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RESEARCH REPORT

Phenotype–Genotype Discrepancy Due to a 5.5-kb Deletion in the *GALT* Gene

Ariadna González-del Angel • José Velázquez-Aragón • Miguel A. Alcántara-Ortigoza • Marcela Vela-Amieva • Nancy Hernández-Martínez

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Abstract Classical galactosemia is an autosomal recessive inborn error of metabolism caused by a deficiency of the galactose-1-phosphate uridyltransferase (GALT). More than 200 mutations have been described in the *GALT* gene. A 5.5-kb *GALT* deletion, first described in patients of Ashkenazi Jewish ancestry, may lead either to an erroneous genotype assignment of classical galactosemia or to discrepancies with parental genotypes and the expected biochemical phenotype. The presence of the 5.5-kb deletion was examined in 27 Mexican nonrelated families with at least one child with reduced GALT activity in erythrocytes and it was detected in the 5.5% ($n = 3$) of the 54 alleles tested. The first molecular studies in three of our families showed that the genotypes of the parents were inconsistent with those of their children, which were considered initially as homozygous p.N314D-Duarte 2, but after analyzing for the presence of the 5.5-kb deletion, were reassigned as compound heterozygotes [5.5-kb deletion] + [p.N314D-Duarte 2]. Identification of the 5.5-kb deletion in Mexican patients suggests that this mutation might not be exclusive to a given ethnic group and should be tested in other populations, especially when there is a discrepancy between the genotypes of patients and parents or by incongruence between biochemical phenotype and *GALT* genotype. Establishing a genotype–phenotype correlation for the 5.5-kb *GALT* deletion and determining the appropriate management will require additional studies in patients with a G/G genotype bearing the 5.5-kb *GALT* deletion.

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Introduction

Classical galactosemia is an autosomal recessive inborn error of metabolism caused by a deficiency of the galactose-1-phosphate uridyltransferase enzyme (GALT; EC 2.7.7.12). More than 200 different mutations have been described in the *GALT* gene (ARUP galactosemia database http://arup.utah.edu/database/galactosemia/GALT_welcome.php; Calderon et al. 2007), and a relationship between genotype, enzymatic activity, and phenotype has been described in the literature (Bosch 2006).

Individuals with the p.N314D variant are no longer diagnosed as galactosemic because p.N314D is now considered a polymorphism (Carney et al. 2009). This variant is in linkage disequilibrium with other variants that distinguish Duarte 1 and Duarte 2 alleles (Trbusek et al. 2001). Heterozygous individuals with a normal and a Duarte 2 allele (N/D2) have 75% of normal enzymatic activity and those homozygous for Duarte 2 (D2/D2) have 50% of normal activity. Furthermore, individuals with a classical galactosemia mutation and a D2 allele (G/D2) have approximately 25% of normal activity. It is known that G/D2 children on regular diet have shown erythrocyte galactose-1-phosphate concentration in normal values but increased concentrations of some galactose metabolites as galactitol and galactonate; these elevations do not cause

clinical manifestations of classical galactosemia at least during early childhood, so it seems that D/G cases do not need a strict galactose-restricted diet (Ficicioglu et al. 2010).

We have performed molecular testing of 27 families from Mexico with at least one child with reduced GALT activity in erythrocytes. In three of these families in which the patient was shown to be homozygous for the D2 allele, only one parent was a D2 carrier, suggesting an exception to the Mendelian recessive autosomal pattern of inheritance.

One possible explanation for the findings observed in these families is nonbiological paternity or uniparental disomy; both possibilities were discarded based on previous molecular analyses (data not shown). Another possibility is that one of the parents was a carrier of a large gene deletion, such as that described by Coffee et al. (2006), who reported a 5.5-kb deletion in the *GALT* gene. This deletion is complex because two fragments of the gene are absent: one is a 3,163-bp fragment at the 5′ extreme that includes the promoter, and the other is a 2,395-bp fragment at the 3′ extreme of the gene and it is conserved an internal 117-bp segment of *GALT* that contains part of exon and intron 8 (Coffee et al. 2006).

In patients with 5.5-kb deletion in the *GALT* gene, molecular analyses designed to determine the presence of a variant in the other allele may lead to an erroneous appearance of homozygosity. Thus, it is important to discard the possibility of a deletion in the *GALT* gene, because its presence could lead to a discrepancy between the biochemical phenotype and the apparent genotype, and create also apparent discrepancies between the genotype of patients and their parents (Coffee et al. 2006).

The aim of this work was to test whether the previously described 5.5-kb deletion in the *GALT* gene was present in the 27 Mexican families under study. A molecular analysis of the *GALT* gene of these families was performed in our laboratory previously to identify G, D1, and D2 alleles; the genotypes of 19 of the 27 cases were previously reported by our group (Velázquez-Aragón et al. 2008).

Patients and Methods

All the Mexican families included in this study were unrelated and had at least one child with reduced GALT activity in erythrocytes. Written informed consent was obtained from all participants.

DNA was purified from peripheral blood leukocytes, dried blood spots in Güthrie cards, or buccal cells using the salting-out method (Puregene Blood kit, Gentra Systems, Minneapolis, MN, USA).

The presence of the 5.5-kb deletion in the *GALT* gene was detected by polymerase chain reaction (PCR) as described by Coffee et al. (2006). Briefly, a three-primer set was used to amplify either the junction of the breakpoints of the deletion or the final portion of the region involved in the deletion and the 3′ near region of the 3′ breakpoint. Using this strategy, we would observe a 680-bp amplicon in the absence of the deletion and a 500-bp amplicon if the deletion were present.

Results and Discussion

Twenty-seven Mexican families with at least one child with reduced GALT activity in erythrocytes were examined for the presence of the 5.5-kb deletion in the *GALT* gene. This deletion was found in three alleles in three different families, representing 5.5% of the 54 alleles tested. A molecular analysis of the *GALT* gene in the patients' families showed discrepancies in these three families between the genotype of patients and their parents.

Patient one was first considered homozygous for the D2 variant, but after analysis for the 5.5-kb deletion was reassigned as a compound heterozygote [del 5.5 kb] + [p.N314D]. At 2 weeks of age, this male patient showed a normal level of galactose in the blood (<14 mg/dL), but exhibited low GALT activity in a qualitative enzymatic activity test (New England Newborn Screening Program). Provision of breastfeeding was suspended and the patient was placed on a restricted galactose diet based on a soya milk formulation. On physical examination, this patient showed only incipient bilateral congenital cataracts. After 4 weeks on a restricted galactose diet (6 weeks of age), GALT activity tests yielded a result of 6 U/gHb (reference $\geq$ 18 U/Hb) and blood galactose levels were 0.3 mg/dL (reference < 2.0 mg/dL, Mayo Clinic Laboratory Service Report). At 4 years old, this patient showed no psychomotor delay or cataracts.

The second patient was also misgenotyped originally, and reassigned as a compound heterozygote [del 5.5 kb] + [p.N314D]. The case is a male who, at 1 month of age, showed no symptoms but lacked detectable enzymatic activity in a Beutler test and had a blood galactose level of 2.8 mg/dL (reference < 14 mg/dL New England Newborn Screening Program). At 5 months of age, GALT activity was 5.5 U/gHb, which was considered moderately deficient (reference 3–18 U/gHb, Mayo Clinic Laboratory Service Report); at 6 months of age, GALT activity was 13.8 U/gHb and blood galactose was 0.1 mg/dL (reference < 2.0 mg/dL Mayo Clinic Laboratory Service Report). A diet based on a soya milk formulation and breastfeeding was given for the first 6 months of life, and was later changed to an unrestricted diet. At 11 months, this patient was asymptomatic.

The third male patient was also reassigned as a compound heterozygote [del 5.5 kb] + [p.N314D]. This

patient presented with only jaundice at 3 weeks of age, but a Beutler test failed to detect enzymatic activity. GALT activity at 1 month of age was 5.6 U/gHb (i.e., moderately deficient) and blood galactose was 2.3 mg/dL (reference 3–18 U/gHb and < 2.0 mg/dL, respectively, Mayo Clinic Laboratory Service Report) so a mild restricted galactose diet was instaured. At 8 months of age, the patient showed no neurological impairment.

The first molecular studies of the three families showed that the genotypes of the parents were inconsistent with those of their children, which were considered initially as homozygous D2/D2. In the first case, the father was D2/N and the mother was N/N. In the second and third cases, the genotypes of both fathers were N/N and the mothers were D2/D2. After analyzing for the presence of the 5.5-kb deletion, the mother of the first case and the fathers of the second and third cases were reassigned as heterozygous [del5.5 kb] + [N] (Fig. 1).

In 2006, Coffee et al. reported a discrepancy between the biochemical phenotype and the genotype of *GALT* in two families. The first case was genotyped as homozygous p.N314D with 35% of normal activity of GALT, which is more consistent with a D2/G phenotype than a D2/D2 phenotype. The father was apparently homozygous for the variant Los Angeles, which is expected to show an elevation in enzymatic activity; however, his enzymatic activity was 51% of normal and the mother had the genotype D2/N. In the second family, the patient showed an apparent homozygosity for the p.N314D variant, but the biochemical activity was more consistent with a D2/G phenotype. No mutations were identified in the mother, but her biochemical activity was consistent with a G/N genotype.

In these two families, Coffee et al. suspected a large deletion in *GALT* that had been previously reported (Elsas and Lai 1998). A subsequent analysis showed that the two patients and their parents with the genotype–phenotype discrepancies were carriers of the 5.5-kb deletion in the *GALT* gene. The conclusion was that the presence of this deletion can lead to the appearance of homozygosity if it is present with a mutation on the other allele causing an erroneous assignment of genotype (Coffee et al. 2006), as occurred in our three Mexican families.

The 5.5-kb *GALT* gene deletion was previously reported by Elsas and Lai in four unrelated patients of Ashkenazi Jewish ancestry (Elsas and Lai 1998). In his work, Coffee identified seven alleles in six cases, including the two cases described above; some of these cases had Hispanic surnames, but the actual ethnic origins were not specific (Coffee et al. 2006). The three families reported in this

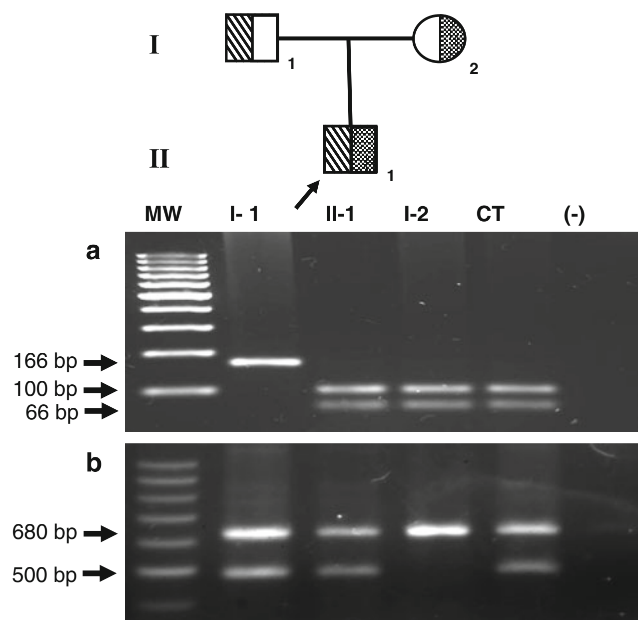

Fig. 1 Analysis of the p.N314D variant (**a**) and the 5.5 kb deletion (**b**) in the second case and his parents. (**a**) The p.N314D variant was analyzed by PCR and restriction with *AvaII* on 3% agarose gel electrophoresis. The p.N314D generates a restriction site for *AvaII* that cuts the 166 bp amplicon in two fragments of 100 bp and 66 bp. The proband (II–1) shows an apparent homozygosity with parental genotype discrepancy due to the absence of p.N314D in I–1. (**b**) Identification of the 5.5-kb deletion by PCR and 2% agarose gel electrophoresis in the same family. A heterozygous genotype with respect to the 5.5-kb *GALT* deletion, represented by a 500-bp amplicon from the deleted *GALT* allele and a 680-bp amplicon from the non-deleted *GALT* allele, was observed in the patient (II–1) and his father (I–1). The presence of only the 680-bp amplicon in the mother (I–2) rules out the presence of the deleted *GALT* allele. Both results in the second case reassigned him as a compound heterozygote [del 5.5 kb] + [p.N314D]. Lane MW: 100-bp DNA ladder. Lane CT: in (**a**) homozygous p.N314D DNA control, in (**b**) heterozygous 5.5-kb *GALT* deletion DNA control; lane (−): blank control.

article were considered Mexicans because they and their grandparents were born in Mexico; the three families also denied that they had different ethnic ancestries. Accordingly, we consider that this mutation might not be exclusive to a given ethnic group and should be tested in other populations, especially when there is a discrepancy between the genotypes of patients and parents or when the patients show discrepancies between biochemical phenotype and genotype, as has been described.

It is not possible to establish a genotype–phenotype correlation for the 5.5-kb deletion because the clinical manifestations of patients with this deletion have not been documented in the literature. Coffee et al. only described the biochemical phenotypes of the two cases previously mentioned. The phenotypes of the other four cases in whom a 5.5-kb deletion was identified were not mentioned (Coffee et al. 2006). Likewise, the phenotype of only one of the four cases reported by Elsas and Lai, was mentioned in a teenager homozygous for the 5.5-kb deletion who was reported to have neurological and ovarian impairment, despite early detection and intervention (Elsas and Lai 1998). In general, it is accepted that patients with G/D2 genotype are asymptomatic and this genotype is considered as benign (Ficicioglu et al. 2010); two of our three cases with the 5.5 Kb deletion had clinical manifestations in the neonatal period, one patient with incipient congenital cataracts and the other with transient jaundice, but we consider that these clinical manifestations were conditioned by other undetermined causes as both patient have a G/D2 genotype and were asymptomatic at 4 years and at 11 months of life, respectively.

