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RESEARCH REPORT

Newborn Screening for Homocystinuria Revealed a High Frequency of MAT I/III Deficiency in Iberian Peninsula

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Abstract Homocystinuria due to cystathionine β-synthase deficiency or "classical homocystinuria" is a rare autosomal recessive condition resulting in altered sulfur metabolism with elevated methionine and homocysteine in plasma and homocystine in urine. This condition is characterized by a high clinical heterogeneity, which contributes to late clinical diagnosis, usually only made after irreversible damage has occurred. Treatment is effective if started before clinical symptoms. The analysis of methionine levels by tandem mass spectrometry (MS/MS) allows the newborn screening for homocystinuria, but false-positive results can be frequently obtained and lead to the unwanted identification of methionine adenosyl transferase (MAT I/III) deficiency. This latter condition is biochemically characterized by isolated persistent hypermethioninemia, accompanied in some individuals with slightly elevated levels of homocysteine in plasma. A dominant form of MAT I/III deficiency, associated with mutation p.R264H, seems to be very frequent in the Iberian Peninsula and usually has a clinically benign course. Both these metabolic disorders are screened in Galicia and Portugal since the introduction of the MS/MS technology, in 2000 and 2004, respectively, resulting in the identification of three patients with classical homocystinuria and 44 patients with MAT I/III deficiency. All but one heterozygous parent of MAT I/III patients, identified with the p.R264H mutation, are healthy adults around the age of 30/40. The implementation of a second-tier test for homocysteine in dried blood spots would considerably reduce the number of MAT I/III-deficient patients identified and improve the specificity and positive predictive value for classical homocystinuria screening.

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Introduction

Homocystinuria due to cystathionine beta-synthase deficiency (CBSD, OMIM #236200) or "classical homocystinuria" is a rare autosomal recessive condition caused by a deficiency in the enzyme cystathionine beta-synthase (CBS, EC 4.2.1.22), the first enzyme in the transsulfuration pathway. Incidence in Europe is estimated to be 1 in 100,000 live births, with an estimated worldwide frequency of 1:344,000 (Mudd et al. 2001). CBS catalyzes the conversion of serine and homocysteine to cystathionine and water and contains three functional domains; the C-terminal domain is responsible for allosteric activation of the enzyme by S-adenosylmethionine (AdoMet); the middle domain contains the catalytic core, which is responsible for the pyridoxal 5′-phosphate-catalyzed reaction; and the N-terminal domain, which contains heme, regulates the enzyme in response to redox conditions (Banerjee and Zou 2005). CBS is mainly expressed in the adult liver and pancreas and is active as a homotetramer (Meier et al. 2001); its deficiency results in altered sulfur metabolism with elevated methionine and homocysteine in plasma and homocystine in urine. The clinical features of untreated

homocystinuria due to CBS deficiency result in defects in a variety of different organs and systems and include myopia, ectopia lentis, mental retardation, skeletal anomalies resembling Marfan syndrome, and thromboembolic events (Mudd et al. 2001). There is a high clinical heterogeneity, which contributes to late clinical diagnosis, usually only made after irreversible damage has occurred. Treatment is effective if started before clinical symptoms and is based on dietary restriction of methionine and supplementation with betaine, vitamin B12, and folic acid. Without treatment, 50% of patients die by the age of 30 years, usually as a result of arterial thromboembolism (Testai and Gorelick 2010).

This condition is due to mutations in *CBS* gene, with more than 170 known mutations, the majority of which being sporadic mutations (http://cbs.lf1.cuni.cz/). A genotype-phenotype correlation is reported, with mutations associated with residual enzyme activity leading to a milder clinical phenotype (Mudd et al. 2001).

Patients with classical homocystinuria can be divided into two important biochemical phenotypes: pyridoxine responsive and pyridoxine nonresponsive. The pyridoxine-responsive patients represent approximately 50% of patients; they usually have milder symptoms and slower disease progression, when on oral pyridoxine (vitamin B6) supplementation (Mudd et al. 2001).

