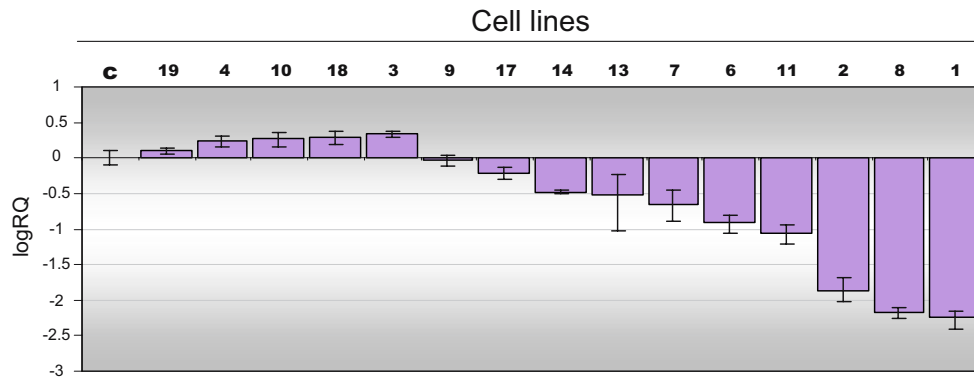


Fig. 1 Gene expression plot for patients with the *MUT* form of MMA. *MUT* mRNA was quantified in control and patient cell lines. The horizontal axis represents the cell lines analysed and the vertical axis represents the results of the relative quantification calculations expressed on a logarithmic scale (log RQ). $RQ = 2^{-\Delta\Delta Ct}$. $\Delta\Delta Ct$ is calculated as the difference between the ΔCt of the target and ΔCt of the internal control gene; the housekeeping gene being GAPDH

in this case. Quantities are shown relative to the expression level of *MUT* mRNA in a control sample, with each increment corresponding to a 10-fold difference in gene expression. The data represent the means of three or four different experiments and the error bars show the calculated maximum (log RQ_{max}) and minimum (log RQ_{min}) expression levels that represent the standard error of the mean expression level (log RQ value).

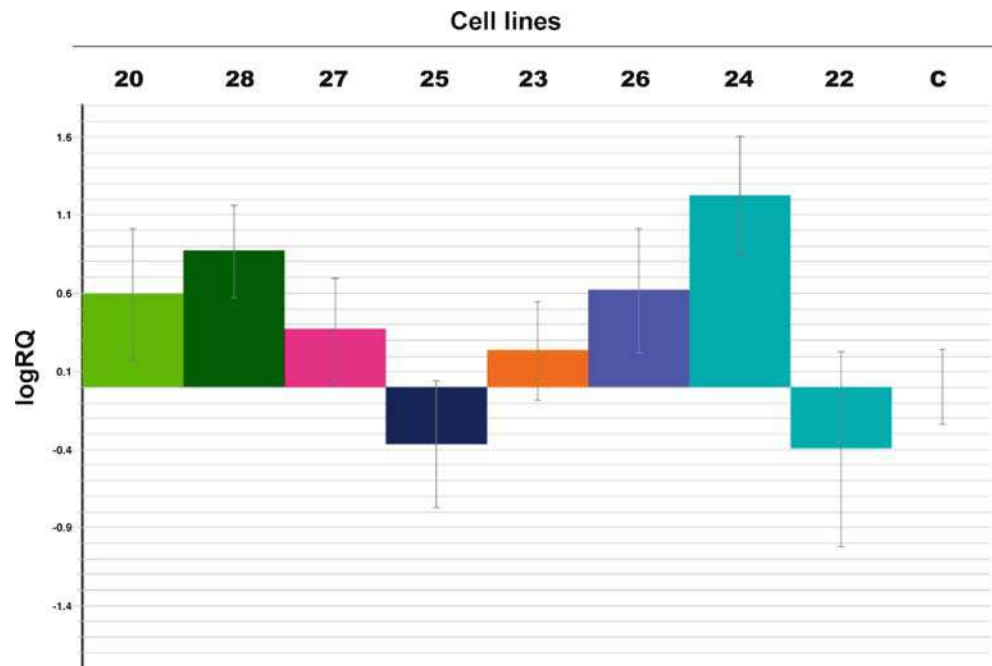
levels within the control range but increased plasma C_3 -carnitine levels (3.1–14.4 $\mu\text{mol/L}$).

