Genetics inMedicine | ORIGINAL RESEARCH ARTICLE

Efficacy of early treatment in patients with cobalamin C disease identified by newborn screening: a 16-year experience

Rebecca C. Ahrens-Nicklas, MD, PhD[1], Ashley M. Whitaker, PhD[2], Paige Kaplan, MD[1], Sanmati Cuddapah, MD[1], Jessica Burfield, RD[1], Jennifer Blair, RD[1], Ligia Brochi, RD[1], Marc Yudkoff, MD[1] and Can Ficicioglu, MD, PhD[1]

Purpose: Despite implementation of newborn screening (NBS), outcomes in cobalamin C disease (cblC) remain poor. Therapy with hydroxycobalamin and betaine is widely used, but dietary recommendations vary among metabolic centers. We present a longitudinal analysis of the relationship between metabolic control, diet, and outcomes in a cohort of cblC patients.

Methods: We completed a retrospective analysis of 12 patients with cblC referred for abnormal NBS results and followed in our center between 1999 and 2015.

Results: Of the patients, 87.5% had intellectual disability and 75% had retinopathy; 16.7% had one episode of mild acidosis. However, no patients manifested major metabolic decompensation. Developmental outcomes correlated more closely with initial metabolic abnormalities than with long-term metabolic control. Increased intake of medical foods resulted in better control but also perturbations in the ratios of essential amino acids and lower *z*-scores for head circumference. We found no relationship between diet and cognitive outcomes.

Conclusions: Although dietary therapy for cblC patients improves metabolic control, few patients experience metabolic decompensation regardless of diet. Increased incomplete protein intake is not correlated with improvements in outcomes. Overall, outcomes are poor despite early initiation of therapy and regardless of the dietary strategy used.

Genet Med advance online publication 2 February 2017

Key Words: biochemical genetics; cobalamin C deficiency; newborn screening; outcomes

INTRODUCTION

Combined methylmalonic acidemia and hyperhomocysteinemia—cobalamin C subtype (or cobalamin C deficiency (cblC)—is the most common disorder involving vitamin B12 metabolism. Mutations in the *MMACHC* gene result in impaired synthesis of both adenosylcobalamin, a cofactor for methylmalonyl-CoA mutase, and methylcobalamin, a cofactor for methionine synthase.[1] Loss of these active forms of vitamin B12 results in the characteristic biochemical findings of elevated serum homocysteine levels with associated low methionine levels and elevated methylmalonic acid (MMA) levels.[2,3]

Clinical features of cblC vary. Some patients manifest with an early-onset phenotype that includes intrauterine growth restriction followed by feeding difficulties, microcephaly, developmental delay, seizures, retinopathy, hemolytic uremic syndrome, cytopenias, and renal failure.[4–8] As in other forms of methylmalonic acidemia, acidosis and acute metabolic crises can occur; however, in general, cblC patients have a lower risk of acute decompensation and lower levels of MMA as compared to patients with the classic *mut* subtype of MMA. A later-onset form of cblC presents with neurologic and thromboembolic complications.[9] Molecular testing has revealed evidence of genotype–phenotype correlations that contribute to the variability of age of onset and clinical severity.[10]

Newborn screening (NBS) for cblC through the detection of elevated propionylcarnitine (C3) on tandem mass spectrometry analysis of dried blood spots has been widely implemented in the United States for more than a decade. Follow-up studies show that, despite early identification and immediate initiation of therapy, outcomes are still poor.[11,12] The ocular and cognitive manifestations of the disease seem especially difficult to prevent.[5,6,8,11–13] The mainstay of treatment includes high-dose intramuscular hydroxycobalamin (OHCbl), which can lower serum MMA and homocysteine levels and increase serum methionine levels.[14,15] Betaine can also be used to increase remethylation of homocysteine to methionine. Some patients are also treated with folinic acid, carnitine, methionine, valine, or other supplements; however, there are limited outcomes data regarding the efficacy of these interventions.

[1]Division of Human Genetics, The Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA; [2]Department of Child and Adolescent Psychiatry and Behavioral Sciences, The Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania, USA.
Correspondence: Can Ficicioglu (Ficicioglu@email.chop.edu)

Submitted 2 September 2016; accepted 15 November 2016; advance online publication 2 February 2017. doi:10.1038/gim.2016.214

Some patients receive formulas that are low in or devoid of amino acids that are propionate precursors. This practice is controversial because most cblC patients have only mild increases in MMA levels as compared to the levels seen in other defects of propionate metabolism.[16] Furthermore, cblC patients are at risk for methionine deficiency due to their inability to convert homocysteine to methionine. Because medical foods deficient in propiogenic amino acids are devoid of methionine, patients may have an iatrogenic exacerbation of their already present methionine deficiency. A recent cross-sectional analysis of medical foods for 28 patients with early-onset cblC raised concerns that incomplete protein intake may induce iatrogenic methionine deficiency and branched-chain amino acid imbalances, which could lead to poor head growth and may influence developmental outcomes.[17]

In this work, we report a retrospective analysis of our 16-year experience with cblC patients identified through NBS. We build upon the previously reported cross-sectional study[17] by investigating the longitudinal relationship between dietary interventions, metabolic control, and outcomes in our cohort. In addition to reporting outcomes for patients that with cblC detected and treated early, we specifically address the following questions:

1. Is there a relationship between initial or long-term metabolite (MMA, homocysteine, and methionine) levels and neurocognitive outcomes?
2. Does dietary protein composition (i.e., natural protein intake or incomplete protein intake) correlate with long-term metabolic control, branched-chain amino acid imbalances, or relative methionine deficiency in an individual over time?
3. Does the long-term protein composition of the diet affect growth or neurocognitive outcomes?