The observation that the mothers of cases two and three are asymptomatic D2/D2 supports the idea that this is a polymorphic variant in the *GALT* gene and not a pathological mutation.

Interestingly, in the three patients where we found the 5.5-kb deletion of *GALT* gene on one allele, the p.N314D variant (D2) was observed on the other allele. Three such cases were also reported by Coffee et al., whereas one case, with clinical manifestations of classical galactosemia, reported by Barbouth et al. was a compound heterozygote [p.K285N] + [del 5.5 kb]. More patients with the 5.5-kb *GALT* gene deletion will need to be studied to establish whether this deletion is associated with other variants besides p.N314D and p.K285N (Coffee et al. 2006; Barbouth et al. 2006).

The frequency of the 5.5-kb deletion in the *GALT* gene in our sample (54 alleles) was 5.5%. Therefore, we suggest that a molecular analysis of this mutation must be conducted in the Mexican population in children identified with low GALT enzymatic activity by neonatal screening. Establishing a genotype–phenotype correlation for the 5.5-kb *GALT* deletion and determining the appropriate management will require additional studies on a larger population of patients that bear genotypes G/G with the 5.5-kb *GALT* deletion.

Take Home Message

The 5.5-kb *GALT* gene deletion was identified in Mexican patients with reduced GALT activity and it was responsible for discrepancies between genotypes of patients and their parents.

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OMIM 230400; Gene symbol GALT; E.C. 2.7.7.12; EntrezGene ID 2592.

Contributions of Individual Authors

José Velázquez-Aragón, Miguel Alcántara-Ortigoza and Ariadna González-del Angel wrote the first draft of the paper. Marcela Vela-Amieva and Ariadna González-del Angel collected the galactosemic patients and clinical data. José Velázquez-Aragón, Miguel Alcántara-Ortigoza, Nancy Hernández Martínez carried out the molecular analysis. José Velázquez-Aragón, Ariadna Gonzalez-del Angel and Miguel Alcántara-Ortigoza analyzed the data. Ariadna González-del Angel and Marcela Vela-Amieva planned, supervised and coordinated the work. All authors contributed to the final version of the paper.

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CASE REPORT

Cardiac Arrest in Kearns–Sayre Syndrome

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Abstract The prognosis of progressive ophthalmoplegia in patients with large-scale mitochondrial DNA deletions is highly variable and almost unpredictable. The risk to develop cardiac involvement and sudden cardiac death is strikingly high, especially in patients with Kearns–Sayre syndrome (KSS). The most typical cardiac complications of the disease are conduction defects, which usually begin with left anterior fascicular block with or without right bundle branch block (RBBB), progressing sometimes rapidly to complete atrioventricular block. Other cardiac manifestations reported are first or second degree of AV block, QT prolongation, torsades de pointes ventricular tachycardia, and rarely dilated cardiomyopathy. Most frequently syncope, sometimes even sudden cardiac death, is the first clinical sign of the cardiac disease in KSS. Due to these life-threatening cardiac conditions, patients should be carefully monitored for cardiac signs and symptoms and pacemaker implantation should be suggested early to avoid sudden cardiac arrest in KSS.

Here, we present two cases of KSS with life-threatening syncope due to complete atrioventricular block. To emphasize the importance of an early pacemaker implantation, we review the literature on cardiac complications in KSS in the last 20 years. In almost all of the reviewed cases, ophthalmoplegia or ptosis was present before the cardiac manifestations. In most of the cases, syncope was the first symptom of the cardiac involvement. There was no correlation between the age of the onset of the disease and the onset of cardiac manifestations.

With our current report, we increase awareness for life-threatening cardiac complications in patients with KSS.

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Introduction

Kearns–Sayre syndrome [KSS; (MIM 530000)] is a mitochondrial syndrome, in most cases caused by large scale mitochondrial DNA deletions or mitochondrial DNA depletion (Maceluch and Niedziela 2007). Multiple organ systems are affected in KSS. The syndrome is defined by the obligatory triad of onset before the age of 20 years, progressive external ophthalmoplegia, and pigmentary retinopathy. In addition, at least one of the following must be present: heart block, cerebellar ataxia, or cerebrospinal fluid protein greater than 100 mg/dL (Rowland et al. 1991). Cardiac manifestations in KSS are as high as 50% and sudden cardiac death reported in up to 20% (Chawla et al. 2008).

Here, we present two cases of KSS with life-threatening syncope due to complete atrioventricular block. Furthermore, we review the literature on KSS with a focus on cardiac complications to increase awareness for this life-threatening complication.

Case Reports

Patient 1

The male patient was born at term as the first child of healthy parents. For the first few years of life, he was healthy and had no clinical symptoms. From the age of 7, he was evaluated for growth delay and a failure to thrive despite normal caloric intake and age-appropriate nutrition. He developed a mild ptosis at the age of 10 years, and complained of easy fatigue. He had a normal IQ and followed regular mainstream education. Laboratory screening revealed a chronic lactic acidemia, elevated serum alanine levels, and GH deficiency. At the age of 11 years, he developed ophthalmoplegia and was diagnosed with reduced mitochondrial ATP production and a complex I deficiency in a surgical muscle biopsy. Southern blot for mtDNA deletions and sequence analysis of the mtDNA in blood revealed no abnormalities. The first cardiac evaluation at the age of 11 years revealed a structurally normal heart with good left ventricular function on the echocardiography. The electrocardiogram (ECG) showed left anterior fascicular pattern with normal PR and QTc intervals. He developed a severe failure to thrive and used a wheelchair for long distances. He was started on tube feeding to optimize his caloric intake combined with GH therapy. Based on the clinical features of KSS sequence, analysis of the mtDNA at the age of 12 years was performed again; this time on mtDNA extracted from muscle, showing the common 5 Mb mtDNA deletion. His cranial MRI at the age of 14 showed cortical atrophy and bilateral signal intensity changes of the posterior cerebellum. An EEG showed diffuse encephalopathy.

During cardiac follow-up at the age of 12 beside the known left anterior fascicular block an incomplete right bundle branch block (RBBB) appeared on the ECG, which remained unchanged up to the age of 15 years. At the age of 16 years, he lost his consciousness three times in a period of 1 month. ECG showed now complete RBBB and a left anterior fascicular block. Echocardiography remained normal. 24 hours Holter ECG showed sinus rhythm, first degree AV block, and the known bifascicular block. An event recorder was given to the patient to register a definitive cardiac cause during syncope, but a week later he was admitted to the intensive care unit and was resuscitated for a complete AV block with a very slow escape rhythm. A DDD pacemaker was implanted successfully.

Patient 2

The female patient was born at term as the first child of healthy parents. After a normal delivery and normal psychomotor development, she remained healthy and besides frequent infections she had no clinical symptoms. From the age of 10 years, she developed growth delay and a failure to thrive despite appropriate nutrition. She was evaluated for muscle weakness, night blindness, and progressive visual loss. She had a normal IQ and followed special education for children with visual loss. MRI showed changes consistent with Leigh syndrome. Ophthalmologic evaluation showed retinitis pigmentosa. She was further evaluated for a chronic lactic acidemia. EMG showed symptoms of myopathy. She was diagnosed with a reduced mitochondrial ATP production and a complex I deficiency in a surgical muscle biopsy. The complex I deficiency was also detected in cultured skin fibroblasts. Southern blot for mtDNA deletions, sequence analysis of the mtDNA in blood, and sequence analysis of the structural nuclear genes of complex I revealed no abnormalities. At that time, KSS was not suspected. At the age of 11, the first cardiac evaluation showed a normal ECG with normal conduction intervals. Echocardiography showed a borderline left ventricular hypertrophy. She developed diabetes at the age of 12 and ptosis at the age of 14 years. In the same year, she presented two episodes of syncope and the ECG showed complete AV block. A pacemaker was implanted with success. She developed bilateral ophthalmoplegia at the age of 15 years. Based on the clinical features of KSS, sequence analysis of the mtDNA was performed in muscle showing the common 5 Mb mtDNA deletion.

Review of the Reported Cases

To summarize the experience in the last two decades, we reviewed the literature on KSS and cardiac complications. A summary of 16 clinical reports on the age of onset, the presentation of cardiac manifestation and the outcome in KSS is given in Table 1. The reported median age of presentation of cardiac manifestations was at the age of 28 years, ranging between 9 and 47 years (Table 1). There was no correlation between the age of the onset of the disease and the onset of cardiac manifestations (not shown in the table). The median time reported between newly diagnosed cardiac conduction defect and first symptoms of cardiac complications (Table 1) was 5.5 years (2–9 years). In one case, the first symptom was syncope with RBBB on the ECG, and 10 days later the patient died of sudden cardiac death at the age of 18 years. In most cases (87%), ophthalmoplegia or ptosis was present before the cardiac manifestations (not shown in the table). In 69% of the cases, syncope was the first cardiac symptom of the disease. Conduction defects are the most typical cardiac manifestations; but also ventricular tachycardia, long QT, and cardiac failure were observed in some cases. In published cases, more female patients were found with KSS involving cardiac complications.

Table 1 Cardiac complications in KSS. Summary of the literature between 1989 and 2009

Reference	Age at cardiac symptoms (in years)	Cardiac symptoms	Cardiac manifestation	Intervention	Gender (M/F)
Remes et al. (1992)	29 38	– Syncope	Intraventricular conduction delay; Complete AV block (infra-His block)	DDD PM	Unknown
Anan et al. (1995)	15	Syncope	Complete AV block (infra-His block)	PM (type is unknown)	M
Kakura et al. (1998)	17	Cardiac failure	Complete heart block	DDD PM	M
Katsanos et al. (2002)	18	Syncope 10 days later SCD	RBBB, Mitral valve prolapse	Died before PM impl.	F
Oginosawa et al. (2003)	29	Syncope	1st degree AV block, LAFB, Polymorphic ventricular tachycardia (normal QT interval)	Dual chamber ICD	F
Hara et al. (2004)	29	Syncope	Mobitz type II 2nd degree AV block, RBBB, LAFB, Ventricular tachycardia	Dual chamber ICD	F
Young et al. (2005)	39	Syncope	1st degree AV block RBBB, LAFB	DDD PM	M
Karanikis et al. (2005)	47	Syncope	Sinus bradycardia 1st degree AV block RBBB, LAFB, Torsades de pointes ventricular tachycardia (long QT)	DDD PM	F
Letsas et al. (2006)	17	–	RBBB, LAFB; Complete AV block		
	20	Syncope		DDD PM	F
Subbiah et al. (2007)	33	Cardiac failure	Complete AV block Torsaides de pointes ventricular tachycardia DCM	DDD PM ICD	F
Skinner et al. (2007)	12 14	– Syncope	RBBB; Complete heart block Torsades de pointes ventricular tachycardia	DDD PM	F
Chawla et al. (2008)	10	Cardiac failure	Complete heart block	VVI PM	F
Riera et al. (2008)	9	–	IRBBB		
	23	–	RBBB, LAFB, LSFB	PM (type is unknown)	M
Yeşil et al. (2009)	22	Syncope	Mobitz type II 2nd degree AV block with RBBB, LAFB	VVI PM (after 12 years total PM dependency)	F
Welzing et al. (2009)	9	Cardiac failure	Complete heart block	VVI PM	F

PM pacemaker, *SCD* sudden cardiac death, *RBBB* right bundle branch block, *LAFB* left anterior fascicular block, *ICD* implantable cardioverter-defibrillator, *DCM* dilatative cardiomyopathy, *IRBBB* incomplete right bundle branch block, *LSFB* left septal fascicular block, *DDD pacemaker* dual chamber pacing and sensing pacemaker, *VVI pacemaker* ventricular pacing and sensing pacemaker

Discussion

Cardiac involvement has been shown to be the most important prognostic factor for life expectancy, and complete heart block is known to be the major cause of death in patients with KSS (Chawla et al. 2008). KSS typically causes cardiac conduction defects. Intracardiac electrophysiologic studies showed that the primary abnormalities are in the AV node-His-Purkinje system (Polak et al. 1989). In cardiac histopathological studies, fatty infiltration and fibrosis of the bundle branches and of the sinoatrial and atrioventricular nodes have been observed (Gallastegui et al. 1987).

The typical conduction defects are fascicular blocks and bundle branch blocks, progressing to complete heart block. In the reviewed literature, the first cardiac complication of the disease was at 9 years, but cardiac manifestation even complete AV block might occur earlier. Unfortunately, there is no effective treatment to prevent the manifestation or slow down the progression of cardiac complications (Welzing et al. 2009).

KSS syndrome is not an easy diagnosis to make in the early phase of the disease. Some of the patients demonstrate Pearson's syndrome initially (Rahman and Leonard 2000), others, like our second patient might have associated Leigh disease. Recent diagnostic guidelines suggest mtDNA deletion analysis in muscle already early in the diagnostic process if KSS is suspected (Finsterer et al. 2009). However, even if the correct diagnosis is made, the adequate therapy could be challenging.

