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ORIGINAL ARTICLE

Carnitine transporter and holocarboxylase synthetase deficiencies in The Faroe Islands

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Summary Carnitine transporter deficiency (CTD) and holocarboxylase synthetase deficiency (HLCSD) are frequent in The Faroe Islands compared to other areas, and treatment is available for both disorders. In order to evaluate the feasibility of neonatal screening in The Faroe Islands we studied detection in the neonatal period by tandem mass spectrometry, carrier frequencies, clinical manifestations, and effect of treatment of CTD and HLCSD. We found 11 patients with CTD from five families and 8 patients with HLCSD from five families. The natural history of both disorders varied extensively among patients, ranging from patients who presumably had died from their disease to asymptomatic individuals. All symptomatic patients responded favourably to supplementation with L-carnitine (in case of CTD) or biotin (in case of HLCSD), but only if treated early. Estimates of carrier frequency of about 1:20 for both disorders indicate that some enzyme-deficient individuals remain undiagnosed. Prospective and retrospective tandem mass spectrometry (MS/MS) analyses of carnitines from neonatally obtained filter-paper dried blood-spot samples (DBSS) uncovered 8 of 10 individuals with CTD when using both C_0 and C_2 as markers (current algorithm) and 10 of 10 when using only C_0 as marker. MS/MS analysis uncovered 5 of 6 patient with HLCSD. This is the first study to report successful neonatal MS/MS analysis for the diagnosis of HLCSD. We conclude that CTD and HLCSD are relatively frequent in The Faroe Islands and are associated with variable clinical manifestations, and that diagnosis by neonatal screening followed by early therapy will secure a good outcome.

Abbreviations

CTD	carnitine transporter deficiency
DBSS	filter-paper dried blood-spot samples
HLCS	holocarboxylase synthetase
HLCSD	holocarboxylase synthetase deficiency
MS/MS	tandem mass spectrometry

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Introduction

The population of The Faroe Islands, a geographically isolated group of islands in the North Atlantic, originates mainly from colonization by a small number of Norwegians about 1000 years ago. Thus, a founder effect probably explains the relatively frequent occurrence of some heritable disorders such as cystic fibrosis and glycogen storage disease type IIIA among the Faroese (Santer et al 2001). We observed that carnitine transporter deficiency (CTD) and holocarboxylase synthetase deficiency (HLCSD) were also quite frequent among patients referred from the islands to our department for diagnosis and treatment.

Carnitine transporter deficiency (CTD; OMIM 212140) is an autosomal recessively inherited disorder of fatty acid β-oxidation caused by mutations in *SLC22A5* (Lamhonwah

et al 2002; Nezu et al 1999; Wang et al 1999). The transporter is found in the plasma membranes of myocytes, renal tubular cells and fibroblasts. Its deficiency results in a gross reduction of carnitine stores, impaired β-oxidation of long-chain fatty acids, and decreased ketogenesis. Clinical manifestations include muscular weakness, hypertrophic cardiomyopathy, sudden death and, in early childhood, hypoketotic hypoglycaemia (Christensen et al 2000; Pierpont et al 2000; Stanley et al 1991). A 95A>G mutation in *SLC22A5* leading to a N32S substitution in the OCTN2 protein has been described in children from The Faroe Islands and Japan, and other populations (Christensen et al 2000; Lamhonwah et al 2002; Wang et al 1999).

Holocarboxylase synthetase (HLCS; OMIM 609018) catalyses the incorporation of biotin into four carboxylases, and HLCSD renders all four carboxylases deficient, hence the synonym multiple carboxylase deficiency (OMIM 253270). HLCSD is caused by mutations in *HLCS* (Suzuki et al 1994), and a common European mutation (IVS10+5G>A) has been described (Yang et al 2001). The most common clinical presentation is that of an infant with breathing difficulty, severe erythematous eczema and a potentially lethal metabolic decompensation with encephalopathy, acidosis and dehydration (Morrone et al 2002; Narisawa et al 1982).

In order to evaluate the feasibility of neonatal screening in The Faroe Islands, we studied carrier frequencies, diagnosis by tandem mass spectrometry (MS/MS), clinical manifestations, and effect of treatment of CTD and HLCSD.

