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ORIGINAL ARTICLE

Clinical presentation and outcome in a series of 88 patients with the cblC defect

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Abstract The cblC defect is the most common inborn error of vitamin B12 metabolism. Despite therapeutic measures, the long-term outcome is often unsatisfactory. This retrospective multicentre study evaluates clinical, biochemical and genetic findings in 88 cblC patients. The questionnaire designed for the study evaluates clinical and biochemical features at both

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initial presentation and during follow up. Also the development of severity scores allows investigation of individual disease load, statistical evaluation of parameters between the different age of presentation groups, as well as a search for correlations between clinical endpoints and potential modifying factors. Results: No major differences were found between neonatal and early onset patients so that these groups were combined as an infantile-onset group representing 88 % of all cases. Hypotonia, lethargy, feeding problems and developmental delay were predominant in this group, while late-onset patients frequently presented with psychiatric/behaviour problems and myelopathy. Plasma total homocysteine was higher and methionine lower in infantile-onset patients. Plasma methionine levels correlated with "overall impression" as judged by treating physicians. Physician's impression of patient's well-being correlated with assessed disease load. We confirmed the association between homozygosity for the c.271dupA mutation and infantile-onset but not between homozygosity for c.394C>T and late-onset. Patients were treated with parenteral hydroxocobalamin, betaine, folate/folinic acid and carnitine resulting in improvement of biochemical abnormalities, non-neurological signs and mortality. However the long-term neurological and ophthalmological outcome is not significantly influenced. In summary the survey points to the need for prospective studies in a large cohort using agreed treatment modalities and monitoring criteria.

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Introduction

The cblC defect is the most common inborn error of vitamin B12 (cobalamin, Cbl) metabolism and is caused by mutations of the *MMACHC* gene as described in over three hundred patients (Lerner-Ellis et al 2006; Nogueira et al 2008; Lerner-Ellis et al 2009).

Cobalamin must be obtained from animal dietary products such as milk and meat (Fowler 1998). Absorption of Cbl requires binding to specific proteins in saliva, proteolytic release and binding to intrinsic factor in the intestine and uptake into the enterocyte via a specific receptor. After release from its complex with intrinsic factor, Cbl enters the circulation bound to transcobalamin and is taken up into lysosomes through endocytosis. After release into the cytosol Cbl is converted to its two active co-enzymes, methylcobalamin in the cytosol and adenosylcobalamin in the mitochondrion. Adenosylcobalamin is the co-enzyme for methylmalonyl-CoA mutase, deficiency of which results in methylmalonic aciduria, one of the biomarkers of the cblC defect. Methylcobalamin is a co-factor for N5-methyltetrahydrofolate: homocysteine methyltransferase, deficiency of which results in an increase of homocysteine with low levels of methionine, other biochemical hallmarks of the cblC defect (Fowler 1998).

Clinical presentation and severity of the cblC defect can vary considerably ranging from severe burden of disease and even death at an early age to late presenting disabilities (Bodamer et al 2001). In particular, patients with early onset of symptoms (<1 year of life) were reported to show an unfavourable outcome with average survival of less than 10 months and mortality exceeding 25 %, whereas patients with late onset (defined by the authors as >4 years of life) survived with a better prognosis following treatment (Rosenblatt et al 1997).

So far, only one study reports the frequency of symptoms within a large series of 50 cblC patients (Rosenblatt et al 1997) and several smaller studies and individual case reports have focused on particular features (Table 1). In late onset or adult cases, clinical features include predominantly neurological disturbances, dementia, myelopathy, and thromboembolic complications (Shinnar and Singer 1984; Thauvin-Robinet

Table 1 Signs and symptoms in patients with cblC defect as reported in the literature