In the case of our own first patient in the differential diagnosis of the episodes of losing consciousness, based on

the severe neurological involvement and history of low blood sugar levels, we initially included the possibility of absence epilepsy, hypoglycemia, and AV block as well. The investigations, however, were inconclusive; the glucose day curve was normal, EEG showed diffuse encephalopathy, and the 24 hours Holter ECG monitoring revealed no definitive cause. At this point of the investigations, cardiac cause of the syncope was not obvious. Due to the severe neurological status, a seizure disorder was also suspected in the patient.

In the case of our second patient, avoiding the life-threatening episode was almost impossible, since the correct diagnosis was not even established by the sudden appearance of the cardiac symptoms. The manifestation of the cardiac involvement played an important role in finding the diagnosis.

The European Society of Cardiology guidelines for cardiac pacing, 2007 recommend pacemaker implantation with any degree of fascicular block in patients with neuromuscular diseases with class IIa indication, level of evidence C, or with second- or third-degree AV block with class I indication, level of evidence B (Vardas et al. 2007).

Our two cases further emphasize the need to consider early pacemaker implantation in patients with known KSS with any sign of conduction impairment and the importance of early diagnosis.

We suggest that all patients with KSS should have ECG screening at least once a year to detect conduction defects, ventricular arrhythmias, and QT prolongation. Because of the progressive character of the disease, prophylactic pacemaker implantation should be considered in patients with KSS earlier than it is now accepted in the cases of fascicular blocks. Furthermore, patients who suffered a syncopal episode should be carefully monitored – especially in those cases when an underlying seizure disorder is unlikely – realizing that complete AV block and cardiac arrest can be the first manifestations of cardiac involvement.

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CASE REPORT

Immune Modulation Therapy in a CRIM-Positive and IgG Antibody-Positive Infant with Pompe Disease Treated with Alglucosidase Alfa: A Case Report

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Abstract Pompe disease is characterized by deficiency or absence of activity of the lysosomal enzyme acid alpha-glucosidase. As a result of ineffective metabolism, glycogen progressively accumulates in muscle tissues. Patients with an aggressive classic infantile-onset form generally rapidly die of cardiorespiratory failure. A cross-reactive immunological material (CRIM)-negative status is predictive of high anti-alglucosidase alfa antibody titers and usually a poor clinical outcome of enzyme replacement therapy (ERT). CRIM-positive patients can also develop robust antibody titers complicating therapeutic management.

We successfully used an immune modulation therapy (IMT) protocol in a CRIM-positive infantile-onset patient with Pompe disease in whom infusions had to be temporarily discontinued because of safety concerns despite administration of pre-infusion medication. Prior to discontinuation, she had shown signs of clinical deterioration and continuous ventilation support through a tracheostomy was required. She was found to be positive for anti-alglucosidase alfa antibodies (1:6,400). IMT (rituximab, methotrexate and intravenous gamma globulin) was started, ERT was safely reintroduced during the IMT induction phase and, subsequently, the enzyme dose was increased, all without any complications. Antibodies disappeared, IMT was tapered and discontinued, and cadiomyopathy steadily improved. During 1 year of follow-up, she remained ventilator dependent and no gains in motor skills were noticed; motor functions will be closely monitored during sustained ERT.

Although the reversal of clinical decline in our CRIM-positive and antibody-positive infant with Pompe disease cannot be solely attributed to IMT, our experiences with this protocol may be helpful to other physicians encountering comparable therapeutic dilemmas.

Abbreviations

CRIM	Cross-reactive immunological material
ERT	Enzyme replacement therapy
GAA	Acid alpha-glucosidase
IAR	Infusion-associated reaction
IMT	Immune modulation therapy
LVMI	Left ventricular mass index

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Introduction

Pompe disease (OMIM #232300), also known as glycogen storage disease type II, is a treatable lysosomal storage disorder caused by the presence of a mutation in the gene encoding acid alpha-glucosidase (GAA) (Hirschhorn and Reuser 2001). Affected individuals have deficient or no activity of lysosomal GAA and are unable to effectively metabolize glycogen. The pathological hallmark of Pompe disease is accumulation of glycogen in muscle tissues

(Hirschhorn and Reuser 2001; van der Ploeg and Reuser 2008).

The spectrum of clinical presentations is continuous and wide. At the most severe end, patients have little, if any, residual GAA activity and usually present with cardiomyopathy, hypotonia and muscle weakness, respiratory distress, feeding difficulties, and failure to thrive during early infancy (Kishnani et al. 2006a). Death from cardiorespiratory failure generally occurs within the first year of life. Patients with a nonclassical infantile, juvenile or late-onset form generally have >1% of normal residual GAA activity and cardiomyopathy is more attenuated or absent. Although the disease course is less aggressive, progressive limb and respiratory muscle involvement can lead to wheelchair and/or ventilator dependency, and ultimately death (van der Ploeg and Reuser 2008). The clinical diversity in Pompe disease can largely be explained by the considerable genotypic variability; more than 350 mutations and sequence variants have been identified in the *GAA* gene (www.pompecenter.nl). The combined incidence of all forms of Pompe disease has been estimated at 1:40,000 (Ausems et al. 1999; Martiniuk et al. 1998).

Until 2006, when cause-specific enzyme replacement therapy (ERT) opened a new era in the treatment of Pompe disease, only supportive care to alleviate symptom could be offered. ERT with recombinant human GAA (rhGAA; alglucosidase alfa, Myozyme®[1]) has shown major beneficial effects in patients throughout the disease spectrum (Kishnani et al. 2006a, b; Nicolino et al. 2009; Kishnani et al. 2007; Amalfitano et al. 2001; van der Ploeg et al. 2010). These benefits included reduction of the risk of invasive ventilation, prolongation of survival, improvement in hypertrophic cardiomyopathy and, among a subset of infantile-onset patients, improvement in motor function, motor skills and functional dependence. It has become apparent that not all infantile-onset patients respond satisfactorily to ERT. A cross-reactive immunological material (CRIM)-negative status has been reported to predict poorer clinical outcome, particularly because of the presence of high titers of anti-alglucosidase alfa IgG antibodies (Kishnani et al. 2010). High antibody titers also increase the likelihood of infusion-associated reactions (IARs) that may complicate therapeutic management (Lipinski et al. 2009). Successful elimination of anti-alglucosidase alfa antibodies with immune modulation therapy (IMT) can play an important role in maximizing the benefits of ERT (Mendelsohn et al. 2009) and in the prevention of severe IARs. We report a case of Pompe disease in a female CRIM-positive and antibody-positive infant in whom ERT had to be interrupted because of safety concerns and could be successfully reintroduced after start of IMT.

[0] Summary of Product Characteristics Myozyme®, http://www.ema.europa.eu/.

Case Report

Feeding difficulties, failure to thrive and muscle weakness were first noticed by the parents of the female infant at the age of 4 months. She had been born after an uneventful pregnancy and delivery, and birth weight (3,752 g), length (52 cm) and Apgar score (10) were normal. The family history was unremarkable and her 5-years-older half sister was healthy. Once admitted to our hospital, clinical examination revealed hypotonia, tachycardia, and macroglossia. Ultrasound examination showed cardiomyopathy (left ventricular mass index (LVMI) 174.4 g/m^2) and hepatomegaly. The diagnosis infantile-onset Pompe disease was suspected and confirmed by demonstration of deficient GAA activity (3% of normal) in lymphocytes, and by genetic studies [Gly309Arg (925 G > A), Gln757X (2269 C > T)].

A month later, ERT was initiated at a dose of 20 mg/kg of Myozyme® administered once every other week. Soon after, she was discharged home and improvements in motor functions, with attainment of new motor milestones, were noticed over a 10-month period. LVMI reduced by 20%. Recurrent upper respiratory tract infections occurred, but the girl remained ventilator-free. At 10 months of ERT, IARs became more frequent despite pre-treatment with diphenhydramine and prednisone, and selection of a slower infusion rate. Infusions were complicated by episodes of tachycardia, hyper- or hypotension, irritability, pallor, tachypnea, wheezing, and oxygen desaturation. The reactions were managed by temporarily slowing or halting the infusion and administering symptomatic treatment.

Her general condition gradually deteriorated with an increase in muscle weakness and hypotonia. Despite intensive conservative treatment, continuous ventilation support through a tracheostomy had to be initiated 1 year after start of ERT. To investigate possible causes of her clinical deterioration, an anti-alglucosidase alfa IgG antibody assay was ordered and a positive test result (antibody titer 1:6,400) was received after 6 weeks; determination of anti-alglucosidase-specific IgE antibodies could not be performed because of logistic reasons. LVMI had significantly increased to 379.8 g/m^2 and IARs (wheezing, oxygen desaturation, bradycardia) had become more serious. Figure 1 depicts the patient's clinical course including changes in LVMI and anti-alglucosidase antibody titers over time. After careful consideration, it was decided to temporarily discontinue ERT. Four weeks later, IMT was started, involving administration of rituximab, methotrexate, and intravenous gamma globulin (IVIG). The IMT protocol (Fig. 2) was approved by the University Hospital

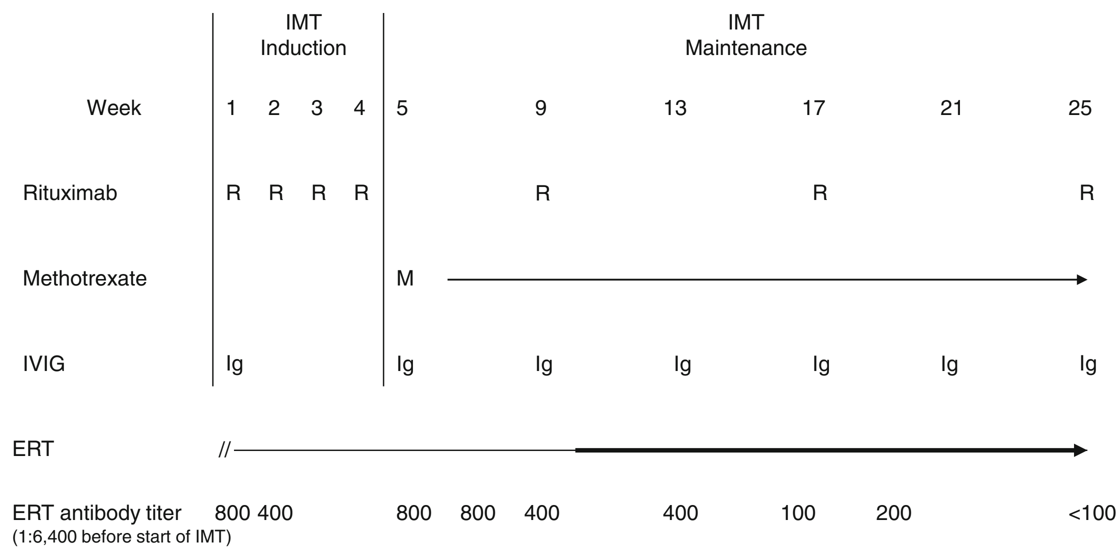

Fig. 1 Immunomodulation therapy (IMT) protocol and anti-alglucosidase alfa antibody titers. Rituximab (R) 375 mg/m2/dose IV; after Wk 25, the patient received one additional administration in Wk 37. Methotrexate (M) 0.5 mg/kg weekly (orally or G-tube) to maintain absolute neutrophil count <500/μL and platelet count <50,000/μL. Discontinued in Wk 46. Intravenous immunoglobulin (IVIG) 500 mg/kg IV every 4 weeks. Monthly administrations continued till Wk 46. Alglucosidase alfa (ERT) restarted at 5 days of IMT at 20 mg/kg every other week (EOW). Dose increased to 40 mg/kg EOW in Wk 10 of IMT (continuing). After Wk 25, anti-alglucosidase alfa (ERT) antibody titers remained undetectable (<1:100)

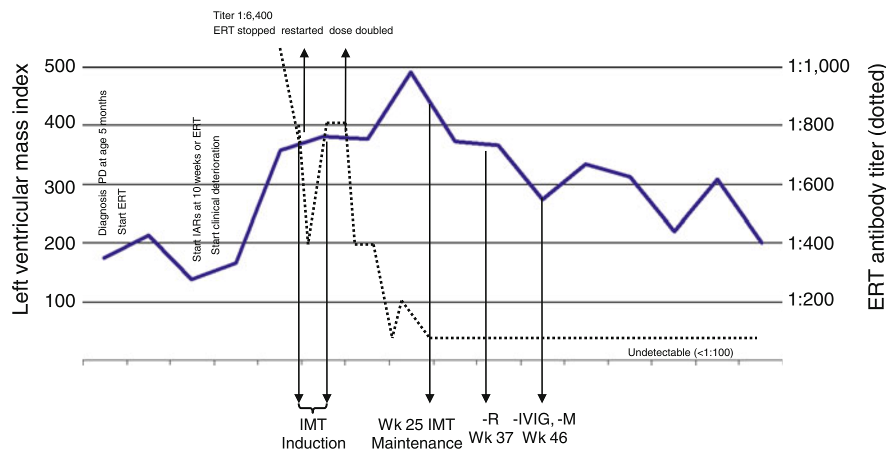

Fig. 2 Clinical course of the patient, including changes in left ventricular mass index and ERT antibodies over time. *IMT* Immunomodulation therapy, – *R* Rituximab stopped, – *IVIG* immunoglobulins stopped, – *M* Methotrexate stopped, *PD* Pompe disease, *IAR* Infusion-associated reaction, *ERT* Enzyme replacement therapy with alglucosidase alfa

of Split Institutional Review Board, and written informed consent was obtained from the parents. At day 5 of IMT, ERT was restarted and diphenhydramine and prednisone were administered only prior to the first ERT infusion. No side effects occurred. Antibody titers ranged between 1:800 at start of IMT (result received 8 weeks after start of IMT). and 1:400 at 10 weeks of ERT. Although the LVMI tended to stabilize, the overall response to ERT was felt to be inadequate. After careful consideration and having sought expert advice, the dose of ERT was increased to 40 mg/kg every other week. Subsequently, antibody titers decreased and LVMI reduced from IMT week 19 onward. At week 25 of IMT (15 weeks after ERT dose increase), antibody titers had become undetectable. No adverse reactions associated with IMT or ERT had occurred. Twelve weeks thereafter, IMT was tapered. Rituximab was discontinued at week 37 of IMT, and the last doses of IVIG and methotrexate were administered in week 46 of the protocol. Currently, more than a year after the IgG antibodies had become undetectable, antibodies are still untraceable. No IARs to ERT have occurred. Although LVMI continues to decrease (a ~60% reduction as compared to the highest value measured), there were no other significant clinical improvements and the girl remained ventilator dependent.