MATERIALS AND METHODS

Study population

In this single-center retrospective study, the clinical features, laboratory values, management, and outcomes of 12 patients with cblC identified through NBS and followed at the Children's Hospital of Philadelphia (CHOP) were analyzed. This study was approved by the CHOP Institutional Review Board.

Inclusion criteria included birth date between 1 August 1999 and 31 December 2015 and the presence of abnormal Pennsylvania (PA), New Jersey (NJ), or Delaware (DE) NBS results with subsequent biochemical and *MMACHC* molecular testing confirming a diagnosis of cblC. All three states use C3 as a primary marker of cblC. PA also uses the C3/C2 ratio and C3/C16 ratio. NBS data were collected at 24–48 hours of life and were typically reported at 4–7 days of life.

Patients were excluded if they had a false-positive NBS result or had not been evaluated in our clinic in the past year. The state screening laboratories refer every neonate born in our geographical region (eastern PA, southern and central NJ, and DE) with an abnormal NBS result to our center. Therefore, all patients confirmed to have cblC through an abnormal NBS result in our referral region have been included.

Investigation of the relationship between metabolite levels and neurocognitive outcomes

All lifetime measurements of methionine, valine, leucine, total homocysteine, and MMA from our institution were reviewed to identify the lowest methionine and valine levels and peak total homocysteine and MMA levels. These data were also used to compute the mean lifetime metabolite levels and mean and peak measurements of the leucine-to-methionine and leucine-to-valine ratios.

Participants underwent neuropsychological evaluation to assess general cognitive ability, language development, visual-motor integration/fine-motor coordination, and academic achievement, and their caregivers completed questionnaires designed to assess adaptive functioning (more details available in **Supplementary Methods** online). Additionally, to conduct analyses containing neurodevelopmental outcomes with such a small sample, an overall impairment score was calculated for each subject using the percentage of neuropsychological domains evaluated that were in the severely impaired range (at least 3 standard deviations (SDs) below the mean).

Investigation of the relationship between dietary protein composition and metabolic control

Dietary records including the amount of incomplete protein (g/kg/day) and complete protein (%RDA) documented in clinic notes were reviewed for all patients at the visit closest to the following ages: 2, 3, 6, 9, 12, 18, and 24 months and then annually (until the last available visit). The homocysteine, MMA, leucine, valine, and methionine levels from each visit were reviewed.

Investigation of the relationship between dietary composition and outcomes

All subjects who underwent neuropsychological assessment were divided into two groups by degree of impairment (severe versus moderate or better) for each domain tested. The average complete and incomplete protein intake of these two groups was then compared.

Statistical analysis

For all statistical analyses, $P < 0.05$ was considered significant. Results are presented as means (±SD). For categorical data, a two-tailed Fisher's exact test was used. For continuous variables, a two-tailed unpaired *t*-test was used. Pearson correlation coefficient and multiple linear regression analyses were used to evaluate correlations between variables.

For statistical analyses of neuropsychological data, continuous dependent variables were tested for normal distribution using Shapiro-Wilk tests of normality as well as visual observation of normality plots due to small sample size. Primary outcome variables, including general cognitive ability (calculated using mental age on either the Woodcock-Johnson III or the

Table 1 Initial patient characteristics

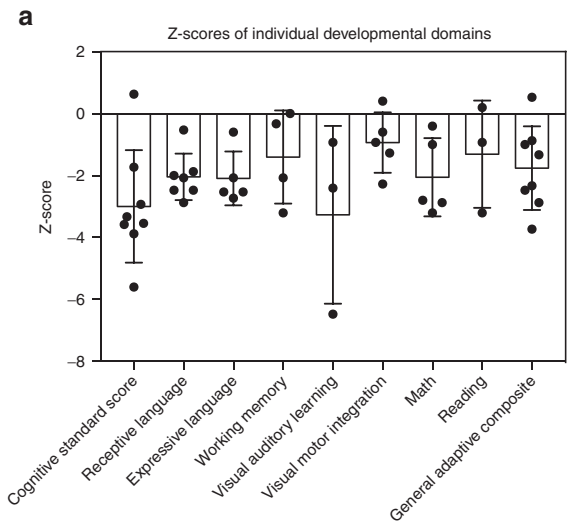
Patient	Sex	Age at diagnosis (days)	Symptoms at diagnosis?	Ethnicity	*MMACHC* Allele 1	*MMACHC* Allele 2	NBS C3 (μmol/l)	Confirmatory C3 (μmol/l)	Initial serum MMA (mmol/l)	Initial urine MMA (mmol/mol Cr)	Initial total homocysteine (μmol/l)	Initial methionine (μmol/l)	Initial CBC
1	M	7	Poor feeding	Caucasian	c.271dupA p.R91KfsX14	c.500delC p.P167QfsX3	Elevated	elevated	70	1,661	128	1.2	Normal
2	M	7	Poor feeding	Caucasian	c.271dupA p.R91KfsX14	c.271dupA p.R91KfsX14	elevated	elevated	122	3,087	183	4.5	Normal
3	F	5	Poor feeding	Hispanic	c.328_331delAACC p.N110DfsX13	c.566G>A p.R189H	4.4	6.7	unknown	834	unknown	15.2	Leucopenia; anemia
4	M	6	Poor feeding	Caucasian	c.440C>A p.G147D	c.468delCT p.G156G_fsX25	7.1	13.8	28	149	123	5.8	Pancytopenia
5	F	4	Poor feeding, lethargy, hypotonia	Ashkenazi	c.271dupA p.R91KfsX14	c.271dupA p.R91KfsX14	9.8	11.2	56	358	108	<2	Leukopenia
6	F	4	Poor feeding, hypothermia, jaundice	Hispanic	c.331C>T p.R111X	c.615C>G p.Y205X	8.7	9.2	46	2,698	225	3.6	Leukopenia
7	F	5	Poor feeding	Hispanic	c.328_331delAACC p.N110DfsX13	c.328_331delAACC p.N110DfsX13	9.1	15	478	3,235	202	4.9	Leucopenia
8	M	6	Asymptomatic	Hispanic	c.482G>A p.R161Q	c.608G>A p.W203X	Normal[a]	6.1	16	388	70	30.3	Normal
9	M	6	Hypothermia, hypotonia	Hispanic	c.430-2A>G	c.481C>T p.R161X	10.8	10.5	162	2,155	210	10	Leukopenia
10	F	34[b]	Poor feeding, hypoglycemia, dehydration, respiratory distress	Hispanic	c.352delC p.Q118RfsX6	c.352delC p.Q118RfsX6	8.4	3.6	31	460	224	2.3	Pancytopenia
11	F	Unknown[c]	Poor feeding, oor growth	Caucasian	c.271dupA p.R91KfsX14	Unknown (normal del/dup analysis)	7.2	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
12	F	6	Poor feeding, acidosis	Hispanic	c.271dupA p.R91KfsX14	c.328_331delAACC p.N110DfsX13	11.3	6.4	22	499	46	3.1	Thrombocytopenia
AVERAGE:		5.6					8.5	9.2	103	1,411	152	8.1	