Frequent signs and symptoms	References
• Feeding difficulties • Haematologic abnormalities • Hypotonia • Developmental delay • Seizures • Failure to thrive • Microcephaly • Pigmentary retinopathy • Decreased visual acuity • Nystagmus	Rosenblatt et al 1997; Robb et al 1984; Ricci et al 2005; Weisfeld-Adams et al 2013
Less frequently reported signs and symptoms	
Eye symptoms • retinal hypopigmentation • strabismus • optic atrophy • maculopathy	Mitchell et al 1986; Tsina et al 2005; Patton et al 2000; Weisfeld-Adams et al 2013
renal involvement including haemolytic uraemic syndrome	Geraghty et al 1992; Sharma et al 2007
Dysmorphic features • macrocephaly • facial dysmorphism • marfanoid features	Cerone et al 1999; Heil et al 2007; Rosenblatt et al 1997
Cardiopulmonary signs • congenital heart disease • cardiomyopathy and left ventricular non-compaction • cor pulmonale • pulmonary hypertension	Andersson et al 1999; Brandstetter et al 1990; De Bie et al 2009; Heinemann et al 2001; Iodice et al 2013; Longo et al 2005; Ogier de Baulny et al 1998; Profitlich et al 2009; Tomaske et al 2001
Neurological signs • neuropsychiatric disturbances • abnormal brain stem auditory and visual evoked potentials • white matter changes/atrophy • hydrocephalus • basal ganglia lesions • sensory peripheral neuropathy	Biancheri et al 2001, 2002; Longo et al 2005; Mamlock et al 1986; Rosenblatt et al 1997; Roze et al 2003; Smith et al 2006; Weintraub et al 1991; Weisfeld-Adams et al 2013
Other presentations: • haemophagocytic lymphohistiocytosis • protein-losing enteropathy • neonatal hyperammonemia • acidosis • microthrombi • Thinned corpus callosum • hyperexia encephalopathy	Ellaway et al 1998; Martinelli et al 2011a; Rosenblatt et al 1997; Weisfeld-Adams et al 2013; Wu et al 2005; Grünert at al 2011

et al 2008). A limitation of many of these studies and case reports is the lack of a clear indication of whether or not symptoms were initially present at diagnosis or if they developed during the subsequent course of the disease while patients were treated.

Treatment with intramuscular hydroxy-Cbl (OH-Cbl), betaine and carnitine is reported to improve biochemical abnormalities including restoration of plasma methionine values to normal (Ribes et al 1990; Bellini et al 1992; Bartholomew et al 1998; Andersson et al 1999). Plasma homocyst(e)ine levels or methionine levels were not improved by the cyano form of Cbl (Andersson et al 1998) given intramuscularly and/or folinic acid (Bartholomew et al 1998). The long term outcome of the cblC defect remains poor in most cases, even when these therapeutic measures were introduced soon after initial presentation and in some cases even prenatally (Huemer et al 2005). Further, there is no consensus on the optimal treatment for this severely debilitating disorder (Martinelli et al 2011b).

This questionnaire based study evaluates clinical findings at initial presentation and during the disease course whilst treated in 88 cblC deficient patients with the following aims: i) to evaluate patients according to age of presentation and to assess possible prognostic indicators; ii) to document the natural history of the cblC defect over time with particular reference to clinical signs both at diagnosis and during the course of the disease; iii) to investigate genotype-phenotype correlations; and iv) to evaluate therapeutic measures.

Patients and methods

Study population and recruitment of patients

The study was approved by the ethics committee of the University of Basel (Ref.Nr.EK:267/04). A total of 88 patients were included in the study. Patients were recruited in three ways. First those from whom cell lines were sent to the University Children's Hospital, Basel for confirmation of diagnosis between January 1987 and May 2005; second, additional patients reported at a workshop held at the 36th EMG Meeting in Rimini 2004 (Bodamer and Fowler 2004); third, further confirmed cases of the cblC defect known to the contacted physicians. Diagnosis was based on the finding of increased homocysteine, either free or total, and methylmalonic acid in plasma and/or urine shown not to be due to nutritional vitamin B12 deficiency. In 75 cases the diagnosis was confirmed by studies of methionine and serine synthesis, propionate incorporation into cell proteins and Cbl uptake and coenzyme synthesis and somatic complementation analysis in fibroblasts performed at the University Children's Hospital Basel (Fowler and Jakobs 1998). Following discovery of the gene for cblC the molecular defect was confirmed by mutation analysis.

