Received: 15 October 2020 | Revised: 27 November 2020 | Accepted: 7 December 2020

DOI: 10.1002/jimd.12338

ORIGINAL ARTICLE

JIMD SSIEM WILEY

Cystathionine β-synthase deficiency in the E-HOD registry-part I: pyridoxine responsiveness as a determinant of biochemical and clinical phenotype at diagnosis

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Funding information
Ministerstvo Zdravotnictví Ceské Republiky, Grant/Award Number: NV19-01-00307

Abstract

Cystathionine β-synthase (CBS) deficiency has a wide clinical spectrum, ranging from neurodevelopmental problems, lens dislocation and marfanoid features in early childhood to adult onset disease with predominantly thromboembolic complications. We have analysed clinical and laboratory data at the time of diagnosis in 328 patients with CBS deficiency from the E-HOD (European network and registry for Homocystinurias and methylation Defects) registry. We developed comprehensive criteria to classify patients into four groups of pyridoxine responsivity: non-responders (NR), partial, full and extreme responders (PR, FR and ER, respectively). All groups showed overlapping concentrations of plasma total homocysteine while pyridoxine responsiveness inversely correlated with plasma/serum methionine concentrations. The FR and ER groups had a later age of onset and diagnosis and a longer diagnostic delay than NR and PR patients. Lens dislocation was common in all groups except ER but the age of dislocation increased with increasing

†E-HOD consortium members are mentioned in Appendix.

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Communicating Editor: Brian Fowler

responsiveness. Developmental delay was commonest in the NR group while no ER patient had cognitive impairment. Thromboembolism was the commonest presenting feature in ER patients, whereas it was least likely at presentation in the NR group. This probably is due to the differences in ages at presentation: all groups had a similar number of thromboembolic events per 1000 patient-years. Clinical severity of CBS deficiency depends on the degree of pyridoxine responsiveness. Therefore, a standardised pyridoxine-responsiveness test in newly diagnosed patients and a critical review of previous assessments is indispensable to ensure adequate therapy and to prevent or reduce long-term complications.

KEYWORDS

homocystinuria, patient registry, natural history, methionine, thromboembolism, developmental delay

1 | INTRODUCTION

Homocysteine (Hcy) is a non-structural amino acid formed during metabolism of the essential amino acid methionine (Met). Hcy can be recycled back to Met by the remethylation pathways or converted to cystathionine by cystathionine β-synthase (CBS, EC 4.2.1.22) and next by γ-cystathionase to the amino acid cysteine. Hcy and its disulfide, homocystine, accumulate in the remethylation disorders and in CBS deficiency, which is also known as classical homocystinuria (OMIM # 236200). CBS deficiency was first identified by selective screening for disorders of amino acid metabolism in patients with cognitive impairment; these patients also had skeletal abnormalities and lens dislocation.[1-3] Further research revealed pyridoxine responsiveness in some patients with CBS deficiency.[4] The *CBS* gene was mapped to chromosome 21q22.3,[5] and its coding sequence and genomic organisation were subsequently characterised,[6,7] allowing the genetic basis of CBS deficiency to be identified.[8,9]

A natural history study of 629 patients revealed the typical clinical features in patients with pyridoxine responsive and non-responsive CBS deficiency.[10] The severe pyridoxine non-responsive form typically presented in childhood with cognitive impairment, lens dislocation, marfanoid features, osteoporosis and thromboembolism, while the fully responsive disease presented later with fewer problems and partially responsive patients exhibited intermediate severity. Measurement of total homocysteine (tHcy) in the 1990s led to ascertainment of adult patients with a milder phenotype. Mudd and co-workers suspected that the mild part of the spectrum was under-represented in this natural history study and that some patients with the fully responsive form might even remain asymptomatic.[11] Recent reports of adults with extreme pyridoxine responsiveness have further expanded the phenotypic spectrum.[12,13]

CBS deficiency is diagnosed either by selective screening of symptomatic patients and high-risk family members or, in some countries, by population-wide newborn screening (NBS).[14] For NBS, the primary markers are usually Met or the methionine-to-phenylalanine ratio followed in some programmes by second-tier tHcy testing.[14-17] Although tHcy can be used as the primary screening marker, this is unlikely to be introduced widely due to the longer analytical procedure and higher cost. The key metabolite changes of CBS deficiency are marked elevation of plasma tHcy with variable increases in Met and decreases in cystathionine (determined by sensitive mass spectrometric methods).[12,18] The diagnosis is usually confirmed by CBS assays in cultured skin fibroblasts or serum/plasma, and/or by sequencing the *CBS* gene.

The European network and registry for Homocystinurias and methylation Defects (E-HOD) started as an EU-funded project in 2013. It is now an international collaborative consortium involving 70 centres from all over the world. One of its core activities is the E-HOD registry, which collects pseudonymized data after obtaining approval from local ethics committees and informed consent from patients/legal representatives. The E-HOD registry has already been used to describe the natural history of remethylation defects[19] and newborn screening practices.[14]

In this study, we have analysed clinical and laboratory data for 328 CBS-deficient patients at the time of enrolment into the E-HOD registry. We stratified this cohort into four groups according to the degree of pyridoxine responsiveness and examined how this influenced the clinical course and biochemical findings. This extended natural history study supports the original notion of Mudd and co-workers that CBS deficiency has an even wider phenotypic spectrum than described before.

2 | SUBJECTS AND METHODS

2.1 | Subjects, ethical issues and E-HOD registry

As of February 28, 2019, the E-HOD registry contained data on 337 individuals with diagnosis of CBS deficiency reported by the different centres. This registry was first approved by the Ethics Committee of University Hospital in Heidelberg (No. S-525/2010; 14.3.2013). All participating centres received approval from their local ethics committees before enrolling patients and all patients provided written informed consent before pseudonymized data were entered into the registry. The EHOD registry documents natural history (symptoms by organs), biochemical and treatment data. It records intelligence and developmental scores (IQ, DQ) from age-appropriate standardised instruments. Standardised self- and proxy report questionnaires inform about neuropsychological development and behaviour. In 2017, the participating centres were asked for permission to use the data of the CBS deficient patients for analysis and publication, and the project was approved by the E-HOD Steering Committee. Analysis of data and publication of results was also approved by the Ethics Committee of the General University Hospital in Prague (No 417/20 S-IV).

