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Relevance of Expanded Neonatal Screening of Medium-Chain Acyl Co-A Dehydrogenase Deficiency: Outcome of a Decade in Galicia (Spain)

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Abstract Neonatal screening of medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is of major importance due to the significant morbidity and mortality in undiagnosed patients. MCADD screening has been performed routinely in Galicia since July 2000, and until now 199,943 newborns have been screened. We identified 11 cases of MCADD, which gives an incidence of 1/18,134. During this period, no false negative screens have been detected. At diagnosis, all identified newborns were asymptomatic. Our data showed that octanoylcarnitine (C8) and C8/C10 ratio are the best markers for screening of MCADD. C8 was increased in all patients and C8/C10 was increased in all but one patient.

The common mutation, c.985A>G, was found in homozygosity in seven newborns and in compound heterozygosity in three, while one patient did not carry the common mutation at all. In addition, two novel mutations c.245G>C (p.W82S) and c.542A>G (p.D181G) were identified. Ten of the 11 identified newborns did not experience any episodes of decompensation. The patient with the highest level of medium chain acylcarnitines at diagnosis, who was homozygous for the c.985A>G mutation, died at the age of 2 years due to a severe infection.

This is the first report of the results from neonatal screening for MCADD in Spain. Our data provide further evidence of the benefits of MCADD screening and contribute to better understanding of this disease.

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Introduction

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD, OMIM #201450) is the most commonly inherited defect of the mitochondrial fatty acid oxidation pathway, with significant morbidity and mortality in undiagnosed patients. The estimated incidence varies between 1/5,000 and 1/68,560 according to the data reported in several studies (Yokota et al. 1991; Andresen et al. 2001; Tran et al. 2007; Wilcken et al. 2007).

MCADD is a recessively inherited disorder and homozygosity for the prevalent c.985A>G (K329E) mutation accounts for up to 80–90% of the detected cases (Yokota et al. 1991; Gregersen et al. 1991; Nennstiel-Ratzel et al. 2005; Giroux et al. 2007). To date, 81 *ACADM* mutations have been described in the Human Genome Mutation database http://www.hgmd.cf.ac.uk, but a clear genotype–phenotype correlation has not been established, and there is a wide phenotypic variability even within the same family (Yokota et al. 1991; Andresen et al. 1997; Lehotay et al. 2004; Waddell et al. 2006; Hsu et al. 2008).

Patients with MCADD have the inability to completely metabolize long chain fatty acids released from adipose tissues during catabolism that usually results in the sequestration of CoA and other biochemical and physiological features of the disorder.

Clinical symptoms are diverse ranging from asymptomatic to hypoketotic hypoglycemic episodes and even sudden death in some patients (Andresen et al. 1993, 2001; Waddell et al. 2006). Symptoms could occur at any age and are typically precipitated by stress in situations of high energy demand such as a prolonged period of fasting or an intercurrent illness, particularly between 3 and 24 months of age. Correct dietary management avoids or minimizes the number of metabolic decompensation episodes. Therefore, early identification of MCADD deficient individuals is important and newborn screening (NBS) by tandem mass spectrometry (MS/MS) for MCADD (Millington et al. 1990) has been initiated in numerous countries over the last decade. In Galicia (northwest Spain), NBS by MS/MS was started in July 2000. The main objective of this study was to evaluate the diagnostic results as well as the outcome of the MCAD deficient individuals after 10 years of screening.

Material and Methods

Analytical Methods

Until 2001, blood spots and urine-impregnated filter paper sampled between the 5th and 8th day of life were collected for neonatal screening in Galicia. In 2002, this recommendation changed and sampling on the third day of life, after 48 h of milk intake, was established.

Medium-chain acylcarnitines C8, C6, C10, C10:1, and the ratios C8/C2, and C8/C10 in blood spots were studied by the usual method of butylation and analysis by MS/MS (Chace et al. 2001). MCADD was suspected in those newborn who presented an elevation higher than the 99.9th percentile of the specific acylcarnitines (C6, C8, C10, C10:1). Cutoff values were obtained through screening of the population (n=199,943 samples): C8>0.52 μM, C6>0.43 μM, C10>0.50 μM, C10:1>0.33 μM, C8/C2>0.02, and C8/C10>1.85

Plasma Free Fatty acids (FFAs) were analyzed as previously described (Martínez et al. 1997) by specific methylation with acetyl chloride/methanol, in one step extraction and derivatization.

Analysis of urinary organic acids was performed by gas chromatography mass spectrometry (GC/MS) by the usual method of solvent extraction and trimethylsilyl derivatization (Tanaka et al. 1980).