The most common *CBS* allele is c.833T>C (p.I278T), which is associated with pyridoxine-responsive homocystinuria; this seems to be rare in Iberian Peninsula (20%; Moat et al. 2004). On the other hand, mutation p.T191M, a pyridoxine nonresponsive mutation, seems to be prevalent in this region (Urreizti et al. 2003; Urreitzi et al. 2006). Mutation p.G307S is also a frequent pyridoxine nonresponsive mutation in northern Europe (Gallagher et al. 1995) and is probably of Celtic origin.

The analysis of methionine levels by tandem mass spectrometry (MS/MS) allows newborn screening (NBS) for homocystinuria, but this method raises two important issues: milder pyridoxine-responsive patients may be missed due to slightly elevated levels of methionine at the screening time, and false-positive results can be obtained due to secondary methionine elevations, resulting from other conditions like liver disease, parenteral nutrition, or methionine adenosyl transferase deficiency (MAT I/III deficiency, MIM#250850). This latter condition is biochemically characterized by isolated persistent hypermethioninemia accompanied, in some individuals, with slightly elevated levels of homocysteine in plasma (Mudd et al. 2001). Methionine adenosyl transferase (MAT, EC 2.5.1.6) is the enzyme responsible for the biosynthesis of S-adenosylmethionine from methionine and ATP and has two hepatic forms (MAT I and MAT III) encoded by *MAT1A* gene. Although usually inherited as an autosomal recessive trait, a dominant form has been reported associated with mutation p.R264H (Chamberlin et al. 1997, 2000). This mutation has been reported in several populations (Chamberlin et al. 1997; Nagao et al. 2013; Chien et al. 2005; Chadwick et al. 2014) and seems to be very frequent in the Iberian Peninsula (Couce et al. 2008, 2013; Martins et al. 2012). The clinical significance of this condition is not clearly elucidated, raising some issues regarding its inclusion in newborn screening programs. Both these metabolic disorders, classical homocystinuria and MAT I/III deficiency, are screened in Galicia (Spanish region) and Portugal since the introduction of the MS/MS technology, in 2000 and 2004, respectively, resulting in the identification of 47 patients, presenting only three of them classical homocystinuria.

Here, we report a high frequency of MATI/III deficiency in Iberian Peninsula detected through neonatal screening for homocystinuria.

Patients and Methods

During the last 10 years, approximately 850,000 newborns were screened in the single Portuguese Newborn Screening Laboratory by MS/MS technology, through the analysis of amino acids and acylcarnitines as butyl esters (Rashed et al. 1995), in dried blood samples. Most samples were collected between the third and sixth days of life. The positive screening criterion for classical homocystinuria and MAT I/III deficiency was a methionine concentration above 50 μmol/L.

Galicia started NBS for both these conditions in 2000, and approximately 374,000 newborns were screened, using the same MS/MS method. If the measured concentration exceeded the 99th centile for the studied population, a second sample was requested. For methionine, this value ranged between 48 and 56 μmol/L over the study period. In Galicia, most of the NBS samples were collected between the second and sixth days of life, and a urine sample was also included in the screening. Homocystine measurement was performed as second-tier test, by MS/MS analysis of urine spots in filter paper (Rebollido-Fernandez et al. 2012).

In both centers, newborns with a persistent, isolated high methionine level in a second sample were sent to a treatment center for further clinical and biochemical evaluation, which included hepatic function evaluation, plasma and urinary amino acid chromatography, plasma total homocysteine, and urinary organic acid analysis.

During this time, 47 patients were identified (Tables 1 and 2); all of them were asymptomatic term newborns. Diagnosis in all patients was confirmed by *MAT1A* or *CBS* gene analysis. Genomic DNA was isolated from blood