2.2 | Definition of pyridoxine responsiveness

Pyridoxine responsiveness was originally defined in the literature as the disappearance of non-protein-bound homocyst(e)ine from plasma after a loading test with pyridoxine.[4,20] The lower limit of detection for this mix of Hcy-containing compounds using an amino acid analyser corresponds to a total homocysteine (tHcy) of about 50-60 μmol/L,[21] and a decrease of tHcy to <50 μmol/L on treatment with pyridoxine was subsequently used to define full responsiveness in a standardised test.[18] The E-HOD registry originally offered selection of pyridoxine responsiveness in categories NO/YES/UNKNOWN, however, the criteria were not defined clearly, and data on tHcy during the pyridoxine loading test were not available. A new means of categorisation of responsivity was needed to ensure consistency, and we have therefore developed new surrogate criteria for assessing the degree of pyridoxine responsiveness even in the absence of a standardised pyridoxine test. We defined pyridoxine non-responsiveness (NR), partial responsiveness (PR), full responsiveness (FR) and, in addition, a new form of extreme responsiveness (ER) requiring only small dose of pyridoxine daily.[12] Consistent with the definition of Morris et al,[18] the major criterion for FR and ER was the lowering of plasma tHcy to <50 μmol/L with pyridoxine administration *alone* (below ≈1 mg/kg/d in ER; above ≈1 mg/kg/d in FR). Treatment with diet and/or betaine were considered indicators of NR or PR. NR patients seldom achieve tHcy <100 μmol/L without a low-methionine diet (ie, a diet extremely low in natural protein with methionine-free amino acid supplements) but some late diagnosed patients cannot manage this diet and are only treated with betaine. The degree of methionine restriction and tHcy levels on therapy were used to help distinguish NR and PR patients. For details see Table 1.

2.3 | Data verification

Following a preliminary analysis of registry data in 2017, we modified the registry entries on pyridoxine responsivity to include all four categories and we sent emails to all collaborators with a request to update their data in January 2019. Data from the registry were extracted on February 28, 2019. Subsequently for each of the 337 patients, we assessed the consistency of the declared pyridoxine responsivity with the dose, additional treatment and tHcy levels achieved; in patients with discrepant findings, plausibility was checked against genotypes with known responsivity. Of the 337 patients we excluded nine individuals in whom we were unable to determine pyridoxine responsivity due to missing data and/or no reply from contributors. Based on communication between the data analysis team and the contributing centres, we reclassified responsivity in 62 patients, with the final approval by the contributors. The reasons for reclassification in each patient are shown in Table S1. In collaboration with contributing centres we also classified responsivity in 71 additional patients for whom the relevant data were originally missing.

Next, we checked the plausibility of the age of onset, age of diagnosis, and of protein and Met intake, betaine and pyridoxine doses, weight and the correct nomenclature of genetic variants. In September 2019, we emailed two-page extracts from records of a subset of 308 patients with specific questions on implausible and/or outlying data, and with a request to provide any missing key data. Clarification of data in question proceeded via bi-directional email communication until October 31, 2019. Despite all these attempts we did not receive the requested information on 26 patients; using a consensus evaluation by VK and JS we excluded implausible or severely outlying data on diet (n = 3, for example, Met and amino acid mixture intake 562 mg/kg/day and 20 g/kg/day, respectively) and plasma tHcy levels (n = 4, for example, free homocystine concentrations of 103 μmol/L instead of tHcy on therapy). The cleaned dataset was deposited into a local database and used for all analyses.

TABLE 1 Criteria for assessing pyridoxine responsiveness in treated individuals with CBS deficiency

	Non-responder	Partial responder	Full responder	Extreme responder
Standardised 6-week test according to Morris 2017				
tHcy	<20% decrease tHcy	>50 μmol/L, but decrease >20%	Decrease to <50 μmol/L (on pyridoxine only, doses > ≈ 1 mg/kg/d)	Decrease to <50 μmol/L at doses of pyridoxine <≈1 mg/kg/d
Surrogate indices to assess pyridoxine responsiveness				
Dietary methionine restriction	Moderate to severe restriction necessary but not always possible	Restriction usually but not always needed	No restriction	No restriction
Betaine	Usually administered in adults and older children	Sometimes used	No betaine	No betaine
Combination of diet and betaine	Often	Can be only diet, only betaine or combination of both		
Pyridoxine doses	Often given, does not imply benefit	Up to 10 mg/kg/d in infants and children; typically in the range of 2–5 mg/kg/d in adults	> ≈ 1 mg/kg/d	<≈1 mg/kg/d

2.4 | Plasma CBS assay

The E-HOD registry contained data on plasma CBS activity in 30 patients. Participating centres were invited to collect plasma from consenting patients and ship them to the Metabolic Centre of Department of Pediatrics and Inherited Metabolic Disorders in Prague for the determination of plasma CBS activity using a published method.[22,23] In this way CBS activity was measured in an additional 34 patients from 10 centres. A total of 71 plasma CBS activities from 64 patients on and/or off treatment with pyridoxine were available for statistical analyses.

2.5 | Statistical analysis

Continuous variables are presented as means with SD or median with interquartile ranges, wherever appropriate. Categorical variables are shown in absolute and relative frequencies.

Differences between responsiveness groups were tested in two ways: (1) for the overall difference between the groups and (b) for the linear trend with the groups ordered as non-responder – partial – full – extreme responder. For categorical variables, the overall difference was tested using Fisher exact test and the linear trend was assessed by the linear-by-linear association test. For continuous variables, the overall difference was tested using Kruskal-Wallis nonparametric ANOVA and the linear trend was tested using the univariate linear regression with responsiveness as a linear factor.

Kaplan-Meier time-to-event graphs were constructed for lens dislocation and thromboembolic events. Since data on the exact occurrence of these events were not available, patients were censored at the time of diagnosis if lens dislocation or a thromboembolic event had already occurred. Overall difference and pairwise differences among responsiveness groups was tested by log-rank test; the Benjamini-Hochberg method was used to control for multiple comparisons.

Statistical language and environment R, version 3.6.3 was used throughout the analysis. The level of statistical significance was set at 0.05.

3 | RESULTS

3.1 | Study population and mode of ascertainment

Data from the E-HOD registry (https://ehod-registry.org/) were analysed for 328 patients with CBS deficiency and verified degrees of pyridoxine responsiveness, entered by 57 centres from 19 countries (Figure 1, panel A and Table S2). Overall, 62% of patients were NR, 20% PR, 12% FR, and 6% ER.

Table 2 shows a summary of data for the patients at diagnosis and at registry enrolment. At registry enrolment the median age of patients was 26 years with a total of 9131 patient-years. The majority of patients were white (92%). The male-to-female ratio of 1.13:1 for the entire cohort was consistent with the known autosomal recessive inheritance, however, sex ratio deviated from 1:1 in different groups of responsiveness ($P = .0069$). Group of NR and PR contained more males while the FR and ER groups contained more females.