[a]CblC missed on NBS due to low carnitine levels, case previously reported (JIMD Rep. 2015; 23: 71–75). [b]Delay in referral for abnormal NBS. [c]Partially mangaged by outside provider until 8 months. CBC, complete blood count; MMA, methylmalonic acid.

Table 2 Current management and outcomes

Patient number	Age at last visit	Current therapy (mg/kg/day excepted as noted)	Current complete protein (%RDA)	Current incomplete protein (g/kg/day)	Weight height OFC BMI Z-scores	Seizures?	Visual acuity/ Nystagmus?	Intellectual Z-score (age tested)	Current and mean (SD) plasma homocysteine (normal <15 µmol/l)	Current and mean (SD) plasma MMA (normal 0.1-0.37 µmol/l)	Current and mean (SD) plasma met (normal 8-49 µmol/l)
1	16 years	OHCbl (1 mg[b]) betaine (289) carnitine (17.8) methionine (7.1) valine (4.2)	129%	0.83	–2.45, –2.64, 0.83, –1.66	–	20/400, +	Unknown	92, 89 (18)	33, 20 (19)	43, 158 (180)
2	16 years	OHCbl (1 mg[b]) betaine (109) methionine (22)	55%	0.4	0.85, 0.7, 0.67, 0.16	–	20/400, +	–3.54 (15 years)	209, 119 (34)	26, 40 (37)	53, 106 (196)
3	11 years	OHCbl (1 mg[c]) betaine (96.8)	105%	0	1.78, 0.08, 0.14, 2.08	–	20/50, -	–1.73 (10 years)	41 29 (10)	12, 51 (47)	23, 32 (12)
4	10 years	OHCbl (1 mg[c]) betaine (133) carnitine (33) methionine (5.9) valine (7.3)	105%	0	–0.56, 0.28, 0.16, –1.36	–	20/300, +	–3.88 (10 years)	46, 52 (23)	15, 10 (9.5)	18, 21 (15)
5	5 years	OHCbl (1 mg[b]) betaine (153) methionine (8.2)	168%	0	0.34, 0.27, –0.57, 0.32	–	20/380, +	Unknown	70, 50 (23)	19, 15 (17)	135, 78 (72)
6	5 years	OHCbl (1 mg[a]) betaine (152) methionine (17.1) valine (9.4)	80%	1.14	1.21, 0.37, –1.03, 1.52	–	Unknown, -	–3.58 (4 years)	78, 57 (38)	38, 9.1 (9.0)	39, 18 (16)
7	5 years	OHCbl (1 mg[c]) betaine (182) carnitine (72.7) methionine (15.2)	105%	1	–0.51, –1.61, –2.32, 0.8	–	Unknown, +	–2.93 (4 years)	68, 69 (18)	23.8, 8.9 (9.1)	13, 21 (12)
8	4 years	OHCbl (1 mg[c]) carnitine (17.5)	184%	0	0.08, –1.27, 0.4, 0.93	–	20/30, -	0.63 (3 years)	20, 14 (2.5)	6.5, 3.0 (4.6)	28, 25 (10)
9	27 months	OHCbl (1 mg[c]) betaine (139) carnitine (16.2) methionine (24.4) valine (11.3)	95%	0.66	1.42, 2.13, –1.45, 0.15	+	20/360, +	–5.6 (25 months)	69, 48 (23)	18, 8.0 (6.2)	8.6, 16 (12)
10	33 months	OHCbl (1 mg[c]) betaine (141) carnitine (57.8) methionine (14.1) valine (5.9)	80%	1.03	–0.19, –1.13, –3.28, 0.02	+	20/260, +	–3.33 (27 months)	54, 47 (14)	11, 4.6 (6.4)	35, 31 (22)
11	18 months	OHCbl (1 mg[a]) betaine (111)	133%	1.1	–0.79, –0.48, –0.73, –0.06	–	Unknown, +	Unknown	24, 26 (5)	1.1, 1.4 (1)	19, 29 (16)
12	7 months	OHCbl (1 mg[c]) betaine (94.3) methionine (8.3) valine (11.1)	92%	1.25	–0.64, –1.8, –0.1, 0.03	–	Unknown, +	Unknown	25 23 (4)	0.5, 0.5 (0.5)	33, 24 (12)
Average or proportion affected			111%	0.62	0.05, –0.43, –0.61, 0.24	2/12 (16%)	20/272 ($n = 8$), 9/12 (75%)	–3 ($n = 8$)	55	16	48