All mutations in the *MMAA* gene have already been described (Martinez et al 2005), except the new variation (c.562G>C) identified in the last nucleotide of exon 2 predicting a homozygous missense mutation (p.G188R). No splicing effect was observed to be associated with this change by RT-PCR using total RNA from fibroblasts. All the mutations identified occurred in the first four exons of the *MMAA* gene. A high frequency of homozygous patients was found (62%), although parental consanguinity was confirmed in only three of six cases.

Through real-time PCR analysis and using a specific probe, we assessed *MMAA* mRNA levels in the *cbIA* cell lines (Fig. 2). According to these levels, the cell lines were divisible into a group showing normal mRNA levels (cases 20, 27, 25, 23, 26 and 22) and a group with slightly higher *MMAA* mRNA levels (cases 28 and 24) ($p < 0.05$).

Four patients from three different families were found to have the *cbIB* form of MMA (Table 3). Fibroblasts derived from these patients exhibited normal mutase activity. Two different *in vitro* response patterns to cobalamin were observed: cell lines 30 and 31 were refractory to cobalamin as noted in mutase-deficient

Fig. 2 Gene expression plot for patients with the *MMAA* form of MMA. *MMAA* mRNA was quantified in control and patient cell lines. *MMAA* gene expression analysis was performed using a specific probe and the results are represented as described above for the *MUT* gene expression assay. The data represent the means of three or four different experiments.



cells, and cell line 32 showed a response to B₁₂. In all four patients, the disease was clinically severe, was of neonatal onset and had a poor prognosis. In addition, no clear biochemical response to vitamin B₁₂ could be confirmed in any *cbIB* patient. Effectively, in the only surviving *cbIB* patient, plasma %OLCFA levels are persistently high.

The mutation spectrum of the *MMAB* gene included four single-nucleotide changes, one in the intron sequence (c.291–1G>A), affecting the acceptor site of intron 4, generating a frameshift deletion of exon 4 (p.G97fs), and three in the coding region, likely missense changes. Cell lines 30 and 31 homozygous for p.G97fs and p.R186W, respectively, showed no OHCbl stimulation of propionate incorporation.

In an effort to determine whether some normal splicing was produced related to the nucleotide change c.291–1G>A, we analysed melting curves for transcripts from control fibroblasts and mutant cell line 30 using a LightCycler instrument and RNA master SYBR green mix. No normal mRNA transcripts were found using specific primers that selectively amplify exon 4-containing sequences.

Discussion

The aim of this study was to analyse clinical data, biochemical features and corresponding genotypes in a cohort of patients with isolated MMA. Most patients were examined after the presentation of clinical symptoms, before the introduction of newborn screening by tandem mass spectrometry (MS/MS). Identification of the defect in each patient was based on determining mutase activity and propionate uptake in cultured fibroblasts along with complementation studies. Further characterization involved identifying the disease-causing mutations in the corresponding affected gene. Nineteen patients were assigned to the complementation group *mut* (14 *mut*⁰ and 5 *mut*⁻), nine to group *cbIA*, and four to group *cbIB*.

All patients with the *mut*⁰ form and two with the *cbIB* form of the disease had the most severe biochemical phenotype, as shown *in vitro* by the unresponsiveness of propionate incorporation to OHCbl in fibroblasts and *in vivo* by the high plasma OLCFA levels exhibited by patients under therapy even when clinically stable. OLCFA levels are an indicator of propionate accumulation *in vivo* (Coker et al 1996). These patients were also the most clinically affected, although some bore missense mutations.

Despite most of the *mut* patients being compound heterozygous for two different mutations and the

limited number of patients with the same genotype, it is tempting to draw the following conclusions. Given that all the MUT mRNAs bearing premature termination codons (PTC) were unstable and were degraded probably by the nonsense-mediated decay (NMD) system and that it is likely that these patients lack MCM protein, we would expect a more severe phenotype. However, we observed some inconsistencies in patients with the same genotype. Patients 1 and 2, who were homozygous for the p.V227fs mutation, exhibited a different clinical course. This suggests that the outcome of disease may depend more on the improved therapy in the last 25 years and on the number of metabolic episodes suffered by the patient at an early age, leading to irreversible cerebral lesions, rather than on the precise genotype.