Table 2 shows the body mass index (BMI) in patients >18 years of age and Figure S1 shows BMI after stratification for different age and pyridoxine responsiveness categories. NR patients aged 18-30 years had the lowest median BMI at 22.6 kg/m^2 whilst ER patients aged >50 years had a median BMI of 31.9 kg/m^2 ($P < .0001$). The increase in BMI with pyridoxine responsiveness remained significant after regression analysis using age and sex as covariates ($P < .001$).

Patients were ascertained mostly by selective screening among symptomatic patients (72%) followed by screening among high-risk family members (15%; this included also one patient diagnosed prenatally), and by NBS (13%). The proportion of patients with different degrees of responsiveness was similar in those ascertained clinically and by high-risk family screening (Figure 1, panel B), however there were no FR or ER among 43 patients ascertained by NBS. Patients were diagnosed between 1962 and 2018; the majority of patients (88%) were diagnosed after year 1983 (Figure S2).

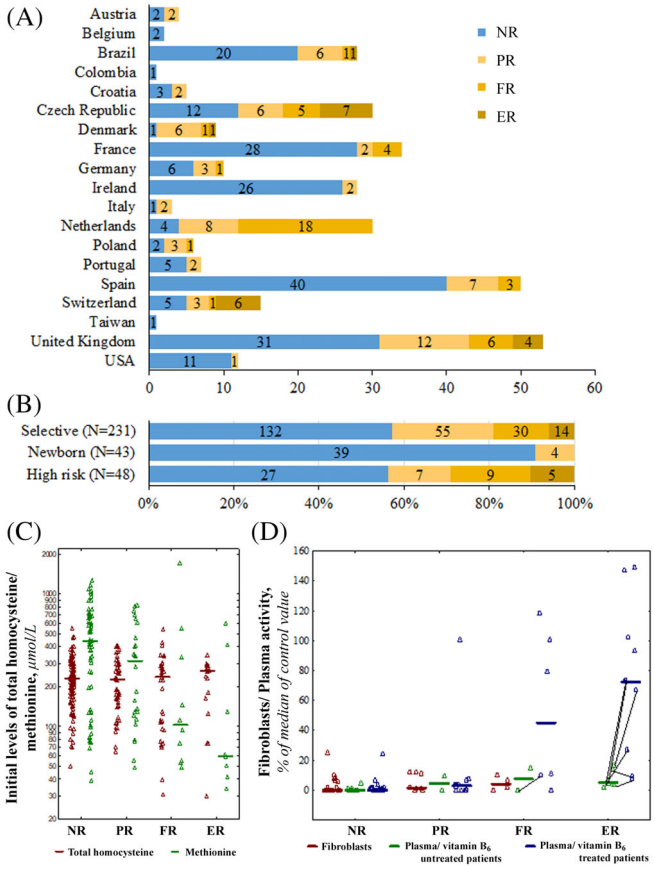

FIGURE 1 Geographical distribution of responsiveness groups and biochemical findings at diagnosis. A, Number of patients per country; B, mode of ascertainment; C, total homocysteine and methionine at diagnosis; and D, CBS activity in fibroblasts and plasma. Data are stratified by degree of responsiveness, NR, non-responders; PR, partial responders; FR, full responders; ER, extreme responders. Bar graphs in Panels A and B show numbers and proportion of patients with different responsivity, respectively. Horizontal bars in Panels C and D indicate the medians. If plasma CBS activities were measured on and off pyridoxine, the results are connected with diagonal lines. Fibroblast and plasma CBS activities are shown as % of median of controls

3.2 | Confirmatory testing and laboratory findings

Solely metabolite analysis was reported in 25% of patients, while confirmatory enzymatic testing and DNA analysis were done in 27% and 69% of patients, respectively. Different combinations of confirmatory laboratory tests were observed in this cohort (Figure S3).

We analysed the biochemical markers at the time of diagnosis in patients for whom the data were available (Table 2 and Figure 1, panel C). Plasma tHcy did not differ significantly among the four responsiveness groups (range of medians 226-262 μmol/L; range of tHcy 29.9–-550 μmol/L) at diagnosis. Similarly, free homocystine with a median of 48 μmol/L did not differ among the responsiveness groups. In contrast plasma or serum Met decreased significantly with increasing responsiveness; the median was 440 μmol/L in NR, 313 μmol/L in PR, 103 μmol/L in FR and 60 μmol/L in ER.

Data on enzymatic activity were available for only 89 patients (33 values in fibroblasts and 71 values in plasma of patients). CBS activity in fibroblasts is not supposed to be influenced by pyridoxine treatment of the patient because the cells will have been exposed to the same pyridoxine concentration in the culture medium. Pyridoxine administration can, however, increase the activity of mutant CBS released from the liver into the circulation probably due to improved folding, particularly in FR and ER (Table 2 and Figure 1, panel D). Thus, although there was an increase in median fibroblast CBS activities from NR, PR and FR (0.0, 1.5, and 4.0% activity in controls, respectively) and in median plasma CBS activity from patients off pyridoxine (either taken at diagnosis or during periods of non-compliance or after short

TABLE 2 Basic characteristics of the cohort at registry enrolment, mode of ascertainment, and biochemical findings at diagnosis