[a]mg/dose given daily, [b]given 2-3 times per week, [c]given once a week. OFC, occipital frontal circumference.



b

Correlation between metabolites and % of developmental domains with severe impairment

Lifetime Worst Metabolite Level	r	R^2	*p* value
Lowest Methionine	−0.76	0.58	0.03*
Lowest Valine	0.16	0.027	0.7
Highest Homocysteine	0.85	0.72	0.0076*
Highest MMA	0.23	0.054	0.58
Lifetime Mean Metabolite Level			
Mean Methionine	0.23	0.053	0.58
Mean Valine	−0.58	0.34	0.13
Mean Homocysteine	0.79	0.62	0.02*
Mean MMA	0.53	0.28	0.18
Amino Acid Ratios			
Peak Leucine:Methionine	0.18	0.031	0.68
Peak Leucine:Valine	−0.32	0.1	0.45
Mean Leucine:Methionine	0.13	0.017	0.76
Mean Leucine:Valine	0.093	0.0086	0.83

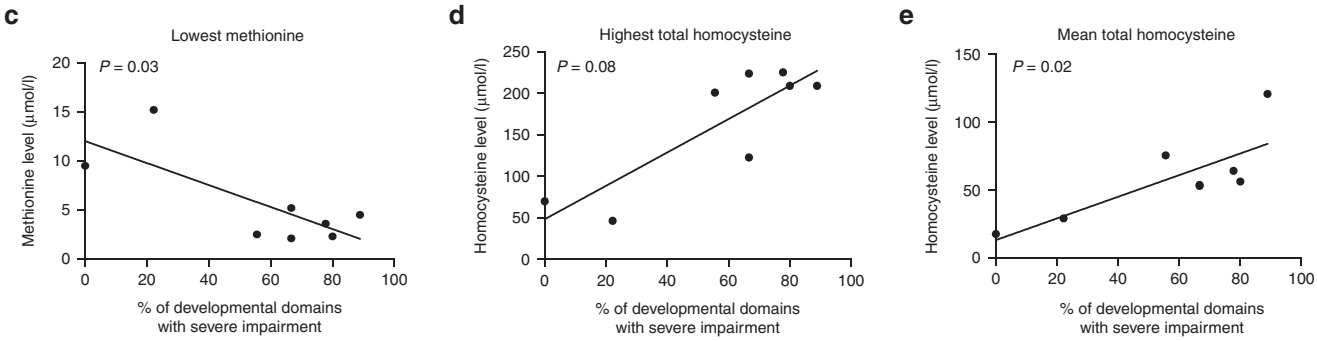


Figure 1 Neurodevelopmental outcomes are correlated with the lowest lifetime methionine and highest peak homocysteine levels and mean lifetime total homocysteine levels. (**a**) Mean (±SD) values for each developmental domain tested are shown. Not all subjects were able to participate in all tests. Closed circles represent each individual for each domain. (**b**) An impairment score, the percentage of neuropsychological domains that were severely impaired, was calculated for each of the eight individuals who underwent neuropsychological evaluation. Pearson correlation coefficient and multiple linear regression analyses were used to evaluate correlations between variables. Statistically significant relationships ($P < 0.05$) are indicated in bold with an asterisk. (**c, d**) Lowest methionine level and highest total homocysteine level correlated with more neuropsychological impairment; 100% of the lowest methionine levels occurred in the neonatal period and 75% of the peak homocysteine levels occurred in the neonatal period. (**e**) Higher mean homocysteine levels were also correlated with poorer neuropsychological test scores. MMA, methylmalonic acid.

Bayley-3 over chronological age instrument), adaptive functioning, language, and visual-motor skills, were converted to *z*-scores and compared to normative age expectations ($z = 0.0$, SD = 1.0).

RESULTS

Initial features of subjects

From 1999 to 2015, 12 patients were referred for an abnormal NBS result and were confirmed to have cblC. All have had regular follow-up in our center and were included in this study. The initial confirmatory metabolite levels drawn on the day of diagnosis are shown in **Table 1**. During the study period in the state of NJ, 1,337,843 infants were screened and 11 cblC patients were identified (incidence of 1:121,622). We had five subjects from NJ in our cohort, representing 45% of the state's cases. Similar total incidence data were not available from PA or DE.

Eleven of 12 (92%) patients presented with symptoms in the neonatal period. The most common neonatal symptom was poor feeding, seen in 83% of patients. Eight of 12 (67%) patients showed decreases of one or more cell lines on initial complete blood count. OHCbl was started for all patients at the time of diagnosis; betaine was started for most patients (100–200 mg/kg/day) (**Supplementary Figure S1** online). Variable dietary plans (including amino acid supplementation in some individuals) were initiated as discussed below.