Considering the propionate uptake results in patients with homozygous or hemizygous missense mutations, we classified the identified missense mutations into *mut*⁻ or *mut*⁰ changes. No missense mutation affected mRNA stability, so it seems that mRNA instability is not a general mechanism that contributes to the *mut*⁰ phenotype as has been described previously (Ogasawara et al 1994). The present *mut*⁰ mutations (p.Q109R, p.A191E, p.L328P, p.L358P, p.R369C and p.L617R) may be considered severe mutations since they were detected in patients with neonatal-onset disease and a severe clinical course. Hemizygous (*mut*⁻) changes (p.Y231N, p.A324T, p.R694W) in *mut*⁻ patients with a less severe phenotype may be classified as mild mutations. The p.R616C mutation was not detected in our group of *mut*⁻ changes, although it has recently been described as *mut*⁻ (Worgan et al 2006). The clinical phenotype of patient 6 bearing p.R616C in hemizyosity could indicate that this change may be a mild defect, but the unresponsiveness of [¹⁴C]propionate incorporation to OHCbl observed in this patient's cells precluded the inclusion of this mutation in the *mut*⁻ group.

Two different biochemical phenotypes were observed in the patients with the *cbIB* form of MMA: two of the corresponding cell lines exhibited *in vitro* B₁₂ unresponsiveness and one cell line was responsive to the vitamin. The unresponsive cells were homozygous for two different mutations [p.R186W and c.291–1G>A (p.G97fs)]. Since the corresponding patients displayed a severe biochemical and clinical phenotype, we can conclude that these two mutations are severe changes. No normal transcripts were found in the cell line bearing the c.291–1G>A change using specific primers which selectively amplify exon 4-containing sequences, indicating a lack of ATR protein which would explain the negative response to vitamin B₁₂. The p.R186W

mutation severely affects the function and structure of the enzyme (Saridakis et al 2004; Schubert and Hill 2006; Zhang et al 2006). The mutation responsible for *in vitro* B₁₂ responsiveness in patient 32 [p.I96T] [p.R191W] is not clear: p.R191W has a slightly elevated K_m for cobalamin as recently reported (Zhang et al 2006), and p.I96T, localized outside the active-site interface, could affect the folding properties of the enzyme (data not shown).

Patients from *mut*⁻ and *cblA* groups had a milder biochemical phenotype, showing OHCbl-enhanced propionate incorporation in fibroblasts and normal or near-normal plasma %OLCFA levels under therapy. OLCFA levels are a good indicator not only of compliance with diet but also of the severity of the disease. The *in vitro* and *in vivo* response may be explained by the milder genotypes found in *mut*⁻ forms, but not in *cblA*. The severe genotypes found in our *cblA* patients and similar cases reported by other authors (Dobson et al 2002b; Lerner-Ellis et al 2004) rendering truncated proteins with loss of function, does not explain the relatively better outcome of these patients (longer-term survival, less neurological effect, no renal disease) compared with the remaining MMA patients. In other metabolic disorders responding to vitamins or cofactors, there seems to be a clear relationship between clinical and biochemical responsiveness and the residual activity of the mutant proteins with missense changes (Chuang et al 2004; Erlandsen et al 2004). Several theories have been proposed to explain vitamin B₁₂ responsiveness based on the hypothetical function of the MMAA protein (Dobson et al 2002b; Korotkova and Lidstrom 2004). However, none of these theories has been confirmed. Through quantitation of MMAA mRNA we detected normal or close to normal mRNA levels in our cell lines. Other expected NMD substrates have been reported to be insensitive to this system, including pathological transcripts. It is unknown how they escape NMD, although RNA editing has been proposed as a mechanism in apolipoprotein B (Holbrook et al 2004). In other cases, translation re-initiation at a downstream ATG of the PTC residue has been proposed as a mechanism for the evasion of NMD related to mutations close to the original initiation codon (Harries et al 2005; Inacio et al 2004). Re-initiation of translation could maintain potentially functional truncated MMAA protein and could explain the phenotype and the B₁₂ responsiveness observed in this disease.