	All patients, n = 328	Non-responders, n = 201	Partial responders, n = 67	Full responders, n = 41	Extreme responders, n = 19	Statistical significance of difference	Statistical significance of trend
Males	174 (53.0%)	114 (56.7%)	40 (59.7%)	15 (36.6%)	5 (26.3%)	0.0069	0.0033
Females	154 (47.0%)	87 (43.3%)	27 (40.3%)	26 (63.4%)	14 (73.7%)		
White	292 (92.1%)	181 (92.3%)	57 (89.1%)	37 (92.5%)	17 (100.0%)	0.6069	0.5826
Other	25 (7.9%)	15 (7.7%)	7 (10.9%)	3 (7.5%)	0 (0.0%)		
Enrolment into registry							
Age at registry enrolment (median, range)	26.0 (0.1-76.3)	22.9 (0.1-67.1)	28.4 (1.9-72.6)	36.7 (7.7-76.3)	42.8 (22.3-74.9)	< 0.0001	< 0.0001
Patient-years at registry enrolment (total for each responsiveness group)	9131	4637	2119	1560	814	–	–
Follow-up time, *years*	3.65 (0.02-6.89)	3.59 (0.02-6.40)	3.52 (0.02–6.89)	4.04 (0.03-5.33)	2.95 (0.25-5.31)	0.2379	0.3407
BMI in patients >18 years of age (median, range) (available data, n = 181)	24.4 (14.0-40.5)	22.9 (14.0-37.0)	25.3 (19.5-38.8)	26.1 (17.2-40.0)	28.4 (22.7-40.5)	<0.0001	<0.0001
Mode of ascertainment (evaluable data, n = 322)							
Neonatal screening	43 (13.4%)	39 (19.7%)	4 (6.1%)	0 (0.0%)	0 (0.0%)	0.0003	Not applicable
Clinically diagnosed patients = selective screening	231 (71.7%)	132 (66.7%)	55 (83.3%)	30 (76.9%)	14 (73.7%)		
High-risk screening	48 (14.9%)	27 (13.4%)	7 (10.4%)	9 (22.0%)	5 (26.3%)		
Method of confirming diagnosis							
Diagnosis by metabolite analysis only	83 (25.3%)	54 (26.8%)	14 (20.9%)	11 (26.8%)	4 (21.1%)	0.7911	0.5658
Confirmatory testing by enzyme analysis	89 (27.1%)	49 (24.4%)	21 (31.3%)	9 (22.0%)	10 (52.6%)	0.052	0.0747
Confirmatory testing by DNA analysis	226 (68.9%)	134 (66.7%)	47 (70.1%)	30 (73.2%)	15 (78.9%)	0.6644	0.1887
Laboratory at diagnosis							
Total homocysteine (n = 239), *μmol/L*	230.0 (29.9-550)	229.0 (50-550)	226.0 (64-408)	236.0 (31-543)	262.0 (29.9-347)	0.6602	0.5965
Free homocysteine (n = 55), *μmol/L*	48.0 (5-384)	46.0 (8-245)	55.0 (5-384)	48.0 (31-101)	38.0 (38-38)	0.9205	0.1085
Methionine (n = 107), *μmol/L*	354.0 (34-1723)	440.0 (39-1280)	313.0 (49-829)	103.0 (49-1723)	60.0 (34-597)	0.0030	0.0388
CBS activity, *% of median control values* (median, range)							
In fibroblasts (n = 33)	1.0 (0.0-25)	0.0 (0.0-25)	1.5 (0.0-12)	4.0 (0.0-10)	–	0.2086	0.7243
In plasma/untreated patient (n = 19)	1.0 (0.0-16)	0.0 (0.0-4.3)	4.5 (0.0-9)	7.5 (0.0-15)	5.0 (2.0-16)	0.0535	0.0722
In plasma/treated patient (n = 52)	2.2 (0.0-149)	0.0 (0.0-24)	3.0 (0.0-100)	45 (0.0-118)	73 (7.5-149)	<0.0001	<0.0001

TABLE 3 Clinical presentation at the time of diagnosis in clinically ascertained patients

	All patients	Non-responders	Partial responders	Full responders	Extreme responders	Statistical significance of difference	Statistical significance of trend
Age of start of symptoms, *year* (median, range; available data, n = 195)	5.9 (0–55)	4.4 (0-38)	7.4 (0-55)	13.2 (0.4-54)	21.0 (3-40)	<0.0001	<0.0001
Age at diagnosis, *year* (median, range; available data, n = 195)	10.0 (0.1-73)	7.0 (0.1-46)	14.0 (2-62)	21.5 (1.2-58)	36.0 (3.9-73)	<0.0001	<0.0001
Patient-years before diagnosis (total for each responsiveness group; available data, n = 195)	2977	1105	823	622	428	NA	NA
Diagnostic delay, *year* (median, range; available data, n = 195)	2.0 (−3-58)	1.6 (−3-28)	2.4 (0-58)	5.1 (0-37)	14.0 (0-49)	0.0018	< 0.0001
Presence of clinical symptoms at diagnosis, number of patients with the symptom							
Central nervous system complications							
Neurologic disease (available data, n = 184)	97 (52.7%)	70 (64.2%)	18 (45.0%)	8 (33.3%)	1 (9.1%)	0.0003	<0.0001
Developmental delay/learning difficulties	74 (40.2%)	58 (53.2%)	12 (30.0%)	4 (16.7%)	0	<0.0001	<0.0001
Seizures/epilepsy	35 (19.0%)	22 (20.2%)	6 (15.0%)	6 (25.0%)	1 (9.1%)	NA	NA
Psychiatric disease (available data, n = 180)	23 (12.8%)	12 (11.2%)	7 (18.4%)	4 (16.7%)	0	0.3634	0.9606
Vascular system complications							
Thromboembolic complication (available data, n = 186)	65 (34.9%)	29 (26.9%)	16 (39.0%)	12 (46.2%)	8 (72.7%)	0.0087	0.0009
Stroke	36 (19.4%)	16 (14.8%)	10 (24.4%)	8 (30.8%)	2 (18.2%)	0.2062	0.1271
Deep venous thrombosis	28 (15.1%)	13 (12.0%)	5 (12.2%)	4 (15.4%)	6 (54.5%)	0.0094	0.0077
Pulmonary embolism	13 (7.0%)	5 (4.6%)	2 (4.9%)	3 (11.5%)	3 (27.3%)	0.0371	0.0109
Rate of thromboembolism per 1000 patient-years	22.2	26.8	20.0	19.3	18.7	NA	NA
Cardiac disease (available data, n = 183)	24 (13.1%)	9 (8.4%)	9 (22.5%)	3 (12.0%)	3 (27.3%)	0.0507	0.0630
Connective tissue abnormalities							
Ocular complication (available data, n = 191)	160 (83.8%)	98 (87.5%)	39 (92.9%)	20 (76.9%)	3 (27.3%)	<0.0001	<0.0001
Lens dislocation	140 (73.3%)	88 (78.6%)	34 (81.0%)	16 (61.5%)	2 (18.2%)	0.0028	0.0024
Rate of lens dislocation per 1000 patient-years	48.0	81.5	43.1	25.7	4.7	NA	NA
Skeletal abnormalities (available data, n = 182)	96 (52.7%)	64 (59.8%)	18 (46.2%)	11 (44.0%)	3 (27.3%)	0.0933	0.0141

Abbreviation: NA, not applicable.

pyridoxine wash-out periods), these did not reach statistical significance (0% control activity in NR, 4.5% in PR, 7.5% in FR and 5% in ER). In plasma from patients taking pyridoxine, however, there was a clear and statistically significant increase of CBS activities, especially in FR and ER (0% of activity of controls in NR, 3% in PR, 45% in FR, and 73% in ER).