Questionnaire based survey

A questionnaire was sent to 49 international metabolic centres with full returns received from 26 centres. The questionnaire items were developed on the basis of a specialist meeting and a review of the literature on cblC. Items covering clinical presentations and features as reported in the literature and open questions on yet unreported organ involvement at diagnosis and during the course were selected. In addition, information was requested on personal data, family history, pregnancy and birth complications, age at first symptoms and at diagnosis, biochemical parameters at diagnosis and during the course, and treatment modalities.

The presence or absence of each specific symptom both at the time of diagnosis and during the course of the disease was recorded. Prenatal symptoms were recorded and different symptoms were grouped according to affected organ systems (supplementary Table 1)

We developed a severity score in order to estimate the disease load in the individual both at presentation and during the course. Thus nutritional symptoms, general development, neurological symptoms, eye problems, renal, cardiac and haematological involvement as well as abnormalities in brain MRI scans were numerically assessed and summed to give a *severity score* ranging from 0 (no symptoms) to 25 (all symptoms present). In addition the physician's *overall impression* on the disease outcome and the present state in each patient was determined by scoring mental status, visual impairment, motor development and quality of life. Zero, one and two points were allocated for normal, moderately impaired and severely impaired, respectively. Deceased patients were allocated a score of 2 points for each category to give a total possible score of 8 (see supplementary Table 1). Questionnaires were completed by the physicians caring for the patient and returned to the University Children's Hospital Basel. Following evaluation of initial returns the submitted information was incorporated into the database and returned to the responsible physician for checking and to reply to additional questions.

In those cases where only free homocysteine disulphide values were available, an estimate of the total plasma homocysteine was made. This was based on the observation in two studies (Moat et al 1999; B. Fowler, unpublished data) which showed that free homocystine is only found in samples in which total homocysteine exceeds 70 μmol/l. Therefore an estimate of total homocysteine was calculated by adding 70 to double the free homocystine concentration. This approach seems to be justified by the observation that there was no significant difference of mean values and standard deviation between the groups of only measured total homocysteine versus measured plus estimated values (two tailed *t*-test).

Statistical analysis

Both severity score and physician's overall impression were compared with various other parameters by statistical analysis. The various statistical methods used for comparisons of data are indicated in the results. For all statistical analysis significance was assigned to p values below 0.05.

Results

Age distribution and demographic data

The age of presentation in all patients ranged from 0 to 162 months with a median value of 0.91 months. As shown in Fig. 1, 43 patients presented below 1 month (neonatal onset), 33 between 1 month and 1 year of age (early onset) and ten above 1 year of age (late onset). Two patients were detected by neonatal family screening. There was evidence of consanguinity in a total of 26 patients.

The ethnic origin was reported as European in 54, unspecified Caucasian in 17, Asian in four with no information in 13. Among the European patients 27 were from Southern countries including Italy, Portugal and Spain. The male to female distribution of 58 to 30 (ratio 1.93) was uneven. A total of ten subjects had died (supplementary Fig. 1). Seven (six male, one female) patients died before 3 months of age, three female patients died at 20 months, 4 years and 16 years respectively. In surviving patients, age at final evaluation ranged from