Table 1 Characterization of patients with MAT I/III deficiency

Patient data				Neonatal screening time (blood spot)		Confirmation time (plasma)			Molecular analysis	
Patient	Origin	Gender	Current age	Age	Met (μM)[a]	Age	Met (μM)[b]	tHcy (μM)[c]	Gene *MAT1A*	References
1	Portugal	F	9 years	6 days	85	12 days	80	13	p.R264H/WT	Chamberlin et al. (1997)
2	Portugal	F	8 years	5 days	123	18 days	176	17	p.R264H/WT	Chamberlin et al. (1997)
3	Portugal	M	8 years	4 days	77	19 days	121	16	p.R264H/WT	Chamberlin et al. (1997)
4	Portugal	M	8 years	4 days	52	19 days	168	16	p.R264H/WT	Chamberlin et al. (1997)
5	Portugal	F	7 years	4 days	103	16 days	85	13	p.R264H/WT	Chamberlin et al. (1997)
6	Portugal	M	7 years	3 days	58	29 days	247	18	p.R264H/WT	Chamberlin et al. (1997)
7	Portugal	F	6 years	4 days	80	30 days	195	14	p.R264H/WT	Chamberlin et al. (1997)
8	Portugal	F	6 years	4 days	85	24 days	254	20	p.R264H/WT	Chamberlin et al. (1997)
9	Portugal	M	6 years	6 days	309	10 days	194	n.a.	p.R264H/WT	Chamberlin et al. (1997)
10	Portugal	M	5 years	6 days	73	23 days	145	14	p.R264H/WT	Chamberlin et al. (1997)
11	Portugal	F	5 years	6 days	77	63 days	157	12	p.R264H/WT	Chamberlin et al. (1997)
12	Portugal	F	5 years	5 days	117	18 days	182	21	p.R264H/WT	Chamberlin et al. (1997)
13	Portugal	F	5 years	5 days	124	24 days	155	15	p.R264H/WT	Chamberlin et al. (1997)
14	Portugal	F	5 years	7 days	67	30 days	85	5	p.L137V/p.S247N	This study
15	Portugal	F	5 years	5 days	92	30 days	79	11	p.R264H/WT	Chamberlin et al. (1997)
16	Portugal	F	5 years	4 days	79	20 days	111	64	p.R264H/WT	Chamberlin et al. (1997)
17	Portugal	F	4 years	3 days	72	19 days	465	n.a.	p.L355R/p.L355R	Couce et al. (2013)
18	Portugal	F	4 years	6 days	80	22 days	147	23	p.R264H/WT	Chamberlin et al. (1997)
19	Portugal	F	4 years	5 days	60	16 days	136	10	p.R264H/WT	Chamberlin et al. (1997)
20	Portugal	M	4 years	4 days	100	13 days	105	n.a.	p.R264H/WT	Chamberlin et al. (1997)
21	Portugal	F	3 years	4 days	63	30 days	117	12	p.R264H/WT	Chamberlin et al. (1997)
22	Portugal	F	3 years	5 days	72	19 days	85	11	p.R264H/WT	Chamberlin et al. (1997)
23	Portugal	F	3 years	3 days	71	16 days	110	15	p.R264H/WT	Chamberlin et al. (1997)

(continued)

Table 1 (continued)