Patient 8 was asymptomatic on initial evaluation and has a milder form of the disease. His NBS did not show a C3 elevation; rather, he was referred to our center for a low carnitine level on NBS and confirmatory testing revealed the diagnosis of cblC (the data for this patient have been previously published[18]). Genotyping confirmed that he carried two different mutations, a previously reported mild missense mutation (p.R161Q) and a more severe nonsense mutation (p.W203X).[10]

Current features of subjects

The mean age at the last evaluation was 6.6 years (7 months–16 years) (**Table 2**); 100% and 92% of patients were treated with OHCbl and betaine, respectively. Dietary therapy ranged from

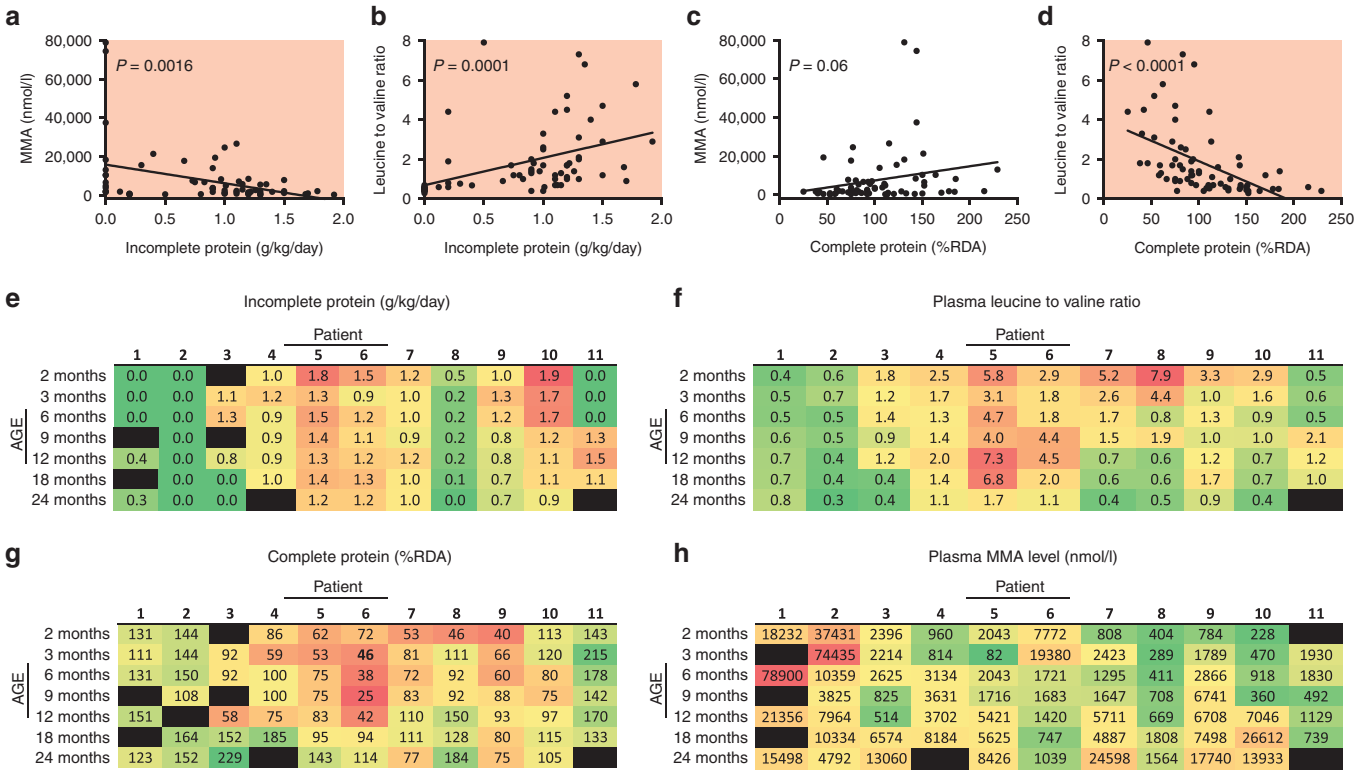
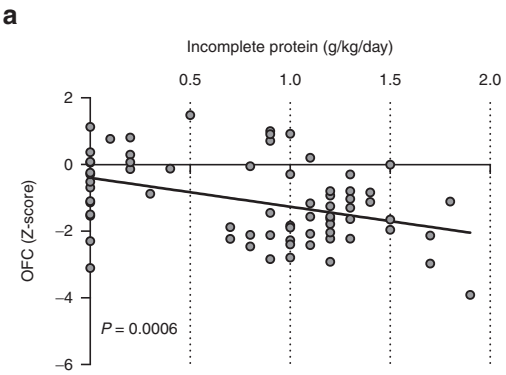


Figure 2 Increased intake of incomplete protein and decreased intake of complete protein is associated with mild improvements in metabolic control, but these dietary interventions are also associated with perturbations of essential amino acid ratios. Retrospective dietary information and serum metabolite values were evaluated for all patients in the study who were at least 18 months old at the last evaluation. Information from the visit closest to selected standardized ages (2, 3, 6, 9, 12, 18, and 24 months) was reviewed. (**a-d**) Pearson correlation coefficient and multiple linear regression analyses were used to evaluate correlations between variables. Statistically significant relationships ($P < 0.05$) are indicated in red. Increased incomplete protein intake was statistically significantly correlated with lower methylmalonic acid (MMA) levels, and there was a nonsignificant trend toward higher MMA levels with higher complete protein intake. Increased incomplete protein and decreased complete protein were associated with higher leucine-to-valine ratios. (**e-h**) Heat map analysis demonstrates differences between individuals in their sensitivity to dietary manipulations as measured by the leucine-to-valine ratio and plasma MMA levels. For incomplete protein (**e**), leucine-to valine-ratio (**f**), and MMA (**h**), the lowest value of the heat map is indicated in green, the highest is in red, and the 50th percentile is in yellow. For complete protein, the lowest value is indicated in red and the highest is indicated in green.