Discussion

ERT with alglucosidase alfa is an essential component of the lifelong clinical care of patients with Pompe disease. However, treatment remains challenging, particularly in patients who have profound deficiency of GAA activity. Early start of ERT has proven to be critical and a better outcome can be anticipated in infants who are CRIM-positive and have remained free of ventilator support (Kishnani et al. 2010; Chien et al. 2008). Our CRIM-positive patient was immediately started on ERT after the diagnosis had been made at the age of 5 months. At 10 months of ERT, severe IARs started to occur and she deteriorated clinically (Fig. 1). The safety and efficacy concerns, and detection of anti-alglucosidase antibodies, forced us to consider other therapeutic approaches. ERT was temporarily discontinued, IMT was started, then ERT was reintroduced, and subsequently the enzyme dose was increased. All these therapeutic changes were well tolerated. In retrospect, it remains unsure to what extent each of the therapeutic measures has contributed to the disappearance of antibodies and reduction in LVMI.

Approximately 90% of ERT-treated CRIM-positive infants with Pompe disease develop IgG antibodies during the first months of treatment, but their functional consequences are still poorly understood (Kishnani et al. 2010). These infants have some endogenous GAA protein which may prevent development of robust antibody titers in most patients (Kishnani et al. 2010). A recent study in a limited number of ERT-treated CRIM-positive infants found that antibodies had no in vivo inhibitory effects on enzyme uptake or activity, also not in patients with the highest titer (1:51,200). Median LVMI decreased to near-normal levels. In contrast, CRIM-negative infants developed a strong immune response with much higher titers (up to 1:1,638,400), but not all had inhibitory activity. If compared to these data, the titer in our patient was only modestly elevated (highest titer 1:6,400). Because samples were not tested for inhibitory antibody activity, a role of immunogenic mechanisms in the patient's clinical decline cannot be rigorously excluded. Until the interruption of ERT, she had received only single doses of prednisone prior to the infusions and no maintenance treatment. It remains unsure whether these biweekly administrations can have reduced the antibody titers.

Clinical studies found that the majority of infantile-onset infants experienced IARs on ERT. However, correlations between IARs and antibody titers are not consistent (Nicolino et al. 2009; Kishnani et al. 2007). IARs are observed in both patients with low and absent antibody titers, as well as in patients with high antibody titers. IARs rarely sustain and generally subside gradually. In most patients, IARs can be prevented by administering premedication and, if they occur, can be controlled by temporarily slowing or halting the infusion and administering symptomatic care (Kishnani et al. 2006a, b; Nicolino et al. 2009; Kishnani et al. 2007; Amalfitano et al. 2001; van der Ploeg et al. 2010). This usually allows continuation of ERT with appropriate pre-infusion medication. In our patient, the serious IARs were uncontrollable despite pre-treatment with diphenhydramine and prednisone and selection of a slower infusion rate. Because of logistic reasons, she could not be tested for anti-alglucosidase-specific IgE antibodies which have been demonstrated in a limited number of ERT-treated patients developing significant allergic reactions. Successful rechallenge with modified infusion regimens has been reported (Lipinski et al. 2009), and there is one case report describing immune modulation with monoclonal anti-IgE antibody (omalizumab) (Rohrbach et al. 2010).

The safety and efficacy concerns made us decide to interrupt ERT and explore alternative therapeutic approaches. We were unaware that the antibody titer had come down spontaneously to 1:800 after the 4-week interruption of ERT but, based on the previously detected titer of 1:6,400, the option of IMT to suppress antibody formation and, thereby, secure safe administration of ERT, was considered. Moreover, IMT could eliminate a theoretical inhibitory effect of antibodies. There is only scarce published experience with tolerance induction protocols in Pompe disease. Methotrexate has been used in the CRIM-negative murine model (Joseph et al. 2008) and Mendelsohn et al. have successfully applied an IMT protocol in conjunction with ERT in a CRIM-negative infant; antibodies disappeared, cardiomyopathy normalized, but the boy became ventilator dependent (Mendelsohn et al. 2009). This protocol involves administration of rituximab (elective depletion of mature CD20+ B-cells), methotrexate (inactivation of rapidly multiplying B- and T-cells) and intravenous gamma globulin (to compensate for B-cell suppression induced by rituximab) (Fig. 2). After having obtained expert advice, it was decided to use this protocol in our patient. The restart of ERT 5 days after initiation of the IMT induction phase was well tolerated. At 10 weeks of IMT, the enzyme dose was increased (as Mendelsohn et al. have done) in an attempt to achieve optimum treatment effect. After having peaked at week 19, LVMI steadily decreased in the absence of antibodies and untoward reactions. Although cardiomyopathy majorly improved, gains in motor development were not observed. This may relate to observations showing that ERT effectively clears lysosomal glycogen from the heart but is less effective in clearing skeletal muscle, and that remodeling of muscle fibers and restoration of muscle function are time-consuming processes (van der Ploeg and Reuser 2008).

In summary, ERT had to be interrupted in our CRIM-positive and antibody-positive infantile patient with Pompe

disease because of safety concerns. Given the observed clinical decline, IMT was introduced, ERT was safely restarted, and subsequently the enzyme dose was increased. Anti-alglucosidase antibodies disappeared and cardiomyopathy gradually improved. Although these observations cannot be solely attributed to IMT, our experiences with this protocol may be helpful to other physicians encountering comparable therapeutic dilemmas. It is advised to first explore all less-invasive options. The knowledge on clinical decline in a subset of CRIM-positive patients needs to be expanded. Encouraging physicians to periodically collect antibody samples in all ERT-treated patients with Pompe disease and to report clinical data will be critical in this endeavor.

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Synopsis

Immune tolerance induction is a viable therapeutic option in poorly responding infantile, antibody-positive patients with Pompe disease.

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CASE REPORT

Carpal Tunnel Syndrome in Fabry Disease

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Abstract Carpal tunnel syndrome (CTS) is a common peripheral mononeuropathy affecting up to 4% of the general population, typically women in late middle age. The incidence in patients with Fabry disease (FD) is unclear, but may affect 25% of patients with this X-linked lysosomal storage disease. We report three cases of CTS in young Caucasian male patients with classical FD, who developed CTS symptoms with supportive nerve conduction study (NCS) findings. Two patients had bilateral CTS and two had evidence of concurrent ulnar nerve neuropathy on NCS, suggesting a systemic process contributed to nerve compression. All were receiving enzyme replacement therapy (ERT) and had a moderate burden of FD complications. It is possible that an increase in connective tissue in the intracarpal canal in FD patients may be incited by injury to fibroblasts, via either accumulation of globotriaosylceramide (GL3) or local ischaemia through endothelial injury. The former hypothesis may be a more plausible explanation for the development of CTS, as histology of the flexor retinaculae from our patients has demonstrated fibroblasts with characteristic vacuolation and excessive myxomatous stroma, despite endothelial clearance of GL3 in these patients receiving ERT. CTS should not be overlooked in FD patients and young patients presenting with CTS should be evaluated for an underlying systemic or genetic disorder. Surgical carpal tunnel decompression was effective in our patients, already troubled by long-standing acroparesthesia, in providing sustained relief of symptoms.

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Introduction

Fabry disease (FD) (OMIM 300644) is an X-linked lysosomal storage disorder due to mutations in the *GLA* gene, which encodes α-galactosidase (EC 3.2.1.22), the enzyme responsible for the degradation of globotriaosylceramide (GL3). Individuals with FD develop multi-system disease via accumulation of GL3 in many tissues, including myocardium, kidney, vascular endothelium, arterial walls and connective tissue (Elleder 2003). We report three cases of carpal tunnel syndrome affecting young male patients with FD.

Patient 1

A Caucasian male, adopted as a neonate, suffered throughout his childhood with undiagnosed pain in his hands and feet, exacerbated by exercise and viral infections. FD was formally diagnosed at age 14 years, after his biological mother mother contacted the adoption agency to inform them of her own diagnosis of FD. α-galactosidase level was reduced in peripheral blood leukocytes (0.11 nmol/min/mg; normal range 0.3–2.1) and in cultured fibroblasts. Subsequent genetic testing revealed an M284T mutation

in the *GLA* gene. When reviewed by a nephrology service at age 20 years, his clinical features included at least 10 years of acroparesthesia, hypohidrosis, umbilical angiokeratoma and intermittent diarrhoea. He had lower limb temperature anaesthesia in a symmetrical stocking distribution to ankle level. Blood pressure, serum creatinine (Se Cr) and 24 hour proteinuria were normal. Nerve conduction studies (NCS) of his lower limbs confirmed mild peripheral neuropathy. He began enzyme replacement therapy (ERT) with Agalsidase alfa (Shire HGT) at age 30 years. Four years later he developed nocturnal pain and tightness in his left thenar eminence with numbness in his left palm and lateral three digits. Nerve conduction studies were diagnostic of severe left carpal tunnel syndrome (CTS) (Table 1). He had low grade proteinuria (0.11–0.4 g/day), but Se Cr and echocardiography were normal. On the Mainz severity score index (MSSI), this patient had a score of 29 (Beck 2006). Left carpal tunnel decompression relieved his symptoms. Histology of the left carpal tunnel flexor retinaculum and connective tissue from within the carpal canal identified fibrous connective tissue with scattered vacuolated cells, a small amount of myxomatous material in the stroma and PAS-positive deposits (Fig. 1). No inflammatory cells or Maltese cross lipid inclusions were identified under polarised light. Unfortunately, electron microscopy (EM) was not possible due to suboptimal specimen processing. Six years later, CTS symptoms have not recurred. Renal function remains normal and proteinuria has not progressed, but he has developed left ventricular hypertrophy with diastolic dysfunction.

Patient 2

After proband identification, this male patient was found to have a reduced WBC α-galactosidase level at age 5 years, and the causative G128E mutation in the *GLA* gene was later defined. Throughout his boyhood he suffered with acroparesthesia, hypohydrosis and chronic diarrhoea. At the commencement of ERT (Agalsidase alfa) at age 34 years, glove-and-stocking sensory neuropathy affected temperature, light touch and pain sensation. Other medical problems included obstructive sleep apnoea, nasal polyps, caecal volvulus and major depression. He worked intermittently with vibratory machinery. Over the next 3 years, he developed bilateral progressive numbness and pain from hands to elbows, initially nocturnal, then during the day, especially when driving. His MSSI score was 30. Motor examination was normal. Light touch and temperature sensation were reduced in a glove distribution in the right upper limb and in the median nerve territory in the left hand. Tinnel's sign was positive at the right wrist. NCS of his upper limbs indicated severe bilateral carpal tunnel syndrome and moderate right ulnar neuropathy at the elbow with mild sensory axonal neuropathy (Table 1). On quantitative sensory testing, the cold detection threshold was elevated but heat pain thresholds indicated hyperaesthesia, consistent with pathology of small myelinated and unmyelinated nerve fibres. Decompression of both carpal tunnels and of the right ulnar nerve relieved all symptoms. Tissue histology showed dense fibrous tissue with an increase in toluidine blue positive extracellular material. EM demonstrated lamellated zebra bodies zebra bodies within the perinuclear cytoplasm of stromal cells (Fig. 2). No cytoplasmic vacuolation of muscle, perineural tissue or vascular endothelial cells was present.

Patient 3

This Caucasian male patient also had the classical phenotype of FD (M284T mutation; belonging to the same pedigree as Patient 1). At the commencement of ERT (Agalsidase alfa) at age 41 years, FD burden included glove-and-stocking sensory neuropathy, left ventricular hypertrophy (LVH) and proteinuria (0.7 g/24 h), but radionuclide glomerular filtration rate (GFR) was normal. At the time, his MSSI score was 26. Five months later, he presented with sudden onset of severe shock-like pains in his right hand and wrist and left arm, after using heavy hand-held drilling equipment over the two preceding days, with previous occupational exposure to machinery. He recalled that similar symptoms 3–4 years earlier had settled with physiotherapy. Investigation confirmed bilateral CTS (see Table 1) and surgery provided sustained and complete symptomatic relief. Light microscopy of specimens from biopsies of the skin and flexor retinaculum of the right palm showed foamy histiocytes in the wall of the blood vessels consistent with FD.

Discussion

FD typically affects both peripheral and autonomic nerves. The mechanism of Fabry neuropathy is poorly understood, but may relate to nerve ischaemia or dysfunction from glycolipid accumulation in the vasa nervorum, or in dorsal root ganglia (Hilz et al. 2000; Schiffmann 2006). Episodic acroparesthesia in the hands and feet, occurring from childhood, affects most males and many females (Galanos et al. 2002). Patients may develop a sensory length-dependent peripheral neuropathy, predominantly affecting small myelinated (A-delta) nerve fibres (resulting in elevated thresholds to cold stimulation) and to a lesser degree, unmyelinated C fibres (transmitting perception of hot stimuli) (Luciano et al. 2002; Torvin Moller et al.