Methods

All patients diagnosed biochemically since 1995 in the Department of Clinical Genetics, Rigshospitalet, Copenhagen, and the Department of Pediatrics, National Hospital, Tórshavn, were included in the study. Biochemical and clinical data were retrieved from clinical files and from regular follow-up by the authors (A. L., F. S. and F. J.). Patients with CTD were prescribed L-carnitine at a dose of 100–200 mg/kg per day in two divided dosages with the aim to keep plasma free carnitine above 20 μmol/L. Patients with HLCSD were prescribed biotin at 5–80 mg daily depending on clinical and biochemical outcome. Normalized excretions of 3-methylcrotonylglycine, 3-hydroxypropionic acid and methylcitrate in urine were regarded as biochemical markers of satisfactory biotin dosage. All patients were seen every 6 months locally and annually in Rigshospitalet.

Biochemical analyses

Biochemical analyses of urine organic acids and urine and plasma carnitines as well as studies in fibroblasts of carnitine uptake and activities of fatty acid β-oxidation were performed as described previously (Christensen and Vikre-Jorgensen 1995; Christensen et al 1981). Reference ranges in plasma were 30–73 μmol/L for total carnitine and 19–60 μmol/L for free carnitine. All other reference ranges are mentioned in Tables 1–4.

Tandem mass spectrometry

Tandem mass spectrometry of filter-paper dried blood-spot samples (DBSS) was performed either as prospective neonatal screening or as selective retrospective analysis of DBSS stored at −25°C from molecularly confirmed cases of CTD and HLSCD. Prospective extended neonatal MS/MS screening has been done since 010202 in approximately 2900 of 3600 Faroese newborns in a project setting. For CTD, disease markers were free carnitine (C_0) < 7.8 μmol/L and acetylcarnitine (C_2) < 6.6 μmol/L. Disease markers for HLCSD were C_5OH > 1.0 μmol/L and C_5OH/C_0 ratio > 0.028. All dried blood-spot samples were taken 4–8 days *post partum*. Acylcarnitines were measured using flow-injection electrospray MS/MS on an API 2000 tandem mass spectrometer equipped with a PerkinElmer Series 200 micro pump and a CTC Analytics HTS PAL System autosampler. Analytes from 3 mm diameter discs were extracted and butylated using the PerkinElmer Neogram amino acid and acylcarnitine MS/MS kit (cat. no. MS-8970 PerkinElmer Life Sciences, Norton, OH, USA). Multiple reaction monitoring was used to measure relevant acylcarnitines and isotope internal standards.

Molecular-genetic analyses

Genomic DNA from patients was isolated from peripheral leukocytes using standard methods. Both the 95 A>G mutation in *SLC22A5* (GenBank AC004628) and the IVS10+5G>A mutation in *HLCS* (GenBank BC060787) were tested using primers designed by Applied Biosystems (primers-by-design), using dual labelling (VIC and FAM) of the probes, allowing the simultaneous analysis of both alleles. The assay was performed as recommended by the manufacturer using the ABI PRISM 7000 Sequence Detection System.

To study carrier frequencies of CTD and HLSCD in the Faroese population, DNA was extracted from DBSS obtained 4–8 days *post partum* from 247 babies born consecutively between 231000 and 310501 on The Faroe Islands and referred for neonatal screening. A 3 mm blood-spot was punched out from the DBSS and heated at 75°C in 20 μl 0.2 mol/L NaOH for 5 min. Subsequently 180 μl 40 mmol/L Tris-HCl was added. Molecular-genetic analyses were performed as above using 5 μl of the extracted DNA.

Table 1 CTD families with members homozygous for the 95 A>G mutation in *SLC22A5*