DNA was isolated and sequenced by standard procedures from blood samples of all the patients and their parents, except one case conceived by in vitro fertilization (IVF) with oocyte donation (patient 9, Table 1), whose biological mother was not studied for obvious reasons.

Patients at Diagnosis

During the period of study, 199,943 newborn samples were tested and 11 cases from ten families were diagnosed (patients 3 and 4 were brothers). Eight cases were Caucasian and three were of Gypsy ethnicity.

At diagnosis, the following parameters were evaluated: age, sex, presence or absence of clinical symptoms, medium chain acylcarnitines and their ratios, plasma FFA, and urinary acylglycines and organic acids. The diagnosis was confirmed by mutation analysis of the *ACADM* gene (OMIM#607008). Clinical course was subsequently monitored.

Patients' Follow-up

Follow-up of all the patients, except one, has been done in our hospital.

Anthropometric evaluation: body weight, body length, and head circumference were measured and expressed as percentiles with respect to the reference population.

Evaluation of cognitive and psychomotor development: we reassessed Psychomotor Development Index (PDI) or Intellectual Quotient (IQ) of survivors using the Brunet Lézine Scale in infants, the Mc.Carthy Scales of Psychomotor Skills (MSCA) in preschool children and Wechler Intelligence Scale for Children Revised (WISC-R) in children older than 6 years. The overall index score of PDI or IQ is considered in the normal range when it is above 85.

Dietary management: the patients received a normal age diet allowing normal growth and development and avoiding prolonged fasting periods and lipolisis by high intake of slow absorption carbohydrates. We used a computer program of dietary calculation developed by ourselves that monitors fat intake and energy (www.odimet.es). Biochemical follow-up includes measurement of plasma-free carnitine at each visit and annually determination of general parameters including transaminases. Carnitine supplement is prescribed if the level is below 12 μM.

Informed consent of the patients' parents was obtained. The study was approved by the Ethics Committee of our hospital.

Results

Since the introduction of the expanded NBS in Galicia, we identified 11 cases with MCADD, which represents an incidence of 1/18,176 newborns. All of them, eight males and three females, were born at term with normal birth weight. The medium age of sample collection for screening was 7.4 days

Table 1 Summary of laboratory investigations, mutations at MCADD diagnosis and evolution.

	Acylcarnitines - mol/L (99.9th percentile)					Free fatty acids -mol/L (control values)			Mutation*	Follow-up (years)	PDI/IQ	Present status
Patients	C8 (<0.52)	C10 (<0.5)	C10:1 (<0.33)	C8/C10 (<1.85)	C8/C2 (<0.02)	C8:0 (≤1-8)	C10:0 (4-9)	C10:1 (≤0.4-1)				
1	1.31	0.20	0.27	6.62	0.12	80	22	10	p.K329E/p.K329E	6	104	FS
2	1.23	0.17	0.20	7.33	0.11	35	10	16	p.K329E/p.K329E	6	98	FS
3	3.06	0.38	0.66	7.94	0.34	200	12	28	p.K329E/p.K329E	5	87	FS
4	5.82	0.56	0.47	10.3	0.67	93	25	38	p.K329E/p.K329E	1	nd	Exitus
5	1.9	0.17	0.22	11.0	0.09	76	88	4.2	p.K329E/p.K329E	3	105	FS
6	9.4	0.71	0.97	13.2	0.74	nd	nd	nd	p.K329E/p.K329E	1	nd	FS
7	4.57	0.40	0.55	11.4	0.55	nd	nd	nd	p.K329E/p.K329E	1	nd	FS
8	0.84	0.50	0.33	1.64	0.07	6.8	6.8	4.2	p.K329E/p.Y67H	6	128	FS
9	1.72	0.22	0.61	7.53	0.22	120	28	6.1	p.K329E/p.W82S	6	142	FS
10	1.26	0.51	0.39	2.45	0.12	nd	nd	nd	p.K329E/p.I416T	0.6	nd	FS
11	5.19	0.81	0.56	6.38	0.6	nd	nd	nd	p.G267R/p.D181G	0.8	103	FS

FS: Free of Symptoms; nd= not done

PDI/IQ: Psychomotor Development Index/ Intellectual Quotient

*All mutations were named according to the precursor enzyme.

(range: 4–23). At diagnosis, all the patients were asymptomatic and presented with increased levels of medium chain acylcarnitines. As shown in Table 1, they exhibited a marked elevation of octanoylcarnitine (C8) with a median value of 3.3 μmol/L. The highest values were observed in patients 4 and 6 and the lowest was observed in patient 8. The C8/C10 ratio showed a clear elevation (median value: 7.79 μmol/L) reaching the highest value in patient 6. In our hands, C8 has demonstrated to be the best parameter for the diagnosis of MCADD; C8/C10 ratio was also good but it was normal in one patient.