In our cohort of patients, the overall survival rate was 53% (17/32), lethality being higher in the *cblB* and *mut*⁰ patients, as found in other series (Fenton et al

2001). At the time of writing, nine surviving patients are older than 12 years, six are between 2 and 10 years old, and two are under 24 months of age. Of these patients, 10 have varying degrees of neurological impairment (attention problems, muscular hypotonia, motor impairment, psychomotor delay), two patients (*mut*⁰ and *mut*⁻) display some degree of renal impairment as the main symptom, and five patients show a good clinical condition. Three *cblA* patients were lost to follow-up. Long-term complications seem to be related to the underlying defect, the age of presentation, compliance with diet, and the development of renal disease in *mut* patients.

Our findings reveal that this disorder, like other monogenic autosomal recessive disorders (Dipple and McCabe 2000a, b), is a complex trait in the sense that it is not only the single product of a mutant gene that renders the metabolic phenotype but modifier genes and nongenetic factors also appear to contribute to the final phenotype of MMA. The knowledge of the genotype and the corresponding molecular mechanisms that give rise to each form of MMA may improve the prognosis of this serious disease and may contribute to finding novel therapeutic targets.

Acknowledgements The authors thank the families included in this study for their cooperation, and M. J. Ecaj, C. Hernández, F. Leal, P. Sanz and A. Sánchez for technical assistance. We thank D. Ortega for the help with the statistical analysis of the data. The study was supported by grants from the Fondo de Investigaciones Sanitarias, Instituto Carlos III (PI060512) and the Ministerio de Educación y Ciencia (SAF 2004–06298). The institutional grant of Fundación Ramón Areces to the Centro de Biología Molecular ‘Severo Ochoa’ is gratefully acknowledged.

Note added in proof The second mutation in patient 11 has been just described by our group in Rincon et al “Propionic and methylmalonic acidemia: antisense therapeutics for intronic variations causing aberrantly spliced mRNA” (*Am J Hum Genet*, accepted for publication).

References

- Acquaviva C, Benoist JF, Pereira S, et al (2005) Molecular basis of methylmalonyl-CoA mutase apoenzyme defect in 40 European patients affected by *mut*⁰ and *mut*⁻ forms of methylmalonic acidemia: identification of 29 novel mutations in the MUT gene. *Hum Mutat* **25**: 167–176.
- Chuang JL, Wynn RM, Moss CC, et al (2004) Structural and biochemical basis for novel mutations in homozygous Israeli maple syrup urine disease patients: a proposed mechanism for the thiamin-responsive phenotype. *J Biol Chem* **279**: 17792–17800.
- Coker M, de Klerk JB, Poll-The BT, Huijman JG, Duran M (1996) Plasma total odd-chain fatty acids in the monitoring of disorders of propionate, methylmalonate and biotin metabolism. *J Inherit Metab Dis* **19**: 743–751.