CTD family		Clinical/paraclinical details	Clinical status at last follow-up (period of follow-up)	Plasma free/total carnitine at presentation last follow-up[a]	Carnitine dose at initial/last follow-up (mg/kg per day)
1	Proband	Diagnosed because of hypoglycaemia, hepatomegaly and hypotonia at age 4 months. Normal ECHO. Asymptomatic on L-carnitine 120 mg/kg per day until age 4 years when muscular weakness was noted. L-Carnitine was increased to 200 mg/kg/day, no effect. Cardiac and intellectual functions normal. He can walk and run short distances and uses wheelchair for longer distances. At age 8 months L-carnitine was not given, resulting in prompt decrease in plasma free carnitine (2.5 μmol/L) without clinical signs Carnitine uptake (fibroblasts): 0.007 pmol/min per mg (0.22–0.64).	Slight muscular weakness (11 years)	Initial: 2.5/2.5 Last: 34/41	120/200
	Younger sister	Laryngitis at age 3 years with respiratory failure, development of cerebral palsy and 4 months later encephalopathy with cerebral oedema and death. She was diagnosed retrospectively via mutation studies of blood from Guthrie card	Dead	–	–
2	Proband	Febrile illness with encephalopathy, seizures, cardiac failure and death at age 14 months. Postmortem studies showed dicarboxylic acids in urine and low carnitine in Guthrie card. She had been treated with pivampicillin	Dead	–	–
	Half-brother	Investigated at age 7 days because of positive family history, and low carnitine was found. Clinically normal. Normal ECHO. L-Carnitine was not given for 5 days, resulting in decrease in plasma free carnitine (2.5 μmol/L) without clinical signs.	Normal (7 years)	Initial: 2.9/6.7 Last: 43/57	100/140

(*Continued on next page*)

Table 1 (*Continued*)

CTD family		Clinical/paraclinical details	Clinical status at last follow-up (period of follow-up)	Plasma free/total carnitine at presentation last follow-up[a]	Carnitine dose at initial/last follow-up (mg/kg per day)
3	Proband	Failure to thrive at age 5 months. Normal development on L-carnitine. Urine excretion of 3-OH-dicarboxylic acids led to mutation studies.	Normal (3 years)	Initial: NA[b] Last 47/57	180/150
	Brother	Diagnosed at 4 years because of positive family history. Slow weight gain in early life. Now hypermobile, otherwise normal.	Normal (3 years)	Initial: 1.7/2.6 Last: 42/55	105/125
	Mother	Diagnosed at 29 years because of positive family history. Reduced physical strength in childhood/adolescence. ECHO normal; two normal pregnancies	Normal (3 years)	Initial: 3.5/4.4 Last: 40/53	110
	Mother's brother	Diagnosed at 19 years because of positive family history. Encephalitis-like episode at age 2 years: full recovery. Complains of fatigue. ECHO normal. Less tired after supplementation with L-carnitine.	Normal (3 years)	Initial: 2.1/2.4 Last: 24/34	175
4	Proband	Asymptomatic. Diagnosed via neonatal MS/MS screening	Normal (0.5 years)	Initial: 2.4/2.9 Last: 22/–	100
	Sister	Diagnosed at 4 years because of positive family history and remains asymptomatic.	Normal (0.5 years)	Initial: 3.5/– Last: 31/–	100

[a]Reference range (μmol/L): free carnitine 19–60; total carnitine 30–73
[b]NA, not available

Table 2 HLCSD families with members homozygous for the IVS10+5G>A mutation in *HLCS*

HLCSD family		Clinical/ paraclinical details	Status at last follow-up (period of follow-up)	Urine organic acids at presentation with elevated excretion	Dose of biotin (mg/day)	Urine 3-hydroxyisovaleric acid during therapy (μmol/mmol creatinine)
1	Proband	This boy presented with encephalopathy and seizures at age 3 months. No eczema. Prompt response to biotin supplementation.	Normal apart from slight fine motor difficulties (11 years)	3-Hydroxyisovaleric acid 3-Methylcrotonylglycine	6	30–100
2	Proband	After vaccination at age 3 months, this girl developed respiratory insufficiency, severe eczema and hypotonia. Prompt response to biotin supplementation.	Normal (6 years)	3-Hydroxyisovaleric acid 3-Methylcrotonylglycine 3-hydroxypropionic acid Methylcitrate	10	300–500
	Father	Diagnosed at age 26 years because of positive family history. Well and without eczema during childhood and adolescence, but increasingly tired in adulthood. He feels less tired after biotin supplementation.	Normal (6 years)	3-Hydroxyisovaleric acid 3-Methylcrotonylglycine Methylcitrate	60	50–100
	Sister	Diagnosed by family history/neonatal MS/MS screening	Normal (1 months)	No data available	10	No data available
3	Proband	Episodic tachypnoea, eczema, fatigue and developmental delay were noted from age 2 years in this boy. He became more active after biotin supplementation, but there was no effect on psychomotor functions.	Psychomotor retardation (4 years)	3-Hydroxyisovaleric acid 3-Methylcrotonylglycine 3-Hydroxypropionic acid Methylcitrate	80	100–250