Table 1 Nerve conduction study findings and grade of carpal tunnel syndrome severity

Patient	Nerve and site	Latency (ms)	Amplitude (mV)	Segment	Conduction velocity (m/s)	Grade of CTS (0–6)[a]
Patient 1	MOTOR					
	Median L					$L = 4$
	Wrist	5.1	8.6			
	Elbow	10	8.5	Wrist–elbow	58	
	Ulnar L					
	Wrist	2.2	11.8			
	Below elbow	6.7	11.5	Wrist-below elbow	56	
	Above elbow	8.6	11.5	Below elbow–above elbow	50	
	SENSORY					
	Mixed ascending L					
	Wrist (median)	4.4	26	Elbow–wrist (median)	69	
	Wrist (ulnar)	5	12	Elbow–wrist (ulnar)	63	
	Digits sensory L					
	Digit 2 (median)	Absent				
	Digit 1 (median)	3.1	2	Wrist–digit 1 (median)	37	
	Digit 5 (ulnar)	2.3	8	Wrist–digit 5 (ulnar)	62	
Patient 2	MOTOR					
	Median R					$R = 4$
	Wrist	5.6	5.6			
	Elbow	11.5	5.7	Wrist–elbow	51	
	Ulnar R					
	Wrist	2.5	7.4			
	Below elbow	6.5	6.4	Wrist–below elbow	53	
	Above elbow	10.3	6.3	Below elbow–above elbow	32	
	Median L					$L = 4$
	Wrist	6.1	6			
	Ulnar L					
	Wrist	2.7	9.9			
	SENSORY					
	Mixed ascending R					
	Wrist (median)	5.4	28	Elbow–wrist (median)	62	
	Wrist (ulnar)	6.6	10	Elbow–wrist (ulnar)	48	
	Digits sensory R					
	Digit 2 (median)	Absent				
	Digit 1 (median)	Absent				
	Digit 5 (ulnar)	2.7	2	Wrist–digit 5 (ulnar)	52	
Patient 3	MOTOR					
	Median R					$R = 3$
	Wrist	5.3	9.5			
	Elbow	11.55	9.1	Wrist–elbow	52.8	
	Ulnar R					
	Wrist	3.15	10.4			
	Below elbow	4.85	10.1	Wrist–below elbow	47.1	
	Above elbow	10.55	10	Below elbow–above elbow	43.9	
	Median L					$L = 3$
	Wrist	4.6	9			
	Elbow	11.4	7.3	Wrist–elbow	48.5	

Table 1 (continued)

Patient	Nerve and site	Latency (ms)	Amplitude (mV)	Segment	Conduction velocity (m/s)	Grade of CTS (0–6)[a]
	Ulnar L					
	Wrist	2.65	9.9			
	Below elbow	4.55	9.5	Wrist–below elbow	52.6	
	Above elbow	9.7	9.1	Below elbow–above elbow	48.5	
	SENSORY					
	Mixed ascending R					
	Wrist (median)	2	10.4	Elbow–wrist (median)	40	
	Wrist (ulnar)	1.15	7.8	Elbow–wrist (ulnar)	62.2	
	Digits sensory R					
	Digit 2 (median)	3.05	1.6	Wrist–digit 2 (median)	44.3	
	Digit 1 (median)	2.85	5.5	Wrist–digit 1 (median)	35.1	
	Digit 5 (ulnar)	2	4.5	Wrist–digit 5 (ulnar)	55	
	Mixed ascending L					
	Wrist (median)	1.9	21	Elbow–wrist (median)	42.1	
	Wrist (ulnar)	1.35	11	Elbow–wrist (ulnar)	59.3	
	Digits sensory L					
	Digit 2 (median)	3.2	3.8	Wrist–digit 2 (median)	40.6	
	Digit 1 (median)	2.8	3.4	Wrist–digit 1 (median)	35.7	
	Digit 5 (ulnar)	2.1	2.9	Wrist–digit 5 (ulnar)	54.3	

CTS carpal tunnel syndrome, *L* left, *R* right

[a] As defined by the "Canterbury Scale" (Bland 2000)

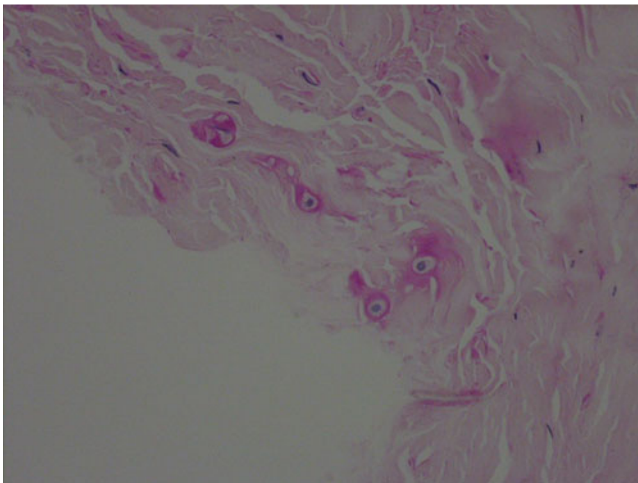

Fig. 1 Light microscopy image showing connective tissue with vacuolated fibroblasts, biopsied from the carpal tunnel of Patient 1

2009). ERT improves small fibre function (Hilz et al. 2004). Light and electron microscopy of peripheral nerve specimens has shown lipid granules within the endothelium of epineural arterioles, perineural fibroblasts, smooth muscle cells and pericytes, but inclusions in Schwann cells, unmyelinated and myelinated axons are less consistent findings (Kocen and Thomas 1970; Sima and Robertson 1978; Kaye et al. 1988; Toyooka and Said 1997).

CTS, a mononeuropathy of the median nerve, was superimposed on these classic FD manifestations in the three patients described. In the general population, CTS is proposed to result from increased pressure within the carpal tunnel, with subsequent median nerve ischaemia, epineural oedema and fibroblast invasion, culminating in the development of constrictive scar tissue around the nerve (Gelberman et al. 1981; Werner and Andary 2002; Uchiyama et al. 2010). While CTS is associated with a number of systemic diseases and some mechanical and occupational factors (Mattioli et al. 2009; Uchiyama et al. 2010), a genetic predisposition for CTS has also been shown (Hakim et al. 2002; Lozano-Calderon et al. 2008). CTS presenting in childhood should prompt consideration of an underlying lysosomal storage disorder or other genetic condition (Van Meir and De Smet 2003; Sri-Ram et al. 2007; Yuen et al. 2007). Patients presenting to Australian general practice with symptoms of CTS are predominantly female (male–female ratio 2 : 3) and 46% are aged 45–65 years (Charles et al. 2009). A similar gender ratio is reported in a Swedish general population cohort, with a population prevalence of "clinically certain" CTS at 4% overall and 2% in males (Atroshi et al. 1999).

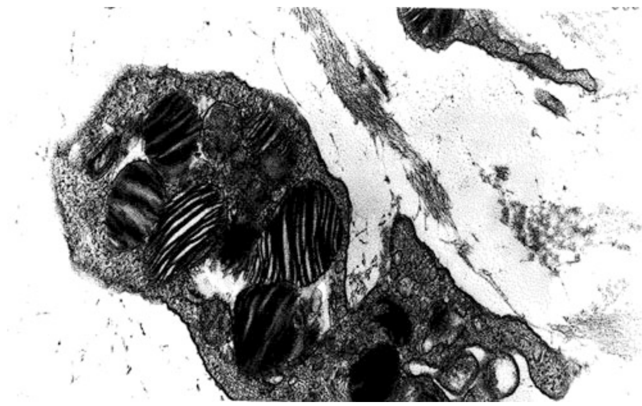

Fig. 2 EM photograph (20,000×) of “zebra bodies” within a fibroblast from tissue taken from the carpal tunnel at the time of surgical decompression from Patient 2

In patients with FD, the prevalence of CTS is unclear but appears to be higher than in the general population, independent of the confounding association between FD and renal failure. In a study of 22 consecutive patients with FD aged 20–52 years with creatinine clearance >50 mL/min, six (27%) had median nerve entrapment at the wrist, as identified by delayed sensory conduction. However, while these patients suffered hand acroparesthesia, they had no clinical features of CTS (Luciano et al. 2002). Another study evaluating peripheral nerve involvement of 12 female FD patients found 3 women (25%), aged between 43 and 60 years, had clinical symptoms of CTS, with confirmatory NCS (Laaksonen et al. 2008).

The 3 male patients described received medical care within a state-wide cohort of 30 male FD patients. Based on their MSSI scores, they had a moderate disease burden at the time of developing CTS. The cohort of 30 male FD patients includes 8 deceased males (all of whom received ERT), who scored a median MSSI score of 36 (range 23–50) at the time of their death. The disease burden of the 3 males with CTS is similar to the remainder of the living FD cohort (which includes 16 patients receiving ERT and 2 participating in a trial of oral chaperone therapy), who scored a median of 28 (range 3–49) on the MSSI. Patients were only investigated for CTS if they had suggestive symptoms; no other individuals in this cohort have had NCS of their upper limbs. The FD patients with CTS developed this condition in their 30s, younger than the typical age of presentation in the general population. Patients 2 and 3 had possible environmental triggers and patient 3 had performed heavy repetitive manual work with vibrating equipment within 24 h of his acute presentation. Although both of these men presented with CTS after receiving ERT for many months, they recalled prior milder symptoms consistent with CTS. Patients 1 and 2 also had evidence of ulnar nerve neuropathy on NCS and Patient 2 required a right ulnar nerve decompression, suggesting that systemic rather than solely local processes were involved in the evolution of nerve compression.

CTS evolved in our patients despite ERT. Histology of the excised flexor retinaculae and perineural tissue demonstrated vacuolation of fibroblasts and an increase in extracellular material. Although nerve biopsies were not performed, rapid improvement in symptoms upon surgical decompression suggests a compressive aetiology for CTS rather than direct neuronal injury. Generally, the endothelium from our carpal tunnel biopsies on patients on long-term ERT was clear of GL3 inclusions, although GL3 storage persisted in tissue fibroblasts. This is consistent with other studies of long-term ERT for FD, where persistent storage of GL3 has been demonstrated in vascular smooth muscle, stromal fibroblasts, pericytes and skeletal muscle fibres (Keslova-Veselikova et al. 2008) and subtotal removal demonstrated in cultured skin fibroblasts (Askari et al. 2007). GL3 clearance varies between different cell types (Murray et al. 2007). Renal, myocardial and dermal endothelium clears after 5–6 months of ERT, and sustained clearance from vascular endothelium has been demonstrated after 30–36 months of ERT (Eng et al. 2001; Wilcox et al. 2004; Keslova-Veselikova et al. 2008; Thurberg et al. 2009). Persistence of inclusions within fibroblasts, despite in vitro evidence that Agalsidase alfa and beta are internalised by fibroblasts with effective lysosomal GL3 clearance (Keslova-Veselikova et al. 2008), may be explained through low expression of cation-independent mannose-6-phosphate (M6P) receptors, as has been demonstrated in Fabry Schwann cells (Kawashima et al. 2007). A different and less effective mechanism of enzyme uptake by fibroblasts compared to endothelial cells has also been hypothesised (Mayes et al. 1982; Hasholt et al. 1988). Endothelial cells may effectively clear GL3 because ERT is delivered at high concentration directly to the endothelium through intravenous administration, which may also protect new endothelium against GL3 accumulation. Therefore, it is plausible that despite ERT, ongoing GL3 accumulation in fibroblasts within the carpal tunnel may continue to incite a response resulting in increased extracellular matrix, culminating in the clinical picture of CTS. The relative absence of endothelial cell inclusions in our patients on ERT suggests that the alternative hypothesis – that endothelial involvement contributes to local ischaemia in the carpal tunnel – is less likely.

Conclusion

CTS may be an under-recognised complication of FD. In this series, three male patients with classical FD (representing 10% of our FD cohort), developed CTS at

a younger age than expected for the general population, despite receiving ERT. The symptomatic benefit of carpal tunnel decompression in these patients, already troubled by long-standing acroparesthesia and sensory neuropathy, highlights the need for appropriate recognition and management of CTS in FD. Histology identified increased extracellular matrix material around collagen fibres as well as inclusions of GL3 in retinacular fibroblasts. An underlying genetic disorder should be considered in the case of children or young adults who develop CTS.

Synopsis

Carpal tunnel syndrome may be a sequela of classical Fabry Disease (through injury to fibroblasts or local ischaemia within the carpal tunnel) despite long-term enzyme replacement therapy, and effective relief of symptoms can be achieved with surgical decompression.

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CASE REPORT

A Zinc Sulphate-Resistant Acrodermatitis Enteropathica Patient with a Novel Mutation in SLC39A4 Gene

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Abstract Acrodermatitis enteropathica (AE) is a rare autosomal recessive disorder of zinc deficiency due to an abnormal intestinal zinc transporter. It is characterized by the triad of acral dermatitis, alopecia, and diarrhoea. Once AE is correctly diagnosed, patients are treated with orally administered zinc sulphate. In some patients, relapses occur during adolescence, despite the regular treatment. Here, we discuss the clinical and molecular features of a 13-year-old adolescent girl with acrodermatitis enteropathica who was resistant to high-dose zinc sulphate therapy. We successfully treated the patient with zinc gluconate and vitamin C, and we detected a novel homozygous c.541_551dup (p.Leu186fsX38) mutation in the exon 3 of her *SLC39A4* gene.