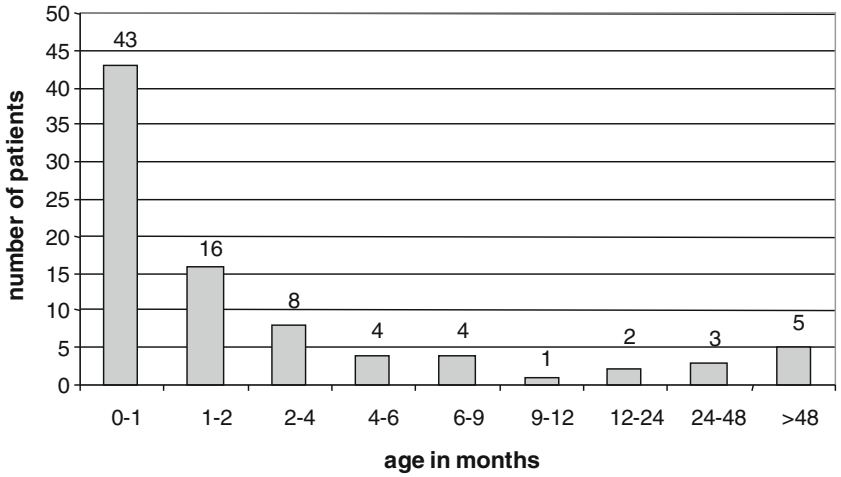

Fig. 1 Age of presentation in patients with the cblC defect

9 months to 47 years and 7 months (mean 123.4 months; S.D. 93.5, median 88.5 months).

Clinical symptoms at onset and during disease course according to subgroups

Figure 2 summarises clinical signs showing the overall frequency of the different findings both at initial clinical presentation and at the last evaluation during the course of the disease. Symptoms such as personality changes and mental retardation were only evaluated after the third month of age.

Comparison of the clinical, biochemical and molecular features between the age groups

Initially all quantitative parameters and individual clinical severity scores listed in supplementary Table 2 were compared for patients with neonatal, early and late onset according to the classification suggested by Rosenblatt et al (1997). Statistical analysis was performed using the Mann-Whitney *U* test for non-parametric variables. Evaluation of data in the neonatal onset and early onset group revealed few statistically significant differences. These were the higher prevalence of microcephaly in the early onset patients both at diagnosis (p=0.0085) and during the course (p=0.049), and a higher severity score for general development at diagnosis in early-onset compared with neonatal-onset patients (p=0.02). Due to the close clinical similarity and overall lack of statistical differences between neonatal and early onset patients, these two groups were combined and termed as "infantile onset" for further comparisons with late onset patients.

Infantile onset patients showed significantly higher scores for nutritional signs and microcephaly (all $p \leq 0.015$) both at diagnosis and during the disease course, when compared with late onset patients. At follow-up alone, significant differences were found between the two groups for total severity score, general development, overall impression and eye-related problems (all $p \leq 0.026$). Considering the biochemical parameters, the infantile onset group showed lower methionine levels at diagnosis (median 8.0 vs. 14.0 μmol/l; p=0.017) and higher homocysteine values at follow-up (55.5 vs. 35.0 μmol/l; p=0.013).

Statistical correlations were analysed using the Spearman Rank test between various parameters and the severity score at diagnosis; the severity score during the course and the overall impression for all patients together and for the different age groups. This test showed a number of correlations. First, age at diagnosis and delay in diagnosis correlates with severity score at diagnosis (p <0.002). Second, total severity score at diagnosis correlates with both severity score and overall impression at follow up (p<0.0001). Third, microcephaly and eye-related signs at diagnosis and during the course correlate with severity score at follow-up (p<0.001). In the late onset patients plasma homocysteine and urine MMA levels correlate with the severity score and with overall impression (p<0.05), whereas in infantile onset patients plasma homocysteine alone at diagnosis correlates with the severity score during the course. Regardless of the age at presentation, plasma methionine negatively correlates with overall impression at follow-up (p=0.048). Plasma homocysteine levels during the course showed a positive correlation with all clinical parameters in late onset patients.