(*Continued on next page*)

Table 2 (*Continued*)

HLCSD family		Clinical/ paraclinical details	Status at last follow-up (period of follow-up)	Urine organic acids at presentation with elevated excretion	Dose of biotin (mg/day)	Urine 3-hydroxyisovaleric acid during therapy (μmol/mmol creatinine)
	Sister	Because of the brother's diagnosis, the mother was given biotin 20 mg daily from week 30 of pregnancy. After birth the girl continued biotin 20 mg daily. She remains asymptomatic.	Normal (3.5 years)	–	20	50–350
4	Proband	Diagnosed by neonatal MS/MS screening, the result of which was reported late at three months. At this time she already had eczema, which disappeared after biotin supplementation. During an intercurrent illness she did not get biotin and promptly developed eczema, hypoglycaemia and dehydration.	Normal (3 years)	3-Hydroxyisovaleric acid 3-Methylcrotonylglycine Methylcitrate	20	250
5	Proband	This boy had a severe, haemorrhagic varicella infection at age 3 years, and at age 7 years developmental delay was noted and diagnosis was made. There was no effect of biotin supplementation on psychomotor functions.	Psychomotor retardation (1 year)	3-Hydroxyisovaleric acid 3-Methylcrotonylglycine 3-Hydroxypropionic acid Methylcitrate	20	No data available

Table 3 Levels of free carnitine and acetylcarnitine determined by prospective/retrospective MS/MS in DBSS

CTD family		Genetic status	Free carnitine (>7.8 μmol/L with median at 27.8)	Acetylcarnitine (>6.6 μmol/L with median at 18.6)	Correct diagnosis at DBSS MS/MS analysis
1	Proband	Homozygous	3.5	5.5	Yes
	Sister	Homozygous	3.4	6.0	Yes
2	Proband	Homozygous	4.4	6.2	Yes
	Half-brother	Homozygous	4.0	9.0	No
3	Proband	Homozygous	2.0	4.9	Yes
	Brother	Homozygous	1.0	4.2	Yes
	Mother's brother	Homozygous	2.9	2.8	Yes
4	Proband	Homozygous	3.7	5.9	Yes
	Sister	Homozygous	4.8	8.21	No
5	Proband	95 A≥G/?[a]	5.6	6.1	Yes

[a]Carnitine uptake in fibroblasts 0.051 pmol/min per mg (reference range 0.32–0.79)

Table 4 Levels of C_5OH and C_5OH/C_0 determined by prospective/retrospective MS/MS in DBSS

HLCSD family		Genetic status	C_5OH (<1.0 μmol/L with median at 0.152)	C_5OH/C_0 (<0.028 with with median at 0.0053)	Correct diagnosis at DBSS MS/MS analysis
1	Proband	Homozygous	1.45	0.049	Yes
2	Proband	Homozygous	1.11	0.059	Yes
	Sister	Homozygous	1.23	0.033	Yes
3	Proband	Homozygous	0.16	0.005	No
	Sister	Homozygous	0.6	0.021	No (substituted with biotin)
4	Proband	Homozygous	1.63	0.049	Yes
5	Proband	Homozygous	1.28	0.0283	Yes

The project was approved by the local ethics committees in Copenhagen, Denmark and Tórshavn, The Faroe Islands ((KF) 01–172/02).

Results

CTD

Four CTD families with 10 patients homozygous for the 95 A>G mutation in *SLC22A5* are known in The Faroe Islands. Clinical and biochemical details are shown in Table 1. One child was diagnosed by prospective neonatal MS/MS screening and the others clinically or by family screening.

Apart from these 10 children, one child with CTD from a fifth family was diagnosed by prospective neonatal MS/MS screening (family 5 in Table 3). Studies of carnitine uptake in fibroblasts documented CTD; the child is heterozygous for the 95 A>G mutation, but the other mutation is unknown.

HLCSD

Five HLCSD families with 8 patients are known in The Faroe Islands, all of whom are homozygous for the IVS10+5G>A mutation in *HLCS*. Clinical and biochemical details are shown in Table 2. All continue to excrete elevated amounts of 3-hydroxyisovaleric acids despite treatment (Table 2). One child was diagnosed by prospective neonatal MS/MS screening, and one other child (known to be at risk, as she was from HLCSD family 2) also had an abnormal result on prospective neonatal MS/MS screening (see Table 4).