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Introduction

Acrodermatitis enteropathica (AE; OMIM 201100) is a rare autosomal recessive disease, first described by Danbolt and Closs in 1942 as an acral rash associated with diarrhoea (Danbolt and Closs 1942). The incidence is estimated as one in 500,000 children (Van Wouwe 1995). The disease seems to be common in sub-Saharan Africa and South East Asia (Van Wouwe 1995). It is caused by a mutation in the *SLC39A4* gene, located on chromosome 8q24.3 (Küry et al. 2002, 2003; Wang et al. 2002). The gene encodes a zinc transporter protein belonging to the ZIP family (Küry et al. 2002, 2003; Wang et al. 2002). About 30 mutations were found up to date (Kilic et al. 2007; Küry et al. 2003; Lehnert et al. 2006; Li et al. 2010; Meftah et al. 2006; Nakano et al. 2003, 2009; Schmitt et al. 2009; Vardi et al. 2009; Wang et al. 2002, 2008). The disease is characterized by a triad of acral dermatitis, alopecia, and diarrhoea (Danbolt 1979). Patients with advanced disease also experience growth delay, mental slowing, poor wound healing, frequent infections, anaemia, photophobia, hypogeusia, anorexia, delayed puberty, and hypogonadism (Cameron and McClain 1986; Maverakis et al. 2007a, b; Prasad et al. 2008). Phenotypic variability was observed among patients and no significant genotype–phenotype correlations could be established (Kharfi et al. 2010; Schmitt et al. 2009; Vardi et al. 2009). Symptoms usually begin during the weaning from breast or formula feeding (PerafÄn-Riveros et al. 2002). Diagnosis is established through a constellation of clinical findings and the detection of low plasma zinc concentration levels (Maverakis et al. 2007a, b). Low levels of serum alkaline phosphatase, a zinc-dependent metalloenzyme, may be a valuable indicator of zinc deficiency (Weismann and Høyer 1985). Treatment of AE includes zinc supplementation at doses of

1–3 mg/kg/day of elemental zinc or 50–150 mg/day of zinc sulphate/acetate or gluconate (Kharfi et al. 2010; Maverakis et al. 2007a, b). In this study, we detected a novel mutation in the human *SLC39A4* gene in 4 patients from related two families.

Patient and Methods

Case Report

A 13-year-old Turkish adolescent girl (individual III.4. in the pedigree of Fig. 1a), born of a nonconsanguineous union, was referred to our clinic for the evaluation of a refractory dermatitis and a diffuse alopecia. She was previously followed by another centre since the 3 months of age, which was the age at onset of the disease. The first symptoms had appeared after a full-term pregnancy and an uneventful neonatal period, during which the infant was breast-fed (breast-feeding was stopped at about 18 months of age). The symptoms observed then were irritability, diarrhoea, symmetrical acral and periorificial dermatitis and alopecia. Oral mucous membranes were fragile and erythematous. Erosions were noted on chest, arms and legs. In perioral and perianal areas, as well as on hands, elbows, feet and knees, skin lesions were erythematous, scaly, crusted, psoriasiform, and vesiculobullous. Given these symptoms characteristic of a severe zinc deficiency, and the infant was diagnosed with acrodermatitis enteropathica and an oral zinc sulphate treatment was immediately started. Since then and during the 13 years that followed, the patient was treated intermittently with high dose of oral zinc sulphate; during these intermittent treatment periods, a partial response was observed, but resolution of the lesions never resolved. The patient did not receive any special diet, and no skin biopsy was performed.

At the time the patient was referred to our clinic, skin lesions were still very significant in spite of high doses of zinc sulphate (300 mg/day, 10 mg/kg/day elemental zinc) therapy. Physical examination revealed symmetrical erythema, erosions, crusts localized on perioral, anogenital and acral regions. In addition, she had alopecia, mild malnutrition (weight: 30 kg (<5th centile), height: 146 cm (10–25th centile), BMI: 14 (<3th centile) and pubertal delay (Fig. 2a, b). Laboratory examinations, including a full blood count and liver and kidney function tests, were within normal range. The serum zinc level was 23.7 μg/dl (70–120), while serum alkaline phosphatase level was only 26 U/L (37–147). High-dose zinc gluconate (300 mg/day, 10 mg/kg/day elemental zinc) and vitamin C (500 mg/day) were started. Her skin lesions almost disappeared within 3 weeks (Fig. 2c, d). Serum zinc and alkaline phosphatase level reached the normal ranges (81 μg/dl and 51 U/L respectively). The patient's younger brother and two of her cousins had a history of similar lesions (respectively noted as individuals III.5., III.9. and III.10 in Fig. 1a). Mutation analyses were performed in the genomic DNA of the patient and her family members (Fig 1b).

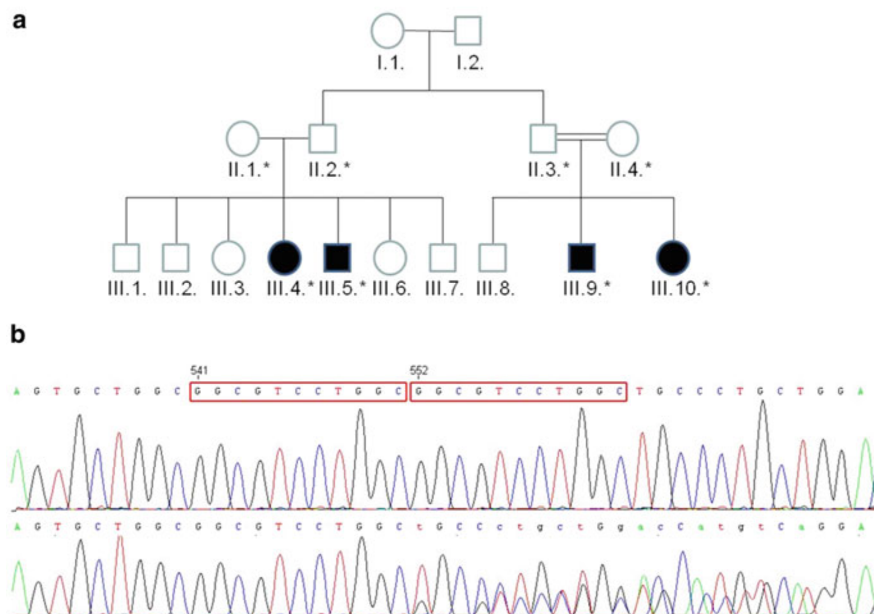

Fig. 1 (**a**) Pedigree of the family of the Turkish patient with acrodermatitis enteropathica. (**b**) Sequence electropherogams of exon 3 of the *SLC39A4* gene showing mutation c.541_551dup (p.Leu186fsX38) mutation carried at homozygous state in the four patients (*upper sequence*), and at heterozygous state in their parents (*lower sequence*)

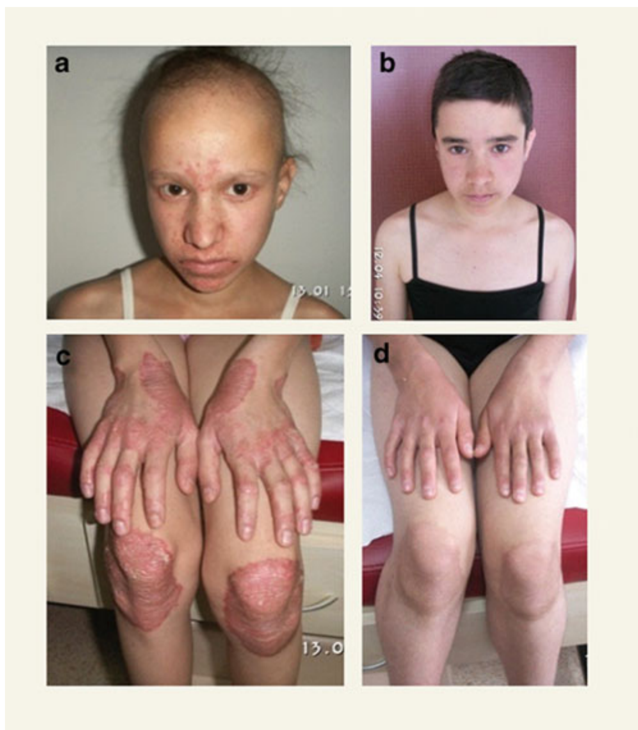

Fig. 2 (**a**, **b**) Alopecia, perioroficial and acral dermatitis (before treatment) (**c**, **d**) Marked improvement of the lesions (after 6 months of treatment)

DNA Sequencing

After having obtained written informed consent, blood samples were collected from peripheral veins of the patients III.4., III.5., III.9. and III.10., and of their respective parents. Genomic DNA was extracted from leucocytes, following a standard procedure with a homemade kit. Samples obtained from the probands, and their extended family, were sent for genetic analysis to the Molecular Genetics Laboratory of the University Hospital of Nantes. A mutation screening of the *SLC39A4* gene was conducted there by polymerase chain reaction (PCR)-amplifying all the 12 exons and their flanking intronic regions, and by sequencing the products on ABIPRISM 3130XL, using Big Dye terminator V1.1 chemistry (Applied Biosystems, Foster City, CA). Primers sequences are available upon request. Permission from the parents was obtained for publication of photographs.

Results and Discussion

The four AE patients described here were found to carry a homozygous duplication in exon 3 of the *SLC39A4* gene (c.541_551dup, according to mRNA reference sequence NM_130849; see Fig. 1b). This duplication was predicted to create a premature termination codon (p.Leu186fsX38). It is therefore very likely that this mutation alters the zinc absorption function of Slc39a4 protein and causes zinc deficiency. The patients' parents were found to be heterozygous for the same mutation, which therefore perfectly segregates within the family according to an autosomal recessive mode of inheritance. The absence of obvious zinc deficiency symptoms in the heterozygous carriers of the duplication might be due to nonsense mediated decay (NMD) targeting the mutant mRNA produced.

Since the Moynahan and Barnes study in 1973, which recognized zinc deficiency as an etiological factor, oral administration of zinc preparation has been the mainstay of treatment (Barnes and Moynahan 1973; Moynahan 1974). Most authors recommend an initial elemental zinc dose of 5–10 mg/kg/day and maintenance doses of 1–2 mg/kg/day (Kharfi et al. 2010; Maverakis et al. 2007a, b). Patients with acrodermatitis enteropathica require lifelong zinc supplementation. Zinc can be administered as acetate, aminoacid chelates, gluconate and sulphate (PerafÄn-Riveros et al. 2002). Zinc sulphate seems to be the best tolerated, and it was successfully used for the treatment since the initial articles were published. (Gartside and Allen 1975; Maverakis et al. 2007a, b; Mortimer et al. 1984). Recurrences were observed with cessation of treatment and during accelerated growth periods such as the adolescent period or pregnancy, which responds to an increase in dosage (Kharfi et al. 2010). No case with a resistance to zinc sulphate treatment has been observed in the literature until now. Our patient represents a case treated with zinc sulphate since 3 months of age; whom severe skin lesions did not respond to increases in dosage of zinc sulphate (300 mg/day elemental zinc), and she continued to have alopecia, malnutrition and pubertal delay. Since acrodermatitis enteropathica is a fatal disease without treatment, we can deduce that the patient had partial response to treatment. For this reason, treatment with zinc gluconate (300 mg/day) and additional vitamin C (500 mg/day) was given to accelerate skin healing (Ellinger and Stehle 2009; Lima et al. 2009). A dramatic response in skin lesions was observed within 3 weeks, and it continued at the 6th month control. No side effect was observed. The patient had better response to zinc gluconate treatment than zinc sulphate treatment. We hypothesize that modifying genes, environmental and/or epigenetic factors could be involved in the variability of biological signs and response to treatment. An increasing number of pharmacogenetic tests are proposed to evaluate the genetic background of response to various treatments (e.g. *KRAS* mutation testing for predicting response to *anti-EGFR* therapy cancer, or analysis of *TPMT* for use of metothrexate in Crohn's disease). It is also

possible that vitamin C treatment helped healing of skin lesions. No challenge to determine which of the substances had played a role in skin lesion healing was carried out.

In conclusion, here we report a novel mutation of *SLC39A4* gene in the family of a Turkish patient with acrodermatitis enteropathica. In patients who are resistant to treatment with increased dosage of zinc sulphate, usage of other forms of zinc and addition of vitamin C may be beneficial. It is worth noting that the present case was resistant to treatment with zinc sulphate, but responded to zinc gluconate treatment. Vitamin C may also have helped for skin lesion healing.

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CASE REPORT

Onset of Adreno-Leukodystrophy After Medulloblastoma Therapy: Causal Connection or Coincidence?

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Abstract X-linked adreno-leukodystrophy (ALD) is a peroxisomal disorder affecting the white matter of the central nervous system and the adrenal cortex. It is caused by mutations in the *ABCD1* gene encoding for a peroxisomal membrane protein. The absent genotype–phenotype correlation implies a contribution by environmental factors to explain the phenotypical heterogeneity. We report on a 4-year-old boy with a biochemically confirmed diagnosis of ALD after birth. At the age of 32 months, the additional diagnosis of a medulloblastoma was made. After treatment of the medulloblastoma, he developed active areas of demyelination representing the characteristic neuroimaging features of ALD. The clinical history of our patient supports the hypothesis that external factors, like neurosurgical intervention as part of medulloblastoma treatment, may accelerate or initiate cerebral ALD-related demyelination. A postsurgical inflammatory reaction may facilitate the inclusion of abnormal fatty acids in myelin. The opening of the blood–brain barrier following neurosurgery may enhance the recognition of previously sequestered antigens considered to play a role in ALD onset. Consequently, neurosurgical disruption of the BBB can precipitate the immune-mediated inflammatory process, which progressively destroys myelin in ALD patients. Tumor-related chemotherapy and/or radiotherapy may also play a contributing role. We suggest that X-ALD patients who undergo neurosurgical intervention need close follow-up imaging to identify active demyelination early.