Patient data				Neonatal screening time (blood spot)		Confirmation time (plasma)			Molecular analysis	
Patient	Origin	Gender	Current age	Age	Met (μM)[a]	Age	Met (μM)[b]	tHcy (μM)[c]	Gene *MAT1A*	References
24	Portugal	M	2 years	4 days	92	28 days	108	12	p.R264H/WT	Chamberlin et al. (1997)
25	Portugal	M	2 years	5 days	101	16 days	94	8	p.R264H/WT	Chamberlin et al. (1997)
26	Portugal	F	2 years	6 days	110	23 days	148	12	p.R264H/WT	Chamberlin et al. (1997)
27	Portugal	F	1 year, 4 months	5 days	79	21 days	156	12	p.R264H/WT	Chamberlin et al. (1997)
28	Portugal	M	1 year	6 days	86	17 days	126	23	p.R264H/WT	Chamberlin et al. (1997)
29	Portugal	F	9 months	5 days	71	15 days	90	n.a.	p.R264H/WT	Chamberlin et al. (1997)
30	Portugal	M	5 months	6 days	71	21 days	113	10	p.R264H/WT	Chamberlin et al. (1997)
31	Portugal	M	3 months	4 days	74	16 days	93	26	p.R264H/WT	Chamberlin et al. (1997)
32	Galicia	F	8 years	6 days	50	35 days	573	23	p.R264H/WT	Chamberlin et al. (1997)
33	Galicia	M	8 years	3 days	100	1 month, 15 days	189	9	p.R264H/WT	Chamberlin et al. (1997)
34	Galicia	F	8 years	5 days	88	2 months, 21 days	341	10	p.R264H/WT	Chamberlin et al. (1997)
35	Galicia	M	8 years	3 days	147	2 months, 13 days	331	12	p.R264H/WT	Chamberlin et al. (1997)
36	Galicia	M	7 years	5 days	100	25 days	164	11	p.R264H/WT	Chamberlin et al. (1997)
37	Galicia	F	6 years	3 days	80	1 month, 15 days	292	n.a.	p.R264H/WT	Chamberlin et al. (1997)
38	Galicia	F	5 years	4 days	115	1 month, 10 days	283	8	p.R264H/WT	Chamberlin et al. (1997)
39	Galicia	F	4 years	2 days	82	1 month, 28 days	106	15	p.R264H/WT	Chamberlin et al. (1997)
40	Galicia	F	2 years	3 days	82	1 month, 20 days	392	n.a.	p.R264H/WT	Chamberlin et al. (1997)
41	Galicia	M	2 years	5 days	76	4 months, 8 days	157	8	p.R264H/WT	Chamberlin et al. (1997)
42	Galicia	M	1 year, 2 months	6 days	45	3 months	255	12	p.R264H/WT	Chamberlin et al. (1997)
43	Galicia	M	2 years	3 days	67	22 days	91	n.a.	p.R264H/WT	Chamberlin et al. (1997)
44	Galicia	M	5 months	3 days	70	2 months, 22 days	144	11	p.R264H/WT	Chamberlin et al. (1997)

n.a. not available, *Met* methionine, *tHcy* total homocysteine
[a] Cutoff value: 50 μM (Portugal) and 56 μM (Galicia)
[b] Normal range: 4–44 μM
[c] Normal range: 2–14 μM; patients 3 and 4 are twin brothers

Table 2 Characterization of patients with classical homocystinuria

Patient data				Neonatal screening (blood spot)		Confirmation time (plasma)			Molecular study	
Patient	Origin	Gender	Current age	Age	Met (μM)[a]	Age	Met (μM)[b]	tHcy (μM)[c]	Gene *CBS*	References
45	Portugal	F	9 years	5 days	130	22 days	744	130	p.V178GfsX23/ p.K523SfsX18	Cozar et al. (2011), Castro et al. (2001)
46	Portugal	M	2 years	6 days	130	14 days	293	149	p.L338P/p.L338P	Urreizti et al. (2003)
47	Galicia	F	9 years	3 days	59	29 days	1,086	148	p.T257M/p.T257M	Sebastio et al. (1995)

Met methionine, *tHcy* total homocysteine
[a] Cutoff value: 50 μM (Portugal) and 56 μM (Galicia)
[b] Normal range: 4–44 μM
[c] Normal range: 2–14 μM

samples and sequenced by standard procedures, based on direct sequencing of PCR-amplified fragments containing all the coding regions, the corresponding intron-exon junctions, and the 5'- and 3′-untranslated regions (see Couce et al. 2008 and Martins et al. 2012 for details; oligonucleotide primer sequences and PCR conditions are available upon request).

Results

More than one million newborns have been tested for CBS and MAT I/III deficiencies, in Galicia and Portugal, since 2000, three patients being identified with classical homocystinuria and 44 patients with MAT I/III deficiency. All patients were confirmed by biochemical and molecular analysis (Table 1), and we had a 0.013% recall rate due to elevated methionine in the screening sample; approximately 50% of these cases were premature neonates with parenteral nutrition.

Thirty-one cases of MAT I/III deficiency (approximate frequency 1:27,400) and two cases of classical homocystinuria (approximate frequency 1: 425,000) were identified in Portugal (Tables 1 and 2). Some of these patients were already reported (Martins et al. 2012; Cozar et al. 2011).

In Galicia, 13 cases of MAT I/III deficiency (approximate frequency 1:28,736) and one classical homocystinuria (approximate frequency 1:374,000) were detected (Tables 1 and 2). These patients were already referred to in previous publications (Urreitzi et al. 2006; Couce et al. 2008, 2013).