Since detailed analysis of genetic variants and of the correlation between genotype and phenotype will be the subject of a future study, we present here only the geographic distribution of the five commonest alleles for which the pyridoxine responsivity is known (ie, p.Thr191Met, p.Gly307Ser, and p.Trp409_Gly453del associated with non-responsiveness, and p.Ile278Thr and p.Ala114Val associated with responsiveness). The p.Thr191Met was most frequent in Spain, p.Gly307Ser in Ireland and p.Trp409_Gly453del in the Czech Republic, p.Ala114Val in Switzerland and p.Ile278Thr in the Netherlands; for details in other countries see Table S2.

3.3 | Natural history in clinically ascertained patients

Two hundred and thirty-one patients were clinically symptomatic and diagnosed by selective screening. We analysed the diagnostic delay and salient clinical features for the complete group as well as within and between the subgroups of patients with different degrees of pyridoxine responsiveness (Table 3, detailed data Table S3).

3.3.1 | Age at presentation, age at diagnosis, and diagnostic delay

The first clinical symptoms were noted at a median age of 4.4 years in the NR group and increased significantly with the degree of responsiveness to 21 years in ER. The degree of responsiveness also correlated significantly with age at diagnosis and diagnostic delay (defined as the time between the age of first symptoms and the age of diagnosis) (Figure 2A). These data clearly show that patients with the milder FR and ER forms present later in life and experience a much longer diagnostic delay compared to patients with the early childhood NR and PR types of CBS deficiency.

3.3.2 | Typical clinical constellations and degrees of pyridoxine responsiveness

The typical clinical symptoms of CBS deficiency include central nervous system (CNS) problems (eg, developmental delay, learning difficulties and psychiatric disturbances); thromboembolic events in various vascular beds; connective tissue abnormalities resulting in skeletal abnormalities (marfanoid features, kyphoscoliosis, genua valga, osteoporosis) and/or abnormal zonular fibres, which cause lens dislocation. We analysed which combinations of clinical symptoms at diagnosis were typically present in the patient subsets. The NR and PR patients exhibited a high proportion of CNS symptoms combined with lens dislocation; FR patients presented most frequently with lens dislocation and thromboembolism while isolated thromboembolism

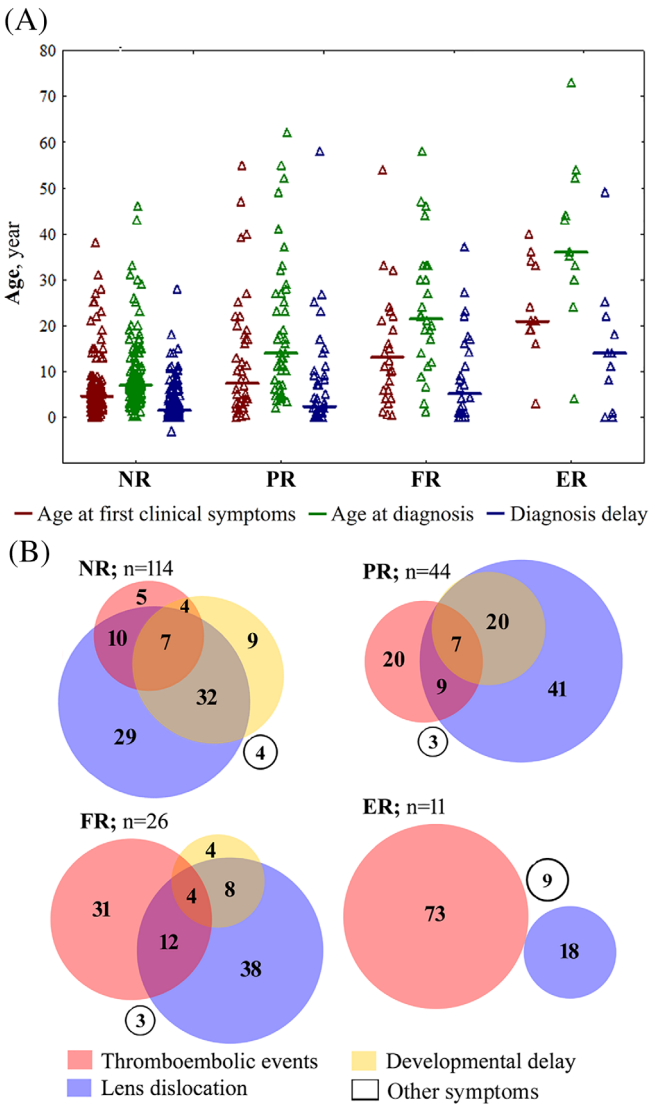

FIGURE 2 Onset of symptoms, diagnostic delay and combination of symptoms in untreated patients. A, Age at presentation and diagnosis, and B, combination of affected systems in clinically ascertained patients. Data are stratified by the degree of responsiveness, NR, non-responders; PR, partial responders; FR, full responders; ER, extreme responders. Horizontal bars in panel A indicate the medians. Panel B, Venn diagrams showing the combination of clinical complications; numbers indicate percentages for each responsiveness group

was the predominant presenting symptom in ER patients (Figure 2B). For more details on the connective tissue abnormalities (ie, skeletal and ocular complications) see Table S3.

3.3.3 | Developmental delay and learning difficulties

Developmental delay and/or learning difficulties were present at diagnosis in 53% of NR, 30% of PR, and 17% of FR patients while none of these symptoms were reported in the ER patients. The differences in the frequency of developmental delay between groups were statistically significant.

3.3.4 | Testing of intelligence & developmental quotients (IQ and DQ)

was done using variable methods in 31 patients following registry entry; no quantitative data at diagnosis were available. Data from six patients were uninterpretable and thus excluded from the analysis. Of the remaining 25 patients, five had normal, 10 below average and 10 low or very low intelligence. Owing to the variable data sources and time points of testing, questionable data quality, skewed distribution towards lower intelligence (indicating a selection bias), and the small number of cases, no further subgroup or other statistical analyses were conducted.

In contrast, there was no significant difference among the responsiveness groups for the frequencies of psychiatric disease (present in 12% of all patients). Data on behavioural testing after registry enrolment were available for 50 patients. The test results followed a similar distribution for all four° of responsiveness (median between four and six behavioural problems on a 20-item scale). Meaningful statistical analysis of correlations with cognitive function test results was not possible as these test results were widely scattered and only available for 31 patients. Details of other neurological, psychiatric and behavioural complications are shown in Table S3.