During the period of study, two individuals only heterozygous for the common c.985A>G mutation were identified, presenting a slight elevation of octanoylcarnitine 0.54 μmol/L, and 0.56 μmol/L, respectively. Other mutations were excluded by sequencing all the exons and intron boundaries of the *ACADM* gene. To our knowledge, no false negative cases have been identified in our region.

Plasma FFA were studied in seven patients (Table 1), the profile showed elevations of C8:0, median value 87.4 μmol/L (Reference <8), C10:0, median value 27.4 μmol/L (Reference <9) and C10:1, median value 15.13 μmol/L (Reference <1). Patient 3 presented the highest C8:0 (200 μmol/L), patient 4 the highest C10:1 (38 μmol/L), and patient 5 the highest C10:0 concentration (88 μmol/L).

Hexanoylglycine (Reference <4.53 mmol/mol creatinine) and suberylglycine (Reference <9.87 mmol/mol creatinine) were increased in all the patients, while the dicarboxylic acid profile was normal in some of them. The heterozygous patients had also normal values of hexanoylglycine and suberylglycine

The diagnosis of MCADD was confirmed by molecular studies. Seven patients (12 alleles, as 2 are siblings) were homozygous for the prevalent c.985A>G (p.K329E) mutation. Three patients were compound heterozygous for the prevalent c.985A>G mutation and for another mutation, and one patient does not carry the c.985A>G mutation in any of the alleles (Table 1). Two of the mutations c.245G>C (p.W82S) and c.542A>G (p.D181G) are novel. The c.1247T>C (p.I416T) has very recently been reported in a US newborn identified by MS/MS-based routine screening (Smith et al. 2010), and we have furthermore observed this mutation in another US newborn identified by MS/MS-based routine screening (personal communication). Among our MCADD individuals (20 alleles), the prevalence of the common allele is 75%.

After diagnosis, all the patients followed the dietary recommendations described above. Cases 3 and 4 were supplemented with L-carnitine due to the low levels of free carnitine (below 12 μM). The evolutionary follow-up showed a normal physical and neurological outcome in all the patients, and until present ten have remained without clinical symptoms. Patient 4 died at 2 years of age in another hospital, due to a serious respiratory infectious, and we cannot identify whether the underlying disease influenced the outcome.

This patient was homozygous for the common mutation, and at diagnosis C8 and C8/C10 were strongly increased (Table 1). The remaining patients have never suffered any episode of decompensation.

All the patients have normal PDI/IQ, and those with the highest PDI/IQ are compound heterozygous for the common and another mutation (patients 8 and 9).

Three newborns are of Gypsy ethnicity and all are homozygous for the prevalent mutation, in agreement with the high prevalence of this mutation in this ethnic group.

Discussion

Although some children with MCADD may remain asymptomatic, even if they are not diagnosed, many papers highlight the importance of an early diagnosis and treatment to reduce the number of decompensation episodes and deaths (Nennstiel-Ratzel et al. 2005; Wilson et al. 1999). Early diagnosis of MCADD is now possible through the implementation of NBS by MS/MS. It allows the establishment of an early treatment and follow-up to prevent the acute metabolic derangement often associated with this disease. In addition, NBS by MS/MS also enables estimates of the prevalence of MCADD in the general population of a defined population to be made.

It has been reported that the incidence of MCADD is similar to that of phenylketonuria (1:10,000 live newborns) or even higher (Grosse et al. 2006). In our population, the frequency is 1/18,847 live births. Since our center receives samples from all the infants born in our area, the NBS results of MCADD reflect the characteristics of the Galician population.

As previously reported by others, we have also found that the combined elevation of C8 and C8/C10 ratio is the most useful tool for most of the MCADD diagnosis (Blois et al. 2005), but the ratio C8/C10 was normal in patient 8. According to Smith, this patient could be considered as having an intermediate phenotype and acylcarnitine results are in agreement with previous descriptions (Smith et al. 2010; Maier et al. 2005). The average value of C8 in our homozygous patients for the common mutation was 3.9 μmol/L, while the value decreased to 1.28 μmol/L in compound heterozygous. These results are similar to those reported by other groups (Al-Hassnan et al. 2010), but lower than those reported by Blois (Blois et al. 2005) with a median value of 13.8 μmol/L for homozygous and of 2.6 μmol/L for compound heterozygous.

Plasma FFA and urine acylglycine profiles are helpful for the diagnosis, but they are not essential for it (Onkenhout et al. 1995; Bonafé et al. 2000; Kobayashi et al. 2007). However, the clearly elevated concentration of cis-4-decenoic acid (C10: 1n-9) (Table 1) in patient 8 with very mild elevation of acylcarnitines and normal C8/C10 ratio, contributed decisively to the diagnosis.