Studies of carrier frequency

Carrier status for CTD could be tested reliably in 235 of 247 DBSS (95%). We found 13 heterozygous and no homozygous samples. Thus, carrier frequency of CTD in The Faroe Islands is 1:18 or 5.5% (95% confidence interval 2.6–8.4%). Assuming survival of all homozygous individuals, this corresponds to a calculated prevalence of the disease of 1:1300 (95% confidence interval 1:560–1:5700).

Regarding HLSCD, 217 of 246 DBSS (88%) could be tested: 10 samples were heterozygous and none was homozygous. Thus, carrier frequency for HLCSD is 1:22 or 4.6% (95% confidence interval 1.9–7.4%), corresponding to a prevalence of 1:1700 (95% confidence interval 1:730–1:12000).

Determination of carnitine and acylcarnitines by MS/MS

After the diagnosis of 4 children with CTD and HLCSD by prospective neonatal MS/MS screening, we did retrospective MS/MS analysis of DBSS from 5 children with molecularly confirmed HLCSD and from 8 CTD patients homozygous for the 95 A>G mutation. The results are shown in Tables 3 and 4, which include the prospectively diagnosed children. Using both free carnitine and acetylcarnitine as disease markers, 8 of 10 CTD patients could be diagnosed, while all 10 CTD patients could be diagnosed if only free carnitine was used. Two heterozygous CTD family members were also examined, both of whom had low-normal C_0 and C_2 compared to normal medians. Five of seven HLCSD patients could be diagnosed using C_5OH and C_5OH/C_0 as disease markers, but one of the two HLCSD patients not diagnosed was receiving biotin when the DBSS was obtained.

Prospective neonatal MS/MS screening has been done since 010202 in approximately 2900 Faroese newborns (about 80% of newborns). No false positive results were obtained for the HLCSD marker. Four cases with raised C_5OH turned out to be caused by 3-methylcrotonyl-CoA carboxylase deficiency in newborns or their mothers. When using carnitine as a marker, one child had a raised level, which proved to be a false positive result; there has been no false positive result concerning low carnitine.

In the period from 010202 to 010107, where prospective neonatal MS/MS screening was done, 5 children with CTD and 3 children with HLCSD were diagnosed among approximately 3600 children born in The Faroe Islands. Two children with CTD were diagnosed by neonatal MS/MS screening, 2 by a positive family history in whom neonatal screening was normal, and one because of clinical symptoms; in the latter case the child's family had not consented to neonatal MS/MS screening, which retrospectively was abnormal. Concerning HLCSD, 2 children were diagnosed by neonatal MS/MS screening, and in one child neonatal screening was normal because of previous supplementation with biotin. The overall incidences of CTD and HLCSD among newborns during this period were 1:720 and 1:1200, respectively, both within the expected range as judged from the calculated frequencies above.

Discussion

We have documented that CTD and HLCSD are prevalent in The Faroe Islands due to high carrier frequencies of about 1:20. The prevalences of CTD and HLCSD were 1:1300 and 1:1700, respectively, and the incidences were 1:720 and 1:1200, respectively. Prevalence data are not available from other European countries. As suggested for glycogen storage disease type IIIA, the high carrier frequencies for CTD and HLCSD are probably due to founder effects in this geographically isolated population (Santer et al 2001).

The 95 A>G mutation in *SLC22A5* and the IVS10+5G>A mutation in *HLCS* are known outside The Faroe Islands. Both mutations are thought to be pathogenic (Lamhonwah et al 2002; Yang et al 2001). The N32 S substitution in the carnitine transporter involves a highly conserved residue and is thought to interfere with the topology or conformation of the protein. A patient with the N32 S substitution (Lamhonwah et al 2002) had a residual carnitine uptake of 20%, somewhat higher than the 3% uptake in fibroblasts from patient 1 (Table 1). However, there does not seem to be any correlation between residual uptake and severity of clinical manifestations (Lamhonwah et al 2002).