b

Correlation between protein intake and growth

Complete protein (%RDA) versus growth parameter	r	R^2	*P* value
OFC	0.21	0.043	0.088
Length	−0.016	0.00027	0.90
Weight	−0.00033	1.1E-07	0.998
Incomplete protein (g/kg/day) versus growth parameter			
OFC	−0.40	0.16	0.0006*
Length	−0.18	0.034	0.15
Weight	−0.24	0.057	0.055

Figure 3 Long-term increased medical food consumption is correlated with lower head circumference measurements, but no other outcomes are correlated with diet in this population. Pearson correlation coefficient and multiple linear regression analyses were used to evaluate correlations between protein intake and growth parameters measured at the standardized time points shown in Figure 2. (**a**) Higher consumption of incomplete protein was correlated with small head circumferences. (**b**) No other growth parameters were correlated with either complete or incomplete protein intake. OFC, occipital frontal circumference.

no or mild protein restriction to the use of medical foods with amino acid supplementation. Mean growth parameters were within two SDs of published norms. Mean (SD) growth *z*-scores included weight, 0.02 (1.1); height, −0.52 (1.3); BMI, 0.23 (1.0); and OFC, −0.54 (1.2). There was no statistical difference between the mean *z*-scores of the different growth parameters.

Biochemical control varied greatly among individuals. Not unexpectedly, all patients had elevated average total

homocysteine and MMA levels. All individuals also had average and current plasma methionine levels that were within the normal range. Eleven of 12 patients (92%) had clinical manifestations of disease (**Table 2**). Two patients (16%) each had a single lifetime episode of acidosis (low serum bicarbonate levels of 13 and 14 μmol/l) in the setting of gastroenteritis. One of these episodes was associated with mild ketonuria; the other did not have associated ketosis. Both patients showed biochemical improvement within 24 hours of initiation of dextrose-containing intravenous fluids. There were no other episodes of acute metabolic decompensation noted in any of the subjects.

Question 1: is there a relationship between initial or long-term metabolite (MMA, homocysteine, and methionine) levels and neurocognitive outcomes?

Eight subjects underwent neuropsychological evaluation. Two older patients refused testing due to scheduling conflicts and two patients were too young to undergo meaningful testing. Deficits were identified across all neuropsychological domains tested, with the cohort as a whole performing below normative expectations (**Figure 1a**). Mean *z*-scores for each domain ranged from −0.9 to −3.3, with no statistical differences between domains, which may reflect global/uniform impairment across domains in this population. The mean general cognitive *z*-score was −3.0 (1.8).

Lifetime peak, trough, and mean metabolite levels were analyzed for each subject who underwent neuropsychological evaluation (**Figure 1**). Correlation of these values with an impairment score (i.e., the percentage of neuropsychological domains tested that showed severe impairment) was completed (**Figure 1b**). The lowest documented methionine, which always occurred in the neonatal period and is a reflection of fetal methionine insufficiency, was correlated with a higher impairment percentage (**Figure 1c**). Peak total homocysteine levels were positively correlated with an increased neuropsychological impairment (**Figure 1d**); 75% of patients had peak total homocysteine levels in the neonatal period.

To investigate the effect of long-term metabolic control on neurodevelopmental outcomes, lifetime mean metabolite levels were examined. Higher mean total homocysteine (**Figure 1e**) levels were correlated with poorer impairment scores. Mean methionine, valine, and MMA levels were not correlated with neurodevelopmental outcomes. These same relationships between mean metabolites and neurodevelopmental outcomes were found when the lifetime area under the curve (normalized for age) was compared to outcomes (**Supplementary Figure S2** online).

The ratios of methionine and valine to leucine were also investigated because relative methionine deficiency and branched-chain amino acid imbalances have been implicated in the pathophysiology of cblC disease. Neither peak nor mean leucine-to-methionine or leucine-to-valine ratios were significantly correlated with the impairment score.

Question 2: does dietary protein composition correlate with long-term metabolic control, branched-chain amino acid imbalances, or relative methionine deficiency in an individual over time?

To evaluate the relationship between diet and metabolic control in each individual and the study population, detailed diet, treatment, and metabolic laboratory data were analyzed. Specifically, for each of the 11 subjects who were older than 18 months of age at the end of the study, data were collected from clinical documentation at uniform ages (2, 3, 6, 9, 12, 18, and 24 months of age) (**Figure 2**).

MMA levels were used as a marker of metabolic control. MMA levels were inversely related to the amount of incomplete protein that a patient ingested ($r = -0.3747$; $R^2 = 0.1404$; $P = 0.0016$) (**Figure 2a**). The correlation between MMA and complete protein intake did not reach statistical significance ($r = 0.2252$; $R^2 = 0.0507$; $P = 0.06$) (**Figure 2c**). There was not a statistically significant relationship between both incomplete or complete protein and total homocysteine (**Supplementary Figure S3A,C** online). However, in addition to diet, homocysteine levels are influenced by betaine and hydroxycobalamin dosages, which varied both between individuals and over time (**Supplementary Figure S1** online).