All MAT I/III-deficient patients had moderately increased levels of methionine, both in screening and confirmation samples, with usually high levels in the confirmation sample. Most patients with p.R264H mutation had slightly elevated levels of total homocysteine (Table 1). In regular annual follow-up, these patients maintain the moderate methionine levels (data not shown). After diagnosis, all patients except patients 35 and 38 received free diets appropriate for their ages with a protein intake within normal limits (FAO/WHO).

The molecular study of MAT I/III-deficient patients revealed that 42 of the 44 patients are heterozygous for p.R264H mutation, with no other mutation identified in *MAT1A* gene. Family studies of these patients lead to the identification of eight new cases and, as expected, one parent (mother or father) of each patient was also confirmed to be heterozygote for p.R264H mutation. All these individuals were clinically well, except the father of patient 15, who died at a young age with a myocardial infarction (Martins et al. 2012). In general, the identified adult patients also had mildly elevated levels of homocysteine and lower levels of methionine, compared with newborns. Two mothers (of twin brother patients 2 and 3 and patient 28) and one great grandmother (of patient 13) even presented completely normal levels of methionine (32, 33, and 49 μM, respectively).

Patient 14 is a heterozygous compound presenting two novel missense mutations: p.L137V (c.409T>G) and p.S247N (c.740G>A). This patient has no Iberian ancestry. Both mutations affect highly conserved amino acid residues, were not found in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) or exome variant database (evs.gs.washington.edu/EVS/), and are predicted to be pathogenic through bioinformatics tools analysis, namely, Condel (bg.upf.edu/condel/), SIFT (sift.jcvi.org/), MutationTaster (www.mutationtaster.org/), and PolyPhen 2 (genetics.bwh.harvard.edu/pph2/index.shtml). In the S247 amino acid residue, another mutation p.S247R (c.739A>C) was recently reported (Nagao et al. 2013).

Patient 17 is homozygous for mutation p.L355R, a recently reported mutation found in a related patient living in Spain (Couce et al. 2013).

All three patients with classical homocystinuria had moderately elevated levels of methionine at screening but extremely high levels of plasma methionine and total homocysteine at confirmation time (Table 2). They have been treated since diagnosis in the neonatal period with dietary restriction of methionine and supplementation with betaine, vitamin B12, and folic acid. In spite of being pyridoxine nonresponsive, they remain asymptomatic to date.

Discussion

Newborn screening has been advocated for classical homocystinuria due to considerable evidence that early detection and treatment can prevent the severe clinical consequences of this condition (Mudd et al. 2001). Nevertheless, newborn screening for classical homocystinuria has been mostly performed by identification of increased methionine concentrations in dried blood spot samples through MS/MS, which is a low-specificity method if a low cutoff value is used for methionine and a low-sensitivity method if, instead, a high cutoff value is used (Gan-Schreier et al. 2010); a compromise value should then be used, but this is never an optimal strategy for screening. In Portugal and Galicia, methionine cutoff values of 50 and 56 μmol/L, respectively, have been used, and, as far as we know, there are no missing cases of homocystinuria. Moreover, we identified a considerable number of patients with a possible benign form of MAT I/III deficiency, a condition not known in the Iberian Peninsula before MS/MS-based newborn screening. All MAT I/III-deficient patients are under biochemical and clinical follow-up in different treatment centers, and all have normal growth, development, and neurological examination. Only patient 7 was found to have myelination abnormalities of unknown clinical significance, and in two families (patients 1 and 5), severe vascular disease histories, without other risk factors, were observed. Careful regular clinical monitoring is being performed for all newborn patients and p.R264H carrier relatives, despite their current healthy state (Martins et al. 2012). This is in accordance with the results of Nagao et al. (2013), who reported 14 clinically well patients with p.R264H mutation, mostly detected through newborn screening. Parents and siblings identified in subsequent family studies were also clinically well, and there was also a tendency to a decrease of methionine values with age.

Only three patients were identified with classical homocystinuria, but they are all free of symptoms proving the usefulness of this screening.