3.3.5 | Behaviour and well-being

Fifty-one patients had at least one self-assessment or parental assessment of 33 behavioural problems between enrolment and data extraction (total: 98 assessments; 1-5 measurements per individual). In total 367 items (21% of possible events) were marked. Symptoms indicated at least once per person were counted as events (repeated items were not counted) to give a general overview of the type of behavioural problems perceived by patients with CBS deficiency or their parents. The main complaints were shyness (53% of 51 patients), anxiety (51%), short attention span (51%), distractibility (47%), sleep problems (51%), hyperactivity (33%), and pain (31%). We did not undertake more detailed evaluation of severity, clustering of symptoms in individuals, differences between self- and parental assessments or correlation with cognitive ability because of the variability of the assessments and the small sample size.

3.3.6 | Detailed analysis of thromboembolism

Thromboembolic complications included stroke (14/36 of which were caused by sagittal sinus thrombosis), deep venous thrombosis and pulmonary embolism (for details see Table S3). The proportion of patients with a history of thromboembolic events reported at the time of diagnosis increased significantly with pyridoxine responsiveness from 27% in the NR to 73% in the ER group ($P = .0087$). This is due to the older age of responsive patients at diagnosis: the number of thromboembolic events per 1000 patient-years was very similar in all four responsiveness groups (ranging from 21.9 to 32.6 events). Indeed, time-to-event analysis showed that the 50% likelihood of having experienced a thromboembolic event by the time of diagnosis was reached at a significantly earlier age in pyridoxine non-responders (18 years) than in the other three groups (33, 30, and 35 years of age in PR, FR, and ER, respectively, Figure 3, panel A).

3.3.7 | Detailed analysis of connective tissue abnormalities

Skeletal anomalies were present in 59% of NR patients and the frequency decreased progressively with pyridoxine responsiveness to 27% in ER. The frequency of lens dislocation by the time of diagnosis also fell with responsiveness (88% in NR decreasing to 27% in ER). A 50% probability of lens dislocation was reached at 8, 14, and 27 years in NR, PR, and FR patients, respectively (Figure 3, panel B). ER patients only reached a 40% probability for lens dislocation at the age of 74 years. Extreme responders and NR differed significantly from all other groups whilst the difference between FR and PR did not reach statistical significance. Data normalised by patient-years show similar results, decreasing from 79.6 per 1000 patient-years in NR to only 4.7 in ER.

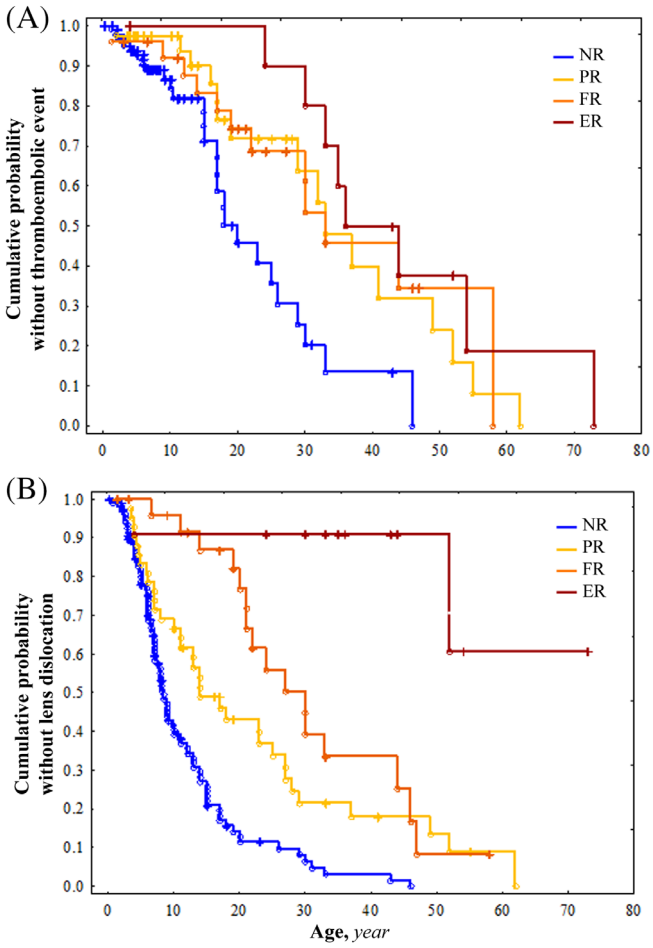

FIGURE 3 Time-to-event graphs of thromboembolism and lens dislocation in untreated clinically ascertained patients. A, Presence and/or history of thromboembolism and B, presence and/or history of lens dislocation. Curves were constructed by the method of Kaplan and Meier, patients were censored at the age of diagnosis if lens dislocation or thromboembolic event were present at diagnosis and/or were recorded in patients´ histories. Data are stratified by degree of responsiveness, NR, non-responders; PR, partial responders; FR, full responders; ER, extreme responders

3.3.8 | Other complications

Feeding problems were reported for 11% of patients, seizures for 19% of patients and cardiac disease (mostly hypertension, mitral valve insufficiency or aortic aneurysm) for 9% of patients in the cohort; there were no statistically significant differences among the responsiveness groups. For details see Table S3.

4 | DISCUSSION

In this paper, we studied the pyridoxine responsiveness and its impact on the biochemical and clinical diversity in 328 individuals with CBS deficiency from the E-HOD registry. The disease shows a wide phenotypic spectrum, ranging from a severe disease with onset during early childhood presenting mostly with CNS, ocular and skeletal involvement to an adult onset disease with predominantly thromboembolic complications.

In many respects, the current cohort of 328 patients resembles the cohort of 629 patients in Mudd's landmark natural history study.[10] Both cohorts were mainly recruited by metabolic physicians, who were asked to collect standardised data, but Mudd's series also included 97 patients from the literature. All of our patients were alive whereas Mudd included 64 deceased patients. Both cohorts were international; most of our patients are from Europe. Mudd did not state the country of origin but a larger proportion from the United States is likely. There is unlikely to be much overlap between the two series as Mudd's data were collected in 1982-3 whereas only 12% of our patients were diagnosed before 1984.

For inclusion in Mudd's series, patients were required to have lens dislocation, hypermethioninaemia or enzymatic confirmation in addition to increased homocystine excretion; CBS deficiency was confirmed by enzymology in one third, but the other patients only had metabolite measurements. We used different criteria to avoid excluding the recently described group of patients with an extremely marked response to pyridoxine.[12,24] CBS deficiency was confirmed by enzymology or DNA analysis in most patients; the clinical features, plasma free Hcy/tHcy and methionine concentrations, and therapy were reviewed in the others to ensure the plausibility of the diagnosis.