To determine whether the incomplete protein found in medical foods can alter the balance of branched-chain amino acids thought to be essential for normal neurologic functioning, the relationship between the leucine-to-valine ratio and diet was examined. Higher incomplete protein consumption was associated with a higher ratio of leucine to valine ($r = 0.4423$; $R^2 = 0.1957$; $P = 0.0001$) (**Figure 2b**). Conversely, increased consumption of complete protein was correlated with lower leucine-to-valine ratios ($r = -0.5078$; $R^2 = 0.2578$; $P \leq 0.0001$) (**Figure 2d**).

Relative methionine deficiency has also been implicated in cblC pathogenesis. Lower leucine-to-methionine ratios were seen when patients were consuming more complete protein ($r = -0.3011$; $R^2 = 0.09067$; $P = 0.01$) (**Supplementary Figure S3D** online). The leucine-to-methionine ratio was not correlated with the daily dosage of incomplete protein (**Supplementary Figure S3B** online). However, blood methionine levels are influenced by betaine dosages, hydroxycobalamin dosages, and methionine supplementation, and not just by diet. These factors varied among individuals and over time (**Supplementary Figure S1** online).

There was individual variation in the sensitivity to alterations in both incomplete and complete protein, as demonstrated by the heat map analysis in **Figure 2e–h** and **Supplementary Figure S3E-H** online. For example, patient 8, who has a mild form of the disease, had a drastic increase in his leucine-to-valine ratio (peak 7.9) at age 2 months, when an incomplete protein (0.5 g/kg/day) and protein-restricted diet were started. His leucine-to-valine ratio quickly normalized as his diet was liberalized and medical food usage was decreased. Patient 9, who has more severe manifestations of disease, was treated with a similar degree of protein restriction and even higher levels of

incomplete protein (1 g/kg/day); at 2 months of age, had a much lower leucine-to-valine ratio as compared to patient 8.

Question 3: does the long-term protein composition of the diet affect growth or neurocognitive outcomes?

Using the diet and growth data obtained at the standard ages described above, the correlation between growth parameter *z*-scores and complete and incomplete protein was evaluated (**Figure 3a**,**b**). There was a statistically significant correlation between increased used of medical foods and lower head circumference *z*-scores. However, it is impossible to determine if this finding is due to increased medical food usage in patients with more severe disease, and therefore smaller head circumferences, or if medical food usage led to impaired head growth. There was no correlation between the intake of complete or incomplete protein and height or weight.

To determine the effect of diet on neurodevelopmental outcomes, the average intake of complete and incomplete protein in the first 2 years was compared between patients with severe neurodevelopmental deficits and those with less severe deficits (mild/moderate impairment) or broad average functioning. Comparisons were made for each of the domains tested. There was no statistically significant difference in average complete or incomplete protein intake among the severity groups for any of the neurodevelopmental areas that were investigated (multiple *t*-test analysis with multiplicity-corrected *P* values using the Holm-Sidak method ranging from 0.37 to 0.99).

DISCUSSION

Newborn screening for cblC has resulted in early detection and institution of therapy for affected infants. Unfortunately, outcomes continue to be suboptimal, with a high residual burden of neurologic and ophthalmologic disease.[5,6,8,11–13] These data again demonstrate that patients identified through NBS still have poor outcomes. The rate of death in our cohort (0%) is lower than that of a historical cohort diagnosed before initiation of NBS (26%).[19] However, it is not clear whether other complications are significantly lower in cblC cohorts diagnosed through NBS.

Concerns have been raised that dietary interventions aimed at improving metabolic markers of disease may actually exacerbate the phenotype by inducing essential amino acid imbalances and deficiencies.[17] In this work, we investigated the relationship between dietary protein intake and both metabolic control and outcomes. By investigating multiple individuals over several years, both intra- and interindividual diet-induced phenomena could be appreciated.

Question 1: is there a relationship between initial or long-term metabolite (MMA, homocysteine, and methionine) levels and neurocognitive outcomes?

Before investigating the influence of diet on outcome, we sought to establish whether superior long-term metabolic control (including both limiting buildup of "toxic" metabolites and avoidance of essential amino acid deficiencies) was associated with better neurodevelopmental outcomes (**Figure 1**). Higher mean homocysteine levels were correlated with more severe neurologic impairment. However, no other longitudinal markers of metabolic control or absolute or relative amino acid deficiencies were correlated with outcomes. This suggests that a factor other than long-term metabolic stability may strongly influence neurodevelopmental manifestations of the disease. It has been suggested that much of the morbidity seen in cblC patients is due to the damaging effects of uncontrolled disease in utero. This has prompted therapeutic trials of hydroxycobalamin in fetuses known to have the disorder.[20,21] In our cohort, the lowest recorded methionine and highest homocysteine values were correlated with neurodevelopmental outcomes. These extreme values generally occurred in the neonatal period prior to therapy initiation and therefore may reflect the degree of metabolic derangement in utero.

Question 2: does dietary protein composition correlate with long-term metabolic control, branched-chain amino acid imbalances, or relative methionine deficiency in an individual over time?