Brain MRI

Brain MRI findings were available from 64 patients (49 infantile and seven late onset cases). Normal findings were present in only 19 % of patients. Brain atrophy and white matter abnormality were seen in both infantile (14/49 and 16/49 respectively) and late onset (4/7 and 1/7 respectively) cases. The most striking differences between the age groups were the finding of hydrocephalus (15/49 patients) and of basal ganglia lesions (6/49 patients) only in infantile onset cases.

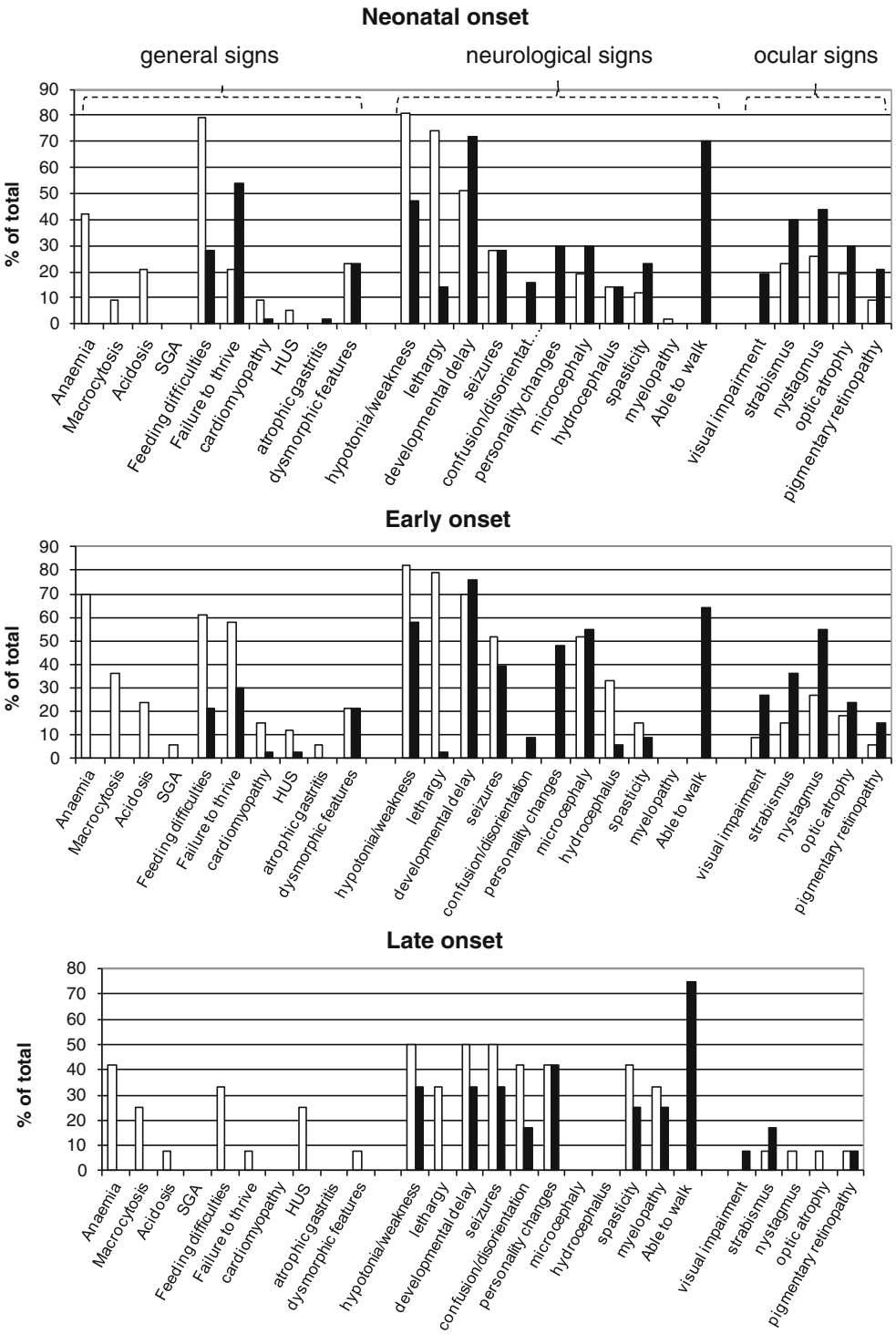

Fig. 2 General signs, neurological signs and ocular signs observed in patients with the cblC defect at onset (light bars) and during course (dark bars). *SGA* small for gestational age, *HUS* haemolytic uremic