Our series differs from Mudd's in two main respects. First, more adult patients are now diagnosed after presenting with thromboembolism; this has led to the recognition of a wider range of pyridoxine responsive forms. The other main difference is the new multifactorial classification of pyridoxine responsiveness in our study. Mudd relied on his collaborators to judge pyridoxine responsivity. At that time, fully responsive patients were generally considered to be those who ceased to have detectable free homocystine in blood when taking pyridoxine. Of Mudd's 629 patients, 231 (37%) were classified as responsive, 231 (37%) as non-responsive, and 67 (11%) as intermediate in response (100 were not classified).

Nowadays, pyridoxine responsiveness can be assessed by a standardised test,[18] but this test has only recently been widely used. Patients in the E-HOD registry had, therefore, been classified as non-responsive, partially or fully responsive using various undefined criteria. Many patients who were said to be pyridoxine responsive had marked fluctuations in tHcy concentrations whilst on long-term pyridoxine or needed additional forms of treatment, indicating that assessments in different centres

were not comparable. We have, therefore, developed a set of biochemical and clinical criteria for defining the degree of responsiveness retrospectively in this series. In particular, supplementation of pyridoxine *alone* should maintain plasma tHcy below 50 μmol/L in FR and ER, whereas a severe methionine restriction is required to control tHcy in NR. According to this classification most of the patients in our cohort were NR (62%) or PR (20%), while FR and ER comprised only 12% and 6%, respectively. The proportion of non-responders was considerably higher than in Mudd's series, which may seem surprising as we would now expect *more* recognition of mild patients presenting as adults, often with thromboembolism. In part, this may be a result of the recent implementation of newborn screening programmes, which detect primarily non-responsive patients and miss less severe cases. The numbers may also reflect the more rigorous assessment of responsiveness in our series. The main reason, however, is probably that the proportions of different responsiveness groups in the E-HOD registry reflects the patients seen by the metabolic centres participating in E-HOD. This is unlikely to be a true reflection of the proportions of different responsiveness groups in the whole population due to a selection bias. Severely affected patients requiring dietary treatment are likely to attend a specialist metabolic centre for detailed monitoring and advice whereas mildly affected adults, who only need pyridoxine to control their disease, will often be managed locally by non-specialist physicians, who are less likely to participate in E-HOD.

The proportions of different responsiveness groups varied markedly between countries in the E-HOD registry (Figure 1A). We hypothesise that two factors may have contributed to the observed geographic differences. The first is the regional variation in the prevalence of alleles conferring different degrees of pyridoxine responsiveness (eg, the high frequency of the pyridoxine-responsive variant p.Ile278Thr in the Netherlands and high frequency of the pyridoxine non-responsive variants p.Thr191Met and p.Gly307Ser in Spain and Ireland, respectively). The second factor relates to the lower likelihood of pyridoxine responsive patients being entered into the E-HOD registry, as discussed in the previous paragraph; the proportion enrolled will vary between countries depending on who manages these patients.

The categorisation into four groups of pyridoxine responsiveness is useful as these groups differ in many phenotypic aspects. In general, the disease severity is milder and age of onset older in pyridoxine responsive patients than in NR and PR, and the combinations of affected organ systems are different.

Lens dislocation was a common presenting symptom in all categories except the ER group but the age at lens dislocation increased with the degree of pyridoxine responsiveness. In his series, Mudd also found earlier lens dislocation in non-responsive than responsive patients, though they occurred at younger ages in both groups (50% probability at 6 and 10 years, respectively).

The risk of thromboembolism is greatly increased in individuals with CBS deficiency compared to the general population. The risk of venous thrombosis in the general population is higher in males and increases substantially with age, from ∼0.03 events per 1000 individuals per year in children to ∼0.2 in young adults, ∼0.9 in middle aged adults and up to ∼12 events per 1000 individuals per year in older adults.[25-27] In the E-HOD cohort, CBS deficient patients experienced 18.7-26.8 thromboembolic events per 1000 patient-years, giving a relative risk at least 20-fold higher than the general population for middle aged adults and ∼1000-fold higher in children. These data indicate that tHcy should be included in algorithms for the investigation of individuals with thromboembolic events. Though thromboembolism was a commoner *presenting* feature in our FR and ER groups, this was just because they were older and did not reflect a higher risk: the number of events per 1000 patient years was similar in all groups. Indeed, thromboembolic events tended to occur at a younger age in the NR group than the others (50% probability at 18 years in NR, 31-36 years in the other groups). Mudd made similar observations in his series (50% probability at 24 years in non-responders, 28 years in responders).

In CBS deficiency, plasma tHcy is generally considered to be responsible for the thrombotic diathesis while the contribution of other metabolic disturbances is largely unknown. Thromboembolism is also a major presenting symptom in adolescent and adult patients with late-onset remethylation defects and comparable tHcy levels.[19] In the cohort of CBS deficient patients, however, tHcy levels at diagnosis were similar in all responsiveness categories, although thromboembolism occurred at a much later age in ER than in NR. If tHcy is the culprit, why should ER patients require a higher long-term tHcy exposure before a thromboembolic event than NR patients? The question cannot be answered from this retrospective study but tHcy values may not be stable in ER patients without treatment, instead varying with nutrition or the intake of commonly available vitamin supplements. The latter often contain pyridoxine doses that may correct the raised tHcy levels in ER and FR patients. By masking the diagnosis, this may contribute to the long delay in diagnosing these patients. While diagnosis is often delayed in all forms of CBS deficiency, the delay was longest in the FR and ER groups. This must also have been partly because thromboembolism is a less specific symptom than lens dislocation.

Newborn screening (NBS) is of outstanding value for severely affected patients, and particularly useful in populations with a high prevalence of severe cases such as Ireland or Qatar. As one might expect, FR and ER patients had higher residual plasma CBS activity than NR and PR patients and lower Met concentrations at presentation. NBS for homocystinuria is almost universally based on initial detection of raised Met concentrations[14,17] and consequently none of the FR or ER patients in this series were detected by NBS.

The lower Met levels and higher residual activity of liver-derived plasma CBS in FR and ER patients suggest that more Hcy is removed via the transsulfuration pathway and less is recycled to Met. These findings support the hypothesis that impaired transsulfuration and decreased cysteine (Cys) formation may play a critical role in the pathophysiology of CBS deficiency,[28] possibly including also CNS involvement.