- Dipple KM, McCabe ER (2000a) Modifier genes convert “simple” Mendelian disorders to complex traits. *Mol Genet Metab* **71**: 43–50.
- Dipple KM, McCabe ER (2000b) Phenotypes of patients with “simple” Mendelian disorders are complex traits: thresholds, modifiers, and systems dynamics. *Am J Hum Genet* **66**: 1729–1735.
- Dobson CM, Wai T, Leclerc D, et al (2002a) Identification of the gene responsible for the cblB complementation group of vitamin B₁₂-dependent methylmalonic aciduria. *Hum Mol Genet* **11**: 3361–3369.
- Dobson CM, Wai T, Leclerc D, et al (2002b) Identification of the gene responsible for the cblA complementation group of vitamin B₁₂-responsive methylmalonic acidemia based on analysis of prokaryotic gene arrangements. *Proc Natl Acad Sci USA* **99**: 15554–15559.
- Erlandsen H, Pey AL, Gamez A, et al (2004) Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations. *Proc Natl Acad Sci USA* **101**: 16903–16908.
- Fenton WA, Gravel RA, Rosenberg LE (2001) Disorders of propionate and methylmalonate metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds; Childs B, Kinzler KW, Vogelstein B, assoc. eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. New York: McGraw-Hill, 2165–2190.
- Harries LW, Bingham C, Bellanne-Chantelot C, Hattersley AT, Ellard S (2005) The position of premature termination codons in the hepatocyte nuclear factor-1 beta gene determines susceptibility to nonsense-mediated decay. *Hum Genet* **118**: 214–224.
- Holbrook JA, Neu-Yilik G, Hentze MW, Kulozik AE (2004) Nonsense-mediated decay approaches the clinic. *Nat Genet* **36**: 801–808.
- Inacio A, Silva AL, Pinto J, et al (2004) Nonsense mutations in close proximity to the initiation codon fail to trigger full nonsense-mediated mRNA decay. *J Biol Chem* **279**: 32170–32180.
- Janata J, Kogekar N, Fenton WA (1997) Expression and kinetic characterization of methylmalonyl-CoA mutase from patients with the mut⁻ phenotype: evidence for naturally occurring interallelic complementation. *Hum Mol Genet* **6**: 1457–1464.
- Korotkova N, Lidstrom ME (2004) MeaB is a component of the methylmalonyl-CoA mutase complex required for protection of the enzyme from inactivation. *J Biol Chem* **279**: 13652–13658.
- Ledley FD, Crane AM, Lumetta M (1990) Heterogeneous alleles and expression of methylmalonyl CoA mutase in mut methylmalonic acidemia. *Am J Hum Genet* **46**: 539–547.
- Leonard JV (1995) The management and outcome of propionic and methylmalonic acidemia. *J Inherit Metab Dis* **18**: 430–434.
- Leonard JV, Vijayaraghavan S, Walter JH (2003) The impact of screening for propionic and methylmalonic acidemia. *Eur J Pediatr* **162**(Supplement 1): S21–24.
- Lerner-Ellis JP, Dobson CM, Wai T, et al (2004) Mutations in the MMAA gene in patients with the cblA disorder of vitamin B₁₂ metabolism. *Hum Mutat* **24**: 509–516.
- Martinez MA, Rincon A, Desviat LR, Merinero B, Ugarte M, Perez B (2005) Genetic analysis of three genes causing isolated methylmalonic acidemia: identification of 21 novel allelic variants. *Mol Genet Metab* **84**: 317–325.
- Moras E, Hosack A, Watkins D, Rosenblatt DS (2007) Mitochondrial vitamin B₁₂-binding proteins in patients with inborn errors of cobalamin metabolism. *Mol Genet Metab* **90**: 140–147.
- Ogasawara M, Matsubara Y, Mikami H, Narisawa K (1994) Identification of two novel mutations in the methylmalonyl-CoA mutase gene with decreased levels of mutant mRNA in methylmalonic acidemia. *Hum Mol Genet* **3**: 867–872.
- Perez-Cerda C, Merinero B, Sanz P, et al (1989) Successful first trimester diagnosis in a pregnancy at risk for propionic acidemia. *J Inherit Metab Dis* **12**(Supplement 2): 274–276.
- Saridakis V, Yakunin A, Xu X, et al (2004) The structural basis for methylmalonic aciduria. The crystal structure of archaeal ATP:cobalamin adenosyltransferase. *J Biol Chem* **279**: 23646–23653.
- Schubert HL, Hill CP (2006) Structure of ATP-bound human ATP:cobalamin adenosyltransferase. *Biochemistry* **45**: 15188–15196.
- Skandalis A, Uribe E (2004) A survey of splice variants of the human hypoxanthine phosphoribosyl transferase and DNA polymerase beta genes: products of alternative or aberrant splicing? *Nucleic Acids Res* **32**: 6557–6564.
- 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**: 42742–42749.
- Worgan LC, Niles K, Tirone JC, et al (2006) Spectrum of mutations in mut methylmalonic acidemia and identification of a common Hispanic mutation and haplotype. *Hum Mutat* **27**: 31–43.
- Zhang J, Dobson CM, Wu X, Lerner-Ellis J, Rosenblatt DS, Gravel RA (2006) Impact of cblB mutations on the function of ATP:cob(I)alamin adenosyltransferase in disorders of vitamin B₁₂ metabolism. *Mol Genet Metab* **87**: 315–322.