Treatment modalities and correlations with clinical outcome

Different treatment modalities were used. Sixty-nine of 76 infantile and nine of 12 late onset patients were treated with parenteral OH-Cbl. Patients not given OH-Cbl were mainly ones who died from the disease. Betaine (oral dose ranged from 100 to 444 mg/kg/day) was used in 67 infantile and six late onset patients, folate or folinic acid were applied in 57 infantile and four late onset cases (oral dose ranged from 56 to 20,000 μg/kg/day. Carnitine was given to 37 infantile and two late onset patients. Supplemental methionine was used only in one patient. No patients were treated with protein restricted diet.

Contingency tables showed no correlation between the use of carnitine, betaine or folate and the total severity score during the course of the disease (two sided Exact Fisher test).

Furthermore, the Spearman rank test revealed no correlation between the dose of betaine and either median methionine levels or total homocysteine levels during the course of the disease.

Genetic analyses

Molecular genetic analysis of *MMACHC* was performed by either the group of D.S. Rosenblatt or B. Fowler (personal communication) in 74 patients (supplementary Table 3). The most common mutations identified were the c.271dupA and c.394C>T mutations. Of a total of 148 mutant alleles 73 were c.271dupA (49 %) and 30 were c.394C>T (20 %). Homozygosity for the c.271dupA mutation was present in 23 patients (22 with infantile onset) and the c.394C>T mutation in ten patients (6 with infantile onset). Two infantile onset patients were homozygous for c.331C>T and a further two were homozygous for c.217C>T. In addition, 15 private mutations were identified.

Discussion

This questionnaire based retrospective survey addresses a comprehensive range of features in the largest number of patients studied so far with the cblC defect, extending the report on 50 patients by Rosenblatt et al (1997). We are aware of a selection bias in the recruitment of either patients from whom cell lines were sent for confirmation of diagnosis to a single metabolic laboratory or patients who were known to the involved metabolic specialists. Nevertheless until now, as in most inborn errors of metabolism no registry or any other unbiased patient reporting system has been implemented for cblC patients. Study patients stemmed mainly from Europe with only four non-Caucasian and a predominance of cases from southern Europe reflecting our catchment area rather than the world-wide ethnicity reported in patients in whom mutation analysis was undertaken (Lerner-Ellis et al 2009).

The degree of consanguinity was high being reported in 26 cases.

Regarding gender of the patients there was a clear imbalance towards males, with a male to female ratio close to 2, which constitutes new information. Whether this is because of lack of detection or increased intra-uterine mortality for females can only be speculated on. Ten patients (nine with infantile onset) died in total, representing 11.4 % of cases compared with 26 % of patients in the previous cohort (Rosenblatt et al 1997) possibly reflecting earlier diagnosis and different or improved treatment modalities.

In contrast to the study by Rosenblatt et al (1997) we differentiate between clinical and biochemical features present at diagnosis and those at follow up. This allows a better understanding of the natural history of the disease and of the response to treatment. Evaluation of variables in the different presenting age groups was backed up by statistical analysis allowing a search for significant correlations between clinical end points and possible modifying factors.