The IVS10+5G>A mutation in *HLCS* has been described previously in Swedish, Danish, French, and German patients, and haplotype analysis indicates that this is a founder mutation in European patients with HLCSD (Suzuki et al 2005; Yang et al 2001). A Swedish child homozygous for the mutation had a residual HLCS activity of 4%, but the K_m for biotin was only slightly increased (Sakamoto et al 2000). The mutation was shown to disrupt normal splicing, but only a moderate decrease of the amount of normal *HLCS* mRNA could be shown. This observation and the finding of asymptomatic patients suggest that this mutation is relatively mild (Sakamoto et al 2000).

Clinical manifestations vary for both CTD and HLCS. The presentation of CTD ranged from failure to thrive, hypotonia and hypoglycaemia to death in cardiac failure, while most of their homozygous family members were asymptomatic despite similarly low plasma carnitine levels. Clearly, the level of plasma carnitine does not correlate with clinical symptoms, which may be because measurements of plasma carnitine levels not necessarily reflect tissue carnitine levels (Evans and Fornasini 2003). To provoke clinical symptoms, modifying factors such as intercurrent infection, poor feeding and vaccination may also be of importance. Our data (Table 1) document that as little as one day without supplementation will lead to a drop of plasma carnitine to pretreatment level. Daily supplementation with L-carnitine in at least two divided doses is therefore vitally important, and we have had no deaths or episodes of encephalopathy, cardiomyopathy or hypoglycaemia among patients on L-carnitine supplementation. All children with CTD on L-carnitine supplementation have also had normal psychomotor development.

Clinical presentations of HLCSD included respiratory insufficiency, hypotonia, mental retardation and acute-onset encephalopathy. Eczema, a classical sign, was present in only 3 cases (in one patient as the only manifestation), and it was absent even in the very late-presenting boy from HLCSD family 3. Although some individuals remain asymptomatic and biochemical data seem to indicate that the *HLCS* IVS10+5G>A mutation is mild (Sakamoto et al 2000), it

may cause both early (e.g. family 4) and severe (e.g. families 3 and 5) manifestations. All patients with normal intellectual development at diagnosis responded to biotin supplementation with disappearance of clinical symptoms, but they continued to excrete elevated amounts of 3-hydroxyisovaleric acid in their urine. As illustrated by HLCSD family 4, the prompt recurrence of eczema and hypoglycaemia after discontinuation of biotin supplementation underscores the importance of compliance.

Prevalence data indicate that 30–35 individuals could be affected with each disorder in the Faroese population of 48 000 inhabitants, i.e. many more than the number on which this report is based. Some patients probably remain asymptomatic and undiagnosed, while others could have died without recognition of their disease. Recently Vijay and colleagues reported four asymptomatic mothers diagnosed because of low carnitine concentrations in their newborns; many individuals with CTD may thus be asymptomatic (Vijay et al 2006), analogously to diseases such as medium-chain acyl-CoA dehydrogenase (MCAD) and 3-methylcrotonyl-CoA carboxylase deficiencies (3-MCC). Only whole-population screening and thorough follow-up of treated and untreated populations will elucidate the natural history of and impact of treatment on disorders such as CTD, HLCSD, MCAD and 3-MCC deficiency.

Until such data become available, the frequency of CTD and HLCSD and their favourable response to therapy make both disorders excellent targets for neonatal screening. Experience from other laboratories indicates that only about 50% of children with CTD can be diagnosed by neonatal MS/MS screening (Wilcken et al 2001). Our prospective and retrospective data of MS/MS analysis of free carnitine and acetylcarnitine in DBSS suggest that such screening for CTD will be more efficient in The Faroe Islands, especially if only free carnitine is used as marker. Screening for HLCSD using MS/MS, to our knowledge not reported in the literature, also seems to be effective (Table 4). The one HLCSD patient we could not diagnose using MS/MS had very low C_5OH and C_5OH/C_0 ratio, and could only have been detected by a very low cut-off resulting in many false positive cases. With current cut-off values, no false positives were found during screening for both CTD and HLCSD. Neonatal screening using direct mutation testing of DNA from a DBSS offers an alternative but more expensive set-up to the one currently used. In addition, newborns with compound heterozygosity (such as the child in CTD family 5) would escape detection by DNA analysis.

Regardless of the method used, neonatal screening with institution of early and lifelong therapy will secure a good outcome for patients with these frequent disorders in The Faroe Islands.

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