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Competing interests: None declared.

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Abbreviations

ALD	Adreno-leukodystrophy
AMN	Adreno-myeloneuropathy
BBB	Blood-brain barrier
CALD	Childhood cerebral ALD
CNS	Central nervous system
MRI	Magnetic resonance imaging
PLP1	Proteolipid protein 1
VLCFA	Very long chain fatty acids

Introduction

X-linked adreno-leukodystrophy (ALD) is a peroxisomal disorder affecting the white matter of the CNS and the adrenal cortex (Moser et al. 2007). Two main phenotypes

affect the CNS: CALD and AMN. CALD typically manifests with behavioral or learning deficits between 4 and 8 years. Progressive impairment of cognition, behavior, vision, hearing, and motor function follows and often leads to total disability and death within a few years. AMN most commonly presents between the age of 20 and 40 with progressive paraparesis and sphincter disturbances. The symptoms are progressive over decades. About 35% of AMN patients additionally develop cerebral symptoms (cerebral AMN) sharing the same poor prognosis as children with CALD. Additional five phenotypes including the "Addison-only" presentation and "asymptomatic patients" are recognized (Moser et al. 2007).

ALD is caused by mutations in the *ABCD1* gene encoding for a peroxisomal membrane protein (Mosser et al. 1993). Significant phenotypical heterogeneity is reported in siblings with identical genotype showing absent genotype–phenotype correlations (Moser et al. 1992). This suggests that modifier genes or environmental factors might be responsible for the phenotypical heterogeneity of ALD. While modifier genes could not be identified, the pathogenetic implication of environmental factors such as head trauma has been shown (Raymond et al. 2010).

We report on a four-year-old boy with biochemically confirmed ALD and a posterior fossa medulloblastoma. Neuroimaging studies showed an "activation" of demyelination after treatment for medulloblastoma.

Case Report

In an asymptomatic male child, the diagnosis of ALD was made shortly after birth because of a positive family history. MRI of the brain at the age of two years was normal. The VLCFA profile was abnormal at this stage (C26:0 1.18 μg/ml, normal 0.23 ± 0.09; C24/C22 1.839, normal 0.84 ± 0.10; C26/C22 0.086, normal 0.01 ± 0.004). Prophylactic treatment with Lorenzo's oil was started. At 32 months of age, he presented to an outside hospital with vomiting. Subsequent brain MRI demonstrated a posterior fossa tumor, which was completely resected. Histopathological examination revealed medulloblastoma with classical features without evidence of metastatic spread. His postoperative course was uneventful and he exhibited no signs of cerebral ALD. He was treated with five cycles of cisplatin, cytoxan, etoposide, and vincristine ("Head Start" II, Dhall et al. 2008) and then received conformal radiation therapy (2,340 Gy to the posterior fossa with 3,660 Gy boost to the tumor bed). MRI after completion was unremarkable without evidence of residual or recurrent tumor. In addition, no signs of ALD-related cerebral demyelination were identified.

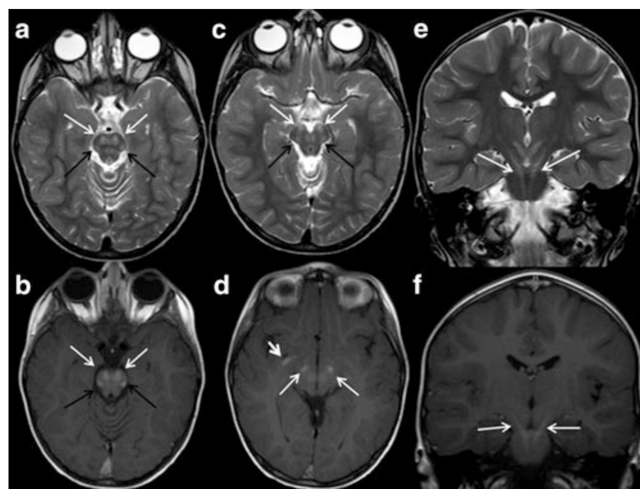

Fig. 1 Axial (**a**, **c**) and coronal (**e**) T2-weighted and contrast enhanced T1-weighted (**b**, **d**, **f**) MR images at 4.3 years show bilateral, symmetrical T2 hyperintensity with matching contrast enhancement of the corticospinal tracts at the level of the midbrain and pons (*white arrows*), the lateral lemnisci (*black arrows*) and red nuclei. Incidental note is made of a contrast enhancing focal lesion in the right temporal medial lobe (**d**, *short white arrow*) representing a metastatic lesion of the posterior fossa medulloblastoma

Unfortunately, 14 months later multiple metastatic lesions were seen within the supratentorial and infratentorial brain as well as in the spinal canal. Again, no lesions suggestive of cerebral ALD were noted. Chemotherapy was started with topotecan and cyclophosphamide. After two cycles, stable disease was noted. Chemotherapy was adjusted to temozolamide and etoposide. He then developed worsening ataxia and loss of speech. Follow-up imaging at the age of 4.3 years revealed a decrease in the size of the contrast enhancing metastatic lesions within the brain and spinal canal (Fig. 1). However, in contrast to the previous MRI studies, bilateral, symmetrical linear foci of enhancement and abnormal T2 high signal had appeared following the course of the corticospinal tracts extending from the posterior limb of the internal capsule to the pontomesencephalic junction (Fig. 1). Additional increased T2 signal with matching contrast enhancement was seen in the splenium of the corpus callosum, lateral lemnisci, and red nuclei (Fig. 1). Signal characteristics and distribution of these lesions were characteristic for cerebral ALD. At this stage, the child presented with truncal ataxia and speech and language difficulties.

Discussion

X-linked ALD is a well-described peroxisomal disorder where a single enzyme defect may result in different clinical phenotypes. There is a significant heterogeneity

in the cerebral ALD neuroimaging phenotype. Loes et al. (2003) defined five different neuroimaging patterns of cerebral ALD based on the primary involvement and distribution of findings as identified on MRI. Our patient most closely fits pattern three of Loes' classification, because of corticospinal tract involvement without abnormalities of the frontal, occipital, or cerebellar white matter. While this neuroimaging pattern is the most frequent distribution of lesions seen in adult patients, it is rarely observed in children. Additionally, the red nuclei also demonstrated signal abnormalities. This finding is rare; having only been reported previously in a single adult patient (Ochi et al. 1998).

External environmental or epigenetic factors may play a pathogenetic role in ALD modulating the clinical phenotype and/or causing the onset of ALD in genetically at-risk patients. Raymond et al. (2010) recently reported the impact of moderate to severe head trauma on ALD progression. In their series, Raymond et al. (2010) reported three asymptomatic patients and two AMN patients who, after head trauma, developed a CALD phenotype. The posttraumatic neurological progression of both AMN patients was brief and severe. This is unusual for AMN patients with cerebral involvement. Based on additional similar cases from the literature (Turpin et al. 1985; Wilkinson et al. 1987; Weller et al. 1992; Carmant et al. 1998; Fatemi et al. 2003), Raymond et al. concluded that head trauma is an environmental factor that can have a modulating–causative role in some patients genetically at risk for ALD.

Our patient, who was genetically at risk for ALD, showed an onset of active demyelination after intense multimodality treatment for medulloblastoma. For reasons outlined below, we consider neurosurgical intervention to be an environmental factor that may initiate ALD-related cerebral demyelination.

The presumed pathogenesis of cerebral myelin injury in ALD includes two stages. In the first phase, the inclusion of an excess of VLCFA in the myelin destabilizes the myelin leading to a dysmyelination, followed by a second phase of an immune-mediated inflammatory process with progressive demyelination (Hudspeth and Raymond 2007).

The first phase is based on the accumulation of excessive saturated, straight VLCFA in myelin lipids and in PLP1 (Raymond et al. 2010). An excess of hexacosanoic acid (C26:0) generates reactive oxygen species altering the physiological properties of myelin and consequently destabilizing the cell membranes. This leads to a spontaneous breakdown of the affected myelin sheaths. Therefore, any factor facilitating the inclusion of VLCFA into myelin will increase myelin instability. Normally, VLCFA are relatively insoluble at normal body temperature. However, their solubility and hence their mobility increase in the case of fever. Both accidental and surgical brain trauma may induce a systemic reaction of the organism, which is triggered by interleukin-6 and characterized by fever, leukocytosis, increased catabolism, and an activation of the coagulation system (Heesen et al. 1996). Therefore, not only post-traumatic fever (as reported by Raymond et al. in head trauma) but also febrile reaction after brain surgery may lead to an increased concentration of abnormal fatty acids in myelin.

The second phase is characterized by an inflammatory-immune demyelinative reaction. The cell membrane components containing VLCFA seem to serve as antigens driving the immune reaction after presentation by CD1 cells (Ito et al. 2001; Hudspeth and Raymond 2007). Factors facilitating the recognition of antigens may intensify the inflammatory reaction. Like head trauma, neurosurgery interrupts and increases the permeability of the BBB. This may enhance the recognition of previously sequestered antigens. Consequently, neurosurgical disruption of the BBB can precipitate the immune-mediated inflammatory process leading to demyelination in CALD patients.

The time interval between head trauma and onset of ALD symptoms varied between three and twelve months in the series published by Raymond et al. (2010). In our patient, the time between neurosurgery and development of ALD findings on MRI was 14 months. This temporal similarity supports the pathogenetic effect of neurosurgery as an environmental factor in the initiation of ALD-related demyelination in predisposed patients.

We are aware that additional factors could play a role in the acceleration/initiation of ALD in this patient. The medulloblastoma itself, by definition, results in a breakdown of the BBB, and could have precipitated this presentation. The patient's chemotherapy and/or radiotherapy could be postulated as an additional contributory factor. Based on its myelosuppressive effect, however, chemotherapy might have a protective rather than a negative effect on the inflammatory-immune component in ALD patients. Unfortunately, so far all studies exploring immunosuppressive or immunomodulating therapies with cyclophosphamide or interferon-β have not been successful (Berger et al. 2010).

In conclusion, this case suggests that, like accidental head trauma, neurosurgical brain injury with disruption of the BBB may act as an environmental factor to initiate or enhance ALD-related white matter demyelination. This finding supports the hypothesis that the phenotypical heterogeneity of ALD patients is at least partially explained by environmental external factors. In addition, our case shows that patients at risk for ALD or patients with a mild clinical course of ALD require close monitoring of their brain involvement after accidental or interventional brain injury.

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CASE REPORT

Successful Plasmapheresis for Acute and Severe Unconjugated Hyperbilirubinemia in a Child with Crigler Najjar Type I Syndrome

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Abstract Crigler–Najjar syndrome type I (CN-I, MIM #218800) is a rare and severe autosomal disorder. It is caused by deficiency of the liver enzyme responsible for bilirubin elimination, the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1; EC 2.4.1.17). Biologically, the disease manifests itself with severe and persistent unconjugated hyperbilirubinemia. Kernicterus is a well-known complication of severe unconjugated hyperbilirubinemia in infants and young children, especially in patients with CN-I.

Few articles have shown the efficiency of plasmapheresis for extreme hyperbilirubinemia.

In this report, we describe the efficiency of plasmapheresis for a rapid control of acute and severe unconjugated hyperbilirubinemia in a 6-year-old CN-I patient who had previously developed kernicterus in the neonatal period. In spite of intensification of phototherapy, the patient developed severe hyperbilirubinemia (up to 830 μmol/l, with bilirubin/albumin ratio at 1.2). With two plasmapheresis procedures, bilirubin serum concentration decreased to 420 μmol/ and bilirubin/albumin ratio to 0.55. Following the acute episode of very severe unconjugated hyperbilirubinemia, the child recovered and neurological examination was unchanged, thus suggesting that plasmapheresis possibly prevented further worsening of kernicterus.

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Introduction

Crigler–Najjar syndrome type I (CN-I, MIM #218800) is a rare and severe autosomal disorder. It is caused by deficiency of the liver enzyme responsible for bilirubin elimination, the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1; EC 2.4.1.17). Biologically, the disease manifests with severe and persistent unconjugated hyperbilirubinemia. CN-I patients require lifelong phototherapy until liver transplantation or, in the near future, gene therapy. These patients are at permanent risk for bilirubin encephalopathy (kernicterus). The latter is due to the deposition of unconjugated bilirubin in the subcortical nuclei (globus pallidus, subthalamic nuclei and brainstem cranial nerve nuclei). Its manifestations include extrapyramidal hypertonia, deafness, mental retardation.

Controlling unconjugated hyperbilirubinemia in CN-I patients relies on daily phototherapy. Bilirubin/albumin ratio is a useful tool in such patients; it has been reported that the high risk values are above 0.7 and that all CN-I patients who developed kernicterus had had values above

1.0 (Strauss et al. 2006). However, acute episodes of severe unconjugated hyperbilirubinemia may occur during infections and surgical procedures. In such situations few drugs have been reported to be useful. Synthetic heme analogues that are competitive inhibitors of heme oxygenase (the rate-limiting enzyme in the production of bilirubin) have been shown to be rapidly efficient, following a single administration. However, in many countries, these new drugs are not available. Plasmapheresis has also been reported to be a useful tool when a rapid decrease in bilirubin serum concentration is mandatory.