In addition to assessment of individual features we also created organ specific and overall severity scores to obtain a quantitative index for comparison with possible modifying factors. We are aware of the shortcomings of the approach of using overall, non standardised scores but it has been shown in other multi-systemic diseases that severity or disease activity scores are extremely helpful in clinical practice and evaluation of treatment and may add to the information deduced from laboratory parameters. Nevertheless the weighting of symptoms and organ damage and the proof of validity and reliability of severity scores is a challenging task which may only be achieved over years and by analysis of larger cohorts (Gladman et al 2002). Therefore the severity score developed for our study constitutes a first attempt to establish the general idea of assessment of patients' symptoms in a more systematic manner. Physician's overall assessment of disease severity is an established parameter for assessing disease severity in other multi-systemic diseases (Gutiérrez-Suárez et al 2007; Seid et al 2013) but has until now only rarely been used in the field of metabolic diseases.

The previous survey by Rosenblatt et al(1997) reported patients below 1 year of age as early onset (44 patients) and above 4 years of age as late onset (six patients). As stated in the results section we combined the neonatal and early onset patients as infantile onset. Thus the infantile onset group contained 88 % of patients, half presenting by 1 month of age and 38 % between 1 month and 1 year of age. The remaining 12% of patients presented after the chosen cut off of 1 year of age and were assigned to the late presentation group. The overall median age of presentation observed in our series is close to the 1 month reported in the previous cohort.

The study of the natural course of the cblC defect underlines that the cblC defect needs to be considered in the differential diagnosis of a wide range of conditions. At initial presentation there are several rather non-specific symptoms occurring in more than half of patients such as hypotonia/weakness, lethargy, feeding difficulties and developmental delay. More specific signs, related to diffuse microangiopathic damage (Martinelli et al 2011a, b), include hydrocephalus, detected in about one third of infantile onset patients, and haemolytic uraemic syndrome. Other symptoms were eye-related signs and cardiomyopathy, present in less than 10 % of patients, psychiatric disorders, spasticity and myelopathy. Since these complications are severe and have only been reported anecdotally in this disorder, our study confirms their importance.

Clinical features were significantly different between the infantile and late onset patients. Hypotonia/weakness, lethargy, feeding difficulties, failure to thrive, nystagmus, dysmorphic features and optic atrophy at diagnosis were all clearly

more prominent in infantile onset patients. Developmental delay and seizures were of similar incidence. In late onset patients, psychiatric changes (personality changes, confusion/disorientation) and spasticity were fairly common features. Myelopathy was almost exclusive to the late onset patients. Apart from the differences in the selection parameters there was a statistically significant better general condition at presentation and lack of microcephaly and cardiomyopathy in late onset patients.

Evaluation of findings during the course of the disease, following treatment reveals that the non-neurological signs resolve after treatment. Thus anaemia, acidosis and macrocytosis all disappeared after treatment. Also hypotonia/weakness, lethargy, feeding difficulties, hydrocephalus, HUS and cardiomyopathy became less frequent. Since some of these complications are potentially fatal (e.g. HUS), treatment not only improves some clinical outcomes but reduces mortality.

In contrast, neurological and ophthalmological problems such as developmental delay, seizures, failure to thrive, microcephaly, dysmorphic features, spasticity and optic atrophy remained at about the same frequency. Also there was an increase of prevalence of nystagmus, strabismus, pigmentary retinopathy, visual impairment despite treatment in infantile onset patients. Therefore the treatment modalities currently employed here appear not to have completely altered the disease course, confirming previous assumptions of a poor long-term neurological prognosis regardless of treatment and biochemical amelioration (Martinelli et al 2011a, b; Weisfeld-Adams et al 2013). On-going decline in IQ over time has been reported by Beauchamp et al (2009) in two patients despite therapy.

We found no correlation between the dose of either OH-Cbl or betaine administered and score parameters during the disease course. A wide variety of treatment modalities have been applied especially concerning the dose and interval of medication (e.g. OH-Cbl, betaine), pointing to the need to understand whether optimisation of known and/or the development of new treatment strategies might positively change the disease course (Dionisi-Vici et al 2013). A recent retrospective literature review reporting on both early and late onset patients suggested that daily treatment with parenteral OH-Cbl combined with betaine results in a better outcome (Carrillo-Carrasco and Venditti 2012).