A second-tier approach for NBS of homocystinuria, by measuring the total homocysteine on the initial dried blood spot, has been developed (Gan-Schreier et al. 2010; Tortorelli et al. 2010). This allows the use of an initial low cutoff value for methionine, with the positive cases being then selected for total homocysteine measurement. With this approach, a higher sensitivity would be achieved, and the identification of newborns with elevated methionine levels due to parenteral nutrition or secondary to other liver disease would be avoided, thus reducing the number of parents affected by the parental anxiety associated with a false-positive result. Some of the newborns with MAT I/III deficiency could also be deliberately ignored, although this could be controversial due to the incomplete knowledge of the natural history of the disease in all patients; the complete lack of MAT I/III activity may represent a risk for development of brain demyelination (Mudd 2011). Some MAT I/III-deficient patients have been reported with myelination disorders (Chamberlin et al. 1996; Furujo et al. 2012; Nagao et al. 2013). Forty-two of the forty-four patients identified in Portugal and Galicia are heterozygous for p.R264H mutation, a dominant form of the disease, associated with a high residual enzyme activity and moderate elevated methionine levels. According to previous reports, some residual activity could be sufficient to prevent clinical symptoms in these patients (Chamberlin et al. 1996). If this is the case, there is no advantage for them to be identified; they will never need treatment and their identification will only lead to unnecessary anxiety for the families. Nevertheless, myelination alterations do not seem to be directly related to methionine levels, and a genotype/phenotype correlation regarding methionine levels and CNS involvement has not yet been established (Chamberlin et al. 1996; Mudd et al. 2001). At least one p.R264H heterozygous 3-year-old girl was reported with myelination abnormalities (Martins et al. 2012), which suggests care must be taken with the assumption that none of these individuals will develop symptoms.

In spite of the improving of the specificity and positive predictive value for classical homocystinuria screening, this second-tier approach may not always allow for the unequivocal differentiation between MAT I/III deficiency and classical homocystinuria since there is the possibility of low levels of total blood homocysteine in the first days of life in patients with classical homocystinuria B6-responsive and mildly elevated values of total homocysteine sometimes observed in MAT I/III-deficient patients. Some patients are initially misdiagnosed as having CBS deficiency due to these mildly elevated values (Nagao et al. 2013). In these cases, the molecular study of *MAT1A* and *CBS* genes or the measurement of other metabolites of the methionine cycle could be important for the correct diagnosis (Stabler et al. 2002).

In conclusion, this report tried to address two main questions: should we maintain NBS for classical homocystinuria through elevated methionine levels, in spite of

the low frequency of this disease in Iberian Peninsula, and the identification of a large number of patients with a possible benign form of MAT I/III deficiency or, on the other hand, should we implement a second-tier test for total homocysteine in dried blood spots? Do patients and families of MAT I/III-deficient patients have any advantage from their identification or, on the contrary, is this diagnosis only a burden to the families?

Considering our present knowledge and results, we decided to implement the second-tier test for total homocysteine. For the MAT I/III-deficient cases which are still to be identified, careful full information regarding the most probable benign clinical course of the disease should be provided to the families, together with the recommendation for regular clinical and biochemical follow-up.

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Synopsis

MS/MS-based NBS in Galicia and Portugal, through the analysis of methionine, revealed a very low birth prevalence for classical homocystinuria (approximately 1:400,000) and a high birth prevalence for MAT I/III deficiency (approximately : 1:28,000).

Compliance with Ethics Guidelines

Conflict of Interest

Ana Marcão, María L. Couce, Célia Nogueira, Helena Fonseca, Filipa Ferreira, José M. Fraga, M. Dolores Bóveda, and Laura Vilarinho declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Additional informed consent was obtained from all patients for which identifying information is included in this article.

Details of the Contributions of Individual Authors

Ana Marcão – Planned, conducted, and reported the work described in the article.

María L. Couce – Planned and reported the work described in the article.

Célia Nogueira – Planned, conducted, and reported the work described in the article.

Helena Fonseca – Conducted the work described in the article.

Filipa Ferreira – Conducted the work described in the article.

M. Dolores Bóveda – Planned the work described in the article.

José M. Fraga – Planned the work described in the article.

Laura Vilarinho – Planned and reported the work described in the article.

All authors have read and approved the final manuscript.

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