Higher levels of incomplete protein intake were correlated with lower MMA levels (**Figure 2**). This suggests that diet does indeed affect metabolic control; however, it is important to note that the absolute MMA levels were much lower than those seen in other forms of methylmalonic aciduria, which are associated with more frequent metabolic decompensations.[22] There was not a clear correlation between diet and homocysteine levels; however, any relationship is likely obscured by the confounding variable of betaine dosage, which varied between individuals and over time (**Supplementary Figure S1** online).

A concern regarding iatrogenic diet–induced methionine deficiency or branched-chain amino acid imbalances prompted calculation of the ratio of leucine to these amino acids. It is important to consider these ratios because medical foods were used in a large percentage of our patients; all patients had been prescribed incomplete protein–containing formula at some point and 67% were still using these therapies at the last evaluation. MMA medical foods contain normal to elevated amounts of leucine and are restricted in methionine, valine, isoleucine, and threonine.

Increased consumption of leucine is thought to lower valine and isoleucine levels through allosteric regulation of the shared branched-chain ketoacid dehydrogenase by the leucine metabolite α-ketoisocaproate.[23,24] In our cohort, the leucine-to-valine ratio was higher in patients consuming higher amounts of medical foods and lower amounts of complete protein (**Figure 2**). Furthermore, the total population mean valine level was at the low end of the normal range at 112.9 ± 38.8 μmol/l (normal 93–321 μmol/l), and 6 of 12 (50%) individuals had mean valine levels that were below the normal range.

Unlike patients with other forms of isolated methylmalonic acidemia, patients with cblC intrinsically have lower methionine levels. Further methionine restriction with MMA/PA medical foods can worsen the methionine deficiency. Nine of

12 (75%) of our patients required methionine supplementation beginning in infancy to maintain normal methionine levels (**Supplementary Figure S1** online). Using this strategy, all patients had current and mean methionine levels within (or above) the normal range.

As in the case of the leucine-to-valine ratios described above, medical foods that are high in leucine and restricted in methionine can lead to relative imbalances of these two amino acids. It has been proposed that increased levels of large neutral amino acids, such as leucine, may impede methionine transport across the blood–brain barrier, thereby exacerbating central nervous system methionine deficiency.[17,25–27] In this work, we used the leucine-to-methionine ratio as a surrogate marker of methionine flux into the brain, as has been done in the work of Manoli et al.[17] Despite normal absolute methionine values with methionine supplementation, the leucine-to-methionine ratio was significantly higher in patients consuming lower amounts of complete protein (**Supplementary Figure S3** online). This may indicate that diet-treated patients have a more profound relative methionine deficiency in the central nervous system.

Question 3: does the long-term protein composition of the diet affect growth or neurocognitive outcomes?

In previous work evaluating growth at a single point in time in a large population of cblC patients, medical food usage was associated with statistically significant lower weight and head circumference *z*-scores.[17] When we replicated this single–time point evaluation, we found no relationship between current dietary therapy status and group mean growth parameters or neurodevelopmental outcomes. However, when we performed a longitudinal analysis of growth at all time points in relation to diet in the first 2 years, we did find a correlation between poor head growth and increased medical food consumption (**Figure 3**). We found no relationship between diet and weight or height in our cohort. With our retrospective data, it is impossible to establish whether dietary therapy induced poor head growth or if more severely affected children, with genetically predetermined poor head growth potential, are more likely to be prescribed medical foods.

Finally, the average complete and incomplete protein intake in the first 2 years was compared between patients with severe neurodevelopmental deficits and those with more moderate impairment. Patients in the two groups did not differ significantly in the protein composition of their diet in the first 2 years of life.

Limitations

Limitations of this work include the single center retrospective study design and the small sample size. The longitudinal nature of the study partially compensated for these limitations because it allowed for evaluation of therapy-induced changes in a single individual over time. Although the study reflects the management practices of physicians at a single center, dietary therapy trends changed over the 16-year timeframe investigated and different physicians used different therapeutic strategies. For example, the oldest patients were not given medical foods in the first 2 years of life, but our younger subjects were. Therefore, this allowed for some variety in the treatment modalities investigated. Another limitation is that in the analysis of correlation between variables, correlation does not imply causation. Also, correlation measurements can be skewed by a few outlier data points.

In summary, retrospective analysis of our center's experience managing children with cblC identified by NBS confirms previous findings that patients generally have few metabolic decompensations but have profound neurodevelopmental and visual disabilities. Higher peak serum total homocysteine and trough methionine levels measured in the neonatal period were correlated with poorer neurodevelopmental outcomes, but postnatal metabolic control had little relationship to outcomes. This suggests that in utero damage may be driving the neurologic manifestations of this disease. Furthermore, although a protein-restricted diet can marginally improve metabolic control, prolonged use of medical foods is not associated with better cognitive outcomes. Longitudinal analysis also showed that higher incomplete protein intake was also correlated with iatrogenic amino acid imbalances and impaired head growth.

It is important to note that there was significant intraindividual variation in the sensitivity (both markers of metabolic control and amino acid ratios) to dietary manipulations. Therefore, when making therapeutic decisions for a patient with cblC disease, a metabolic physician needs to consider the individual's history as they weigh the possible modest benefit of medical food–induced metabolic control against the risk of diet-induced poor head growth and amino acid imbalances. Prospective, controlled trials of dietary practices are needed for patients with cblC disease. Given the longitudinal data reported here and in previous works,[27,28] there is little evidence supporting the use of dietary therapy for cblC deficiency.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENTS

We thank all CHOP metabolism doctors, fellows, nurses, and genetic counselors who contributed to the care of these patients.

DISCLOSURE

The authors declare no conflict of interest.

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