Patients with CBS deficiency were originally described as being lean and thin, especially in childhood.[29] In a more recent small study patients showed significantly lower lean mass index but the fat percentage was not significantly different from controls.[30] Another study found that accelerated growth ceases before puberty and does not result in an increased final height or an abnormal BMI in adulthood.[31] In our study the BMI data collected on registry enrolment (during treatment) showed that the BMI in adult individuals increases with degree of pyridoxine responsiveness from a median of 22.9 kg/m^2 in NR to a median of 28.4 kg/m^2 in ER. It is unclear whether this observation is caused by differences in the duration of normal diet prior to diagnosis, differences in the severity of natural protein restriction or possibly by different degrees of Cys depletion in the various forms of CBS deficiency. In the general population, it has been shown that the plasma total cysteine concentration (tCys) correlates positively with BMI and fat mass content.[32] In several mouse models CBS deficiency was associated with decreased body fat mass[33,34]; this change in body composition was corrected by methionine restricted diet or enzyme replacement therapy but not by N-acetylcysteine administration.[35,36] Due to the absence of data on tCys concentrations in the E-HOD registry we were unable to explore whether BMI was related to the extent of Cys depletion in various forms of homocystinuria.

CBS deficiency has a continuous wide spectrum of symptoms and pyridoxine responsiveness is also a continuous variable. Responsiveness has been categorised in this and earlier studies for the sake of simplicity and clarity and to allow recommendations on treatment. The mainstays of treatment for CBS deficiency are still a methionine-restricted diet in non-responsive patients and pyridoxine administration in responsive patients. Betaine may be added to lower tHcy (at the cost of increasing Met). Precise assessment of pyridoxine responsiveness at diagnosis is mandatory to tailor the available treatment options to the patient's needs and to avoid overtreatment, for example with pyridoxine in non-responders or diet in patients adequately controlled by pyridoxine alone.

This work has some limitations related to selection bias. First, the E-HOD research initiative encompasses centres that are especially interested in the homocystinurias and methylation disorders and willing to invest time entering their patients' data into the registry. Moreover, E-HOD was not originally a worldwide project and coverage of cases within countries remains variable. Second, subjects have been enrolled at variable ages and times after diagnosis, when centres joined the registry. Due to the retrospective nature of the study and despite careful data cleaning, a certain amount of inaccuracy of the history, clinical observation and treatment data must be expected. Furthermore, the lack of harmonisation and standardisation within laboratories probably creates some inaccuracy in biochemical parameters. Finally, data on cognitive abilities, behavioural problems and quality of life have only been collected in a very limited number of subjects because psychological assessments and patient reported outcomes have not yet been fully integrated into the care for patients with classical homocystinuria.

In summary, data from our study show that the new proposed categories of pyridoxine responsiveness are associated with different clinical severities and patterns of symptoms. Re-assessing pyridoxine responsiveness may have important therapeutic consequences.

ACKNOWLEDGEMENTS

This work was supported by the grant NV19-01-00307 (Agentura Pro Zdravotnický Výzkum České Republiky), and institutional programs RVO-VFN 64165 (General University Hospital in Prague) and Progres Q26 (Charles University). The authors would like to thank patients, data managers and nutritionists for providing data for the registry.

Several authors of this publication are members of the European Reference Network for Rare Hereditary Metabolic Disorders (MetabERN)—Project ID No 739543.

The European network and registry for homocystinurias and methylation defects (EHOD) project has been established with funding from the European Union in the framework of the Health Program (No. 2012_12_02). From the end of the EU-project phase, the E-HOD project has received ongoing support from SOBI, Recordati Rare Disease Foundation, Vitaflo, and Nutricia Metabolics Germany.

CONFLICT OF INTEREST

Viktor Kožich, Jitka Sokolová and Jakub Krijt declare that Charles University-First Faculty of Medicine received a partial reimbursement for preclinical testing of OT-58 from Orphan Technologies. Andrew A.M. Morris received honoraria for lectures from Recordati & Nutricia and Advisory Board fees from Nutricia; he is an Expert Witness for a relevant medicolegal case. Stefan Kölker received funding from Recordati Rare Diseases for post-marketing authorization studies on Cystadane (betaine anhydrous). Martina Huemer has received honoraria for a lecture from Aeglea and research grant from Nutricia Metabolics. Carlo Dionisi-Vici received a research fund and reimbursement for attending a symposium from Medifood, honoraria from Recordati-Orphan Europe and Nutricia, and consulting fee from Nutricia. Markéta Pavlíková, Florian Gleich, Henk Blom and Matthias M.R. Baumgartner declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Viktor Kožich: Study design, verification of data quality and consistency, data analysis, writing of the first draft, manuscript revision and final approval. Viktor Kožich is the guarantor for the article.

Jitka Sokolová: Verification of data quality and consistency, data analysis, preparation of figures, tables and of supplementary materials, manuscript revision and final approval.

Andrew A.M. Morris: Study design, data analysis and interpretation, writing of the first draft, manuscript revisions and final approval.

Markéta Pavlíková: Statistical analysis and interpretation, preparation of figures, tables and of supplementary materials, manuscript revision and approval.

Florian Gleich: Study design, concept and maintenance of E-HOD registry, data verification, manuscript revision and final approval.

Stefan Kölker: Study design, concept and maintenance of the E-HOD registry, interim data interpretation, manuscript revision and final approval.

Jakub Krijt: Study design, analysis and interpretation of CBS activity in plasma, manuscript revision and final approval.

Carlo Dionisi-Vici: Study design, interim data interpretation, manuscript revision and final approval.

Matthias Baumgartner: Study design, interim data interpretation, manuscript revision and final approval.

Henk J. Blom: Study design, data analysis and interpretation, writing of the first draft, manuscript revision and final approval.

Martina Huemer: Study design, data analysis and interpretation, writing of the first draft, manuscript revision and final approval.

E-HOD consortium members: Data acquisition, data entry into registry, verification of data quality and consistency, manuscript revision and final approval.

DATA AVAILABILITY STATEMENT

Only aggregated data are being published in this study to comply with the GDPR rules.

ETHICS STATEMENT

This study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The E-HOD registry was first approved by the Ethics Committee of the University Hospital in Heidelberg (No S-525/2010; 14.3.2013). All participating centres received approval from their local ethics committees before enrolling patients and all patients provided written informed consent before pseudonymized data were entered into the registry. Analysis of data and publication of results was also approved by the Ethics Committee of the General University Hospital in Prague (No 417/20 S-IV).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Kožich V, Sokolová J, Morris AAM, et al. Cystathionine β-synthase deficiency in the E-HOD registry-part I: pyridoxine responsiveness as a determinant of biochemical and clinical phenotype at diagnosis. *J Inherit Metab Dis*. 2021;44:677–692. https://doi.org/10.1002/jimd.12338

APPENDIX A: E-HOD consortium members

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