Similar to other studies, the prevalent mutation in our population is c.985A>G (p.K329E), which was found in 75% of the alleles with MCADD, but was not as high as previously anticipated (Yokota et al. 1991; Giroux et al. 2007; Waddell et al. 2006; Blois et al. 2005), although it is rather high if we consider other neonatal screening studies (Andresen et al. 2001; Maier et al. 2005; Nichols et al. 2008; Smith et al. 2010) as in the screened individuals it is usual to find milder mutations, while the common mutation c.985A>G is most frequently found in the clinically presenting patients.

It seems that there is no clear relationship between genotype and phenotype (Andresen et al. 1997; Lehotay et al. 2004; Waddell et al. 2006; Hsu et al. 2008), but patients homozygous for the common mutation have higher levels of C8 (Andresen et al. 2001; Waddell et al. 2006; Smith et al. 2010), and it is associated with a greater predisposition to suffer decompensation in situations of metabolic stress (Arnold et al. 2010). Thus, compound heterozygous for the prevalent c.985A>G mutation and a milder mutation in the other allele may have a risk reduction due to their greater residual enzyme activity (Lehotay et al. 2004). Among our population patient 4, homozygous for the c.985A>G, mutation died at the age of 2 years in the context of a severe intercurrent illness. The remaining patients, including his brother, who is also homozygous for c.985A>G, have not suffered any episode of metabolic decompensation. Therefore, the control of environmental factors seems to be essential, as they could play an important role in the clinical development and in the expressiveness of the MCADD (Gregersen et al. 2008).

In addition to the common mutation, five other mutations have been detected in our group of patients. The c.799G>A (p.G267R) mutation has been previously reported in symptomatic patients (Yokota et al. 1991; Andresen et al. 1997; Zschocke et al. 2001). Glycine 267 is highly conserved in humans and other organisms and are found at the equivalent positions in human short-chain and branched-chain acyl-CoA dehydrogenases (Zschocke et al. 2001). Expression studies in *Escherichia coli* revealed a decrease of the enzyme activity that could be increased by co-overexpression of the GroESL chaperonins (Andresen et al. 1997).

Mutation c.199T>C (p.Y67H) found in patient 8, has also been previously reported, and is the second most prevalent mutation of MCADD (Andresen et al. 2001). This mutation has never been identified in patients with a clinical phenotype, but in several newborns identified by neonatal screening in the United States, Australia, and Germany (Maier et al. 2005; Smith et al. 2010; Zschocke et al. 2001). These newborns had a relatively mild acylcarnitine profile, like our patient 8, that was compound heterozygous for this and for the common mutation and only presented a slight elevation of metabolites. Expression studies of the p.Y67H mutation in *E. coli* revealed that the biogenesis and/or stability are compromised (Andresen et al. 2001), but the catalytic activity of the enzyme was minimally affected (O'Reilly et al. 2004). However, there is several reason to believe that this mutation is not clinically neutral, particularly at high body temperatures (O'Reilly et al. 2004).

In addition, two novel mutations c.245G>C (p.W82S) and c.542A>G (p.D181G) were identified. Expression studies of these mutations have not been done, but several lines of evidence suggest that they may be pathogenic: (1) the nucleotide changes were not present in more than 100 control chromosomes analyzed, (2) tryptophan in position 82 is a residue highly conserved among different species and among other acyl-CoA dehydrogenases (http://coot.embl.de/PolyPhen/), (3) in addition, p.W82S is homologous to the p.G101R mutation in GCDH protein, which may indicate that changes at this position of acyl-CoA dehydrogenase proteins are not tolerated. Concerning p.D181G mutation, its pathogenecity is uncertain and further studies are necessary to elucidate whether it could be disease causing.

From this study, and in agreement with other authors (Pollitt and Leonard 1998; Blois et al. 2005), we can conclude that NBS for MCADD must be strongly recommended due to its relatively high incidence coupled with an easy and specific detection and simple treatment.

Sypnosis

This study presents the first published data about neonatal screening for MCADD and evolution of the diagnosed patients in Spain (region of Galicia) describing the prevalence of mutations, two novel mutations and the correlation phenotype–genotype.

Ethical Statement

- All the authors have contributed equally to the planning and execution of this work.
- M.L. Couce serves as guarantor for the article, accepts full responsibility for the work and the conduct of the study, had full access to the data, and controlled the decision to publish.
- Competing interest statement: All authors declare that they have no competing interests.
- The authors confirm independence from the sponsors; the content of this article has not been influenced by the sponsors.
- The patients' parents were fully informed about this study and gave informed voluntary consent to participation.

The authors

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