In this regard only one patient in our survey was treated with supplemental methionine, which has been claimed to be successful in individual cases of other disorders of defective remethylation (Kvittingen et al 1997; Abeling et al 1999). The recent observation both in vitro and in vivo of increased oxidative stress and of abnormal glutathione status in cblC defect (Jorge-Finnigan et al 2010; Pastore et al 2013; Richard et al 2009), may suggest the use of antioxidant therapy to correct redox state abnormalities and its potentially related clinical signs.

Of the biochemical features, plasma methionine was higher at diagnosis and plasma homocysteine was lower during the disease course in late onset patients. The finding that only plasma methionine levels were correlated with overall impression is supported by the correlation of methionine levels and assessment of adaptive behaviour skills by parents/caregivers (Weisfeld-Adams et al 2013), suggesting a potential contribution of reduced methionine availability in the pathogenesis of some of the clinical signs of the disease (Martinelli et al 2011a, b). Nevertheless, severity scores showed no significant correlation with methionine.

The only correlation observed with factors affected by treatment, was the aforementioned relationship of total homocysteine measured during the course of the disease and overall impression.

Regarding possible genotype-phenotype correlations, results of mutation analysis showed that the homozygosity for the c.271dupA mutation correlates with infantile presentation. These findings support the previously suggested idea that c.271dupA is associated with infantile onset. Homozygosity for the c.394C>T mutation was seen in six infantile presenting patients and in four late presenting patients This apparent contradiction with the previously reported correlation between late onset and this mutation (Morel et al 2006; Nogueira et al 2008) may be related to differences in the classification of patients.

Recently, the cblC defect has been included in some countries in the panel of diseases screened for by expanded newborn screening (Carrillo-Carrasco et al 2012). However, neither the management nor the outcome of asymptomatic patients with cblC detected by newborn screening has been extensively studied. At present there is only anecdotal data suggesting that earlier diagnosis of affected subjects through expanded newborn screening improves overall outcome but not neurological and ocular symptoms (Weisfeld-Adams et al 2013; C. Dionisi-Vici personal observation). The report of a family showing a non-neurological phenotype with fatal isolated pulmonary hypertension at the age of 2 years in one sibling and with haemolytic uremic syndrome at the age of 3 years in the eldest one (Iodice et al 2013), highlights the possibility that if expanded newborn screening would have been performed, these two brothers might have experienced a different clinical outcome. Early intervention preceding the appearance of severe signs related to micro-angiopathy might well be expected to also avoid the occurrence of hydrocephalus, one of the more severe abnormalities occurring in the cblC defect.

Taken together, these findings confirm that an exact comprehensive understanding of the pathophysiology of cblC defect has still not been achieved. Lack of good developmental and neurological outcome may reflect possible irreversibility of pathogenetic mechanisms in this multifactorial defect. Additionally, it is likely that the synergistic effect of different

mechanisms, which include the accumulation of putatively toxic metabolites and the deficiency of products downstream of the enzymatic defect(s), is responsible for the multisystem organ involvement (Martinelli et al 2011a, b).

In summary, this survey points to the need for careful prospective multi-centre studies as recently started with the European Network and Registry for Homocystinurias and Methylation Defects (E-HOD) using agreed treatment modalities and monitoring parameters since present treatment regimes do not fully improve the disease course.

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Conflict of interest Sabine Fischer, Martina Huemer, Federica Deodato, Diana Ballhausen, Avihu Boneh, Alberto B. Burlina, Roberto Cerone, Paula Garcia, Gülden Gökçay, Stephanie Grünewald, Johannes Häberle, Jaak Jaeken, David Ketteridge, Martin Lindner, Hanna Mandel, Esmeralda G. Martins, Karl O. Schwab, Sarah C. Gruenert, Bernd C. Schwahn, László Sztriha, Maren Tomaske, Friedrich Trefz, Laura Vilarinho and David S. Rosenblatt declare that they have no conflict of interest

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