We report on a 6-year-old CN-I patient who had developed kernicterus in the neonatal period, in whom very severe unconjugated hyperbilirubinemia occurred following a surgical procedure. Plasmapheresis was rapidly efficient in this patient, allowing a quick decrease in serum bilirubin concentration.

Case Report

The patient was a 6-year-old girl who was diagnosed with CN-I syndrome at the age of 3 months. The diagnosis was based on very high levels of serum-unconjugated bilirubin (621 μmol/l). She was the third child of consanguineous Tunisian parents. The first two children were healthy. She was born after an uneventful, full-term pregnancy, and was delivered vaginally. Weight and height were 3,950 g and 49 cm, respectively.

She had developed jaundice during the first 24 h of life and had been treated with classical phototherapy during 3 days, then discharged, after trans-cutaneous control of bilirubin serum concentration had shown an estimated value of 180 μmol/l. The infant was 3-months old when she was taken to the hospital for permanent and severe jaundice. Save marked jaundice, physical examination revealed peripheral hypertonia and axial hypotonia; the infant was reactive to sound stimulations, had an appropriate smile and the liver was not enlarged. Severe unconjugated hyperbilirubinemia was isolated, with neither evidence for hemolysis (normal RBC count, normal G6PD and pyruvate kinase activities, mother's blood group A+, baby's blood group A+) nor other hepatic dysfunction (normal levels of serum transaminases and γGT, PT and factor V at 100%). The serum bilirubin/albumin molar ratio was 1.15. The infant was placed under continuous intensive phototherapy and received albumin infusion (1 g/kg in 2 h).

Bilirubin serum concentrations quickly decreased to 500 (within 24 h), then 400 μmol/l. (within 48 h), while bilirubin/albumin ratio had the same evolution (0.8 at 24 h, 0.65 at 48 h). During the following weeks, the duration of phototherapy was progressively decreased, under both clinical and biological controls, to10 h a day with a good control of serum bilirubin concentrations (between 270 and 320 μmol/l). Brain MRI proved normal and EEG did not show any abnormality. Auditory function was tested and found to be normal. The child was then discharged with at-home phototherapy and regular follow-up. Molecular study showed that the child was homozygous for the c.1070A > G mutation within exon 3 of the UGT1A1 gene, a previously reported mutation in CN-I patients (Petit et al. 2008) thus confirming the diagnosis of CN-I syndrome.

Unfortunately**,** the diagnosis of kernicterus, initially suspected, was confirmed in the following months with extrapyramidal hypertonia, axial hypotonia, swallowing troubles, and face and mouth dyspraxia. In spite of intensive physiotherapy, peripheral hypertonia continuously worsened resulting in partial dislocation of both hips that required surgery at the age of 6 years. After surgical procedure, both hips were immobilized by a hip-to-foot cast, thus reducing the efficiency of phototherapy, half of the body surface being hidden by the cast. Despite increasing the duration of phototherapy up to 18 h a day and the use of albumin infusions every 10 days (1 g/kg of body weight) , serum bilirubin concentrations increased a week after surgery to reach 659, then 830 μmol/l (bilirubin albumin ratio at 1.2). The patient was transferred to a specialized unit and a central line was inserted in an internal jugular vein. The girl was treated with one session of plasmapheresis that resulted in a decrease of bilirubinemia to 430 μmol/l, followed, 24 h later by an increase of bilirubinemia to 630 μmol/l (Fig. 1). A second session of plasmapheresis was performed, allowing a rapid decrease of bilirubinemia to 420 μmol/l, while the bilirubin/albumin ratio decreased to 0.55 (Fig. 1). The evolution was then uneventful and the girl was able to come back to our unit.

Two months later, when the cast was taken off, the duration of phototherapy was reduced to 10–12 h a day. Neurological examination proved unchanged, thus suggesting that plasmapheresis possibly prevented further worsening of kernicterus.

Discussion

Kernicterus is a neurological syndrome that is due to the accumulation of unconjugated bilirubin in the brain, and more precisely in the subcortical nuclei, e.g., subthalamic nuclei, globus pallidus, and brainstem cranial nerve nuclei. Kernicterus is a well-known complication of severe unconjugated hyperbilirubinemia in infants and young children (Wang et al. 2008), especially in patients with CN-I (Blaschke et al. 1974; Chalasani et al. 1997; Labrune et al. 1992; Shapiro 2010). Our patient , when admitted at 3 months of age, already had clinical manifestations of

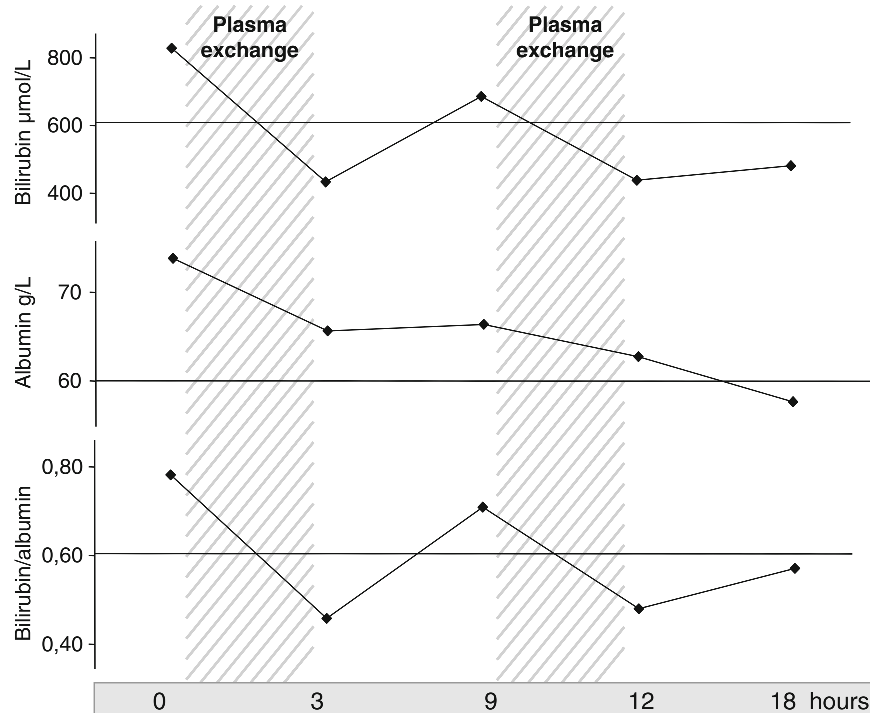

Fig. 1 Changes in bilirubin and albumin serum concentration during plasmapheresis. Plasma exchanges were performed on a Hématé machine, using a Gambro PF 1000 filter. Each exchange was performed during 120 min, with a volume of 60 ml/kg body weight, against albumin (50 g/l). The flow of extracorporal circulation was 100 ml/min. Anticoagulation with enoxaparine (50 U/kg body weight) was used

kernicterus, obviously related to the very high levels of bilirubin serum concentrations she had had since birth.. This complication should have been prevented and thus avoided, should this neonate have been carefully followed up after being discharged from the hospital at 4 days (Ahlfors et al. 2009; Strauss et al. 2006). Once the diagnosis had been ascertained, usual daily phototherapy allowed a good control of serum bilirubin concentration, as it is the case in most patients with CN-I.

The production of bilirubin physiologically increases during fasting periods, infectious episodes. Thus, patients with CN-I and their families are told to increase the duration of phototherapy should such events occur. Despite these precautions, kernicterus has been reported in such patients, following infections (Labrune et al. 1992), or surgical procedures (Walmsley et al. 2010), even in adults. In our patient, we tried to forecast the postoperative evolution by trying to reproduce the consequences of the hip-to-foot cast (we covered the lower half of the patient's body with a sheet, thus reducing the available skin surface for phototherapy), with a careful monitoring of bilirubin serum concentrations. We had concluded that (data not shown) increasing the duration of daily phototherapy up to 16–18 h combined with regular albumin infusions should be efficient for preventing the risks related to increased bilirubin serum levels. This therapeutic management was enough during a week, and then failed to control the severe and rapid increase of bilirubinemia (there was no clear explanation for such a rapid increase in bilirubin serum concentration, but several factors may have contributed such as reduction of food intakes, physical stress, …). Few reports had shown the efficiency of plasmapheresis for extreme hyperbilirubinemia . A few years ago, a paper reported the successful use of plasmapheresis in an 18-year-old patient who developed acute EBV infection with a peak bilirubin level of 75.7 mg/dl (1,287 µmol/l, 50% of unconjugated and 50% of conjugated bilirubin). Twenty-four hours after plasmapheresis, the bilirubin concentration had decreased to 24.4 mg/dl (Place et al. 2007). In patients with CN-I syndrome, plasmapheresis has been successfully used in three adults (48, 18, and 25 years old) who developed hyperbilirubinemia after surgical procedures (Blaschke et al. 1974; Chalasani et al. 1997; Walmsley et al. 2010).

In our case, as heme oxygenase inhibitors are currently unavailable in our country (Abraham and Kappas 2008; Drummond and Kappas 2004; Kappas 2004) plasmapheresis appeared to be the only way for a rapid biological efficiency. Two sessions had to be performed for correct control of hyperbilirubinemia. We believe that this treatment has been efficient and possibly prevented the development of new sequellae in our patient in whom

physical and neurological examinations remained unchanged.

The prevention of such situations is mandatory. Intensification of phototherapy, avoiding fasting periods (and thus providing regular and increased caloric intakes) and contraindicated drugs (Strauss et al. 2006) is highly important. However, prevention may not be efficient enough, as in our case. Liver transplantation, which is, to date, the only "radical" treatment of CN-I had been discussed in this patient but, owing to kernicterus, its indication had been rejected.

In conclusion, plasmapheresis may be a useful treatment for extreme acute unconjugated hyperbilirubinemia occurring in children with CN-I syndrome when phototherapy is transiently impaired for any adverse complication.

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RESEARCH REPORT

Treatment with Lactose (Galactose)-Restricted and Medium-Chain Triglyceride-Supplemented Formula for Neonatal Intrahepatic Cholestasis Caused by Citrin Deficiency

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Abstract Citrin plays a role in the transfer of NADH-reducing equivalent from cytosol to mitochondria as part of the malate–aspartate shuttle in liver. Citrin deficiency may cause an impairment of glycolysis due to an increase in the cytosolic NADH/NAD ratio leading to an energy shortage in the liver. Mutations of the *SLC25A13* gene are responsible for neonatal intrahepatic cholestasis (NICCD) and adult-onset type II citrullinemia (CTLN2). Most patients with NICCD show a resolution of symptoms within the first year of life, but some patients present with severe symptoms and require liver transplantation. We treated four patients including three siblings with NICCD by lactose (galactose)-restricted and medium-chain triglyceride (MCT)-supplemented formula. This formula rapidly improved the clinical condition and laboratory findings. Early treatment was more effective and did not require long-term administration. Lactose (galactose)-restriction can avoid further increase in the cytosolic NADH/NAD ratio in the liver and MCT supplementation can provide energy to hepatic cells by producing an excess of acetyl-CoA in mitochondria. Early treatment with lactose (galactose)-restricted and MCT-supplemented formula is recommended for patients with NICCD and possibly for patients with CTLN2.

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Introduction

Citrin, encoded by *SLC25A13*, is a mitochondrial inner membrane aspartate–glutamate carrier that transfers cytosolic NADH-reducing equivalent into the mitochondria as part of the malate–aspartate shuttle in the liver (Kobayashi et al. 1999; Saheki et al. 2010). Human citrin deficiency causes neonatal intrahepatic cholestasis (NICCD: OMIM 603471) and adult-onset type II citrullinemia (CTLN2: OMIM 605814). NICCD presents in the first few weeks of life with prolonged cholestasis, liver dysfunction and metabolic abnormalities including aminoacidemia and galactosemia (Tazawa et al. 2001; Tomomasa et al. 2001). Some patients receive liver transplantation (Tamamori et al. 2002; Shigeta et al. 2010), but in most patients symptoms resolve within the first year of life and they subsequently remain healthy (Ohura et al. 2007). One or more decades later, some of the patients develop neurological symptoms of CTLN2 showing behavioral aberrations, restlessness, disorientation and coma (Kobayashi et al. 1999; Tomomasa et al. 2001; Saheki et al. 2010).

Patients with NICCD complicated with galactosemia or cholestasis have been treated with lactose (galactose)-free formula or one containing medium-chain triglyceride (MCT) supplemented with fat-soluble vitamins (Ohura et al. 2007; Chew et al. 2010). For CTLN2, clinical benefit has been demonstrated with liver transplantation (Saheki et al. 2010). Recently, a favorable result was reported in one patient with therapy using sodium pyruvate and arginine under the low carbohydrate formula (Mutoh et al. 2008).

We now present a promising therapy with lactose (galactose)-restricted and MCT-supplemented formula in four cases including three siblings with NICCD and

recommend this therapy in order to avoid an increase of cytosolic NADH/NAD ratio and supply an energy source to the mitochondria in liver.

Materials and Methods

Formula

MCT formula supplemented with essential fatty acids (Meiji Milk Product Co., Ltd., Tokyo) contains protein 13.2 g, MCT 20.5 g, linoleic acid 2.0 g, alpha-linolenic acid 0.5 g, soluble polysaccharide 30.5 g, lactose 26.1 g and 504 kcal per 100 g powder. Lactose-free formula (Morinaga Milk Industry Co., Ltd., Tokyo) contains protein 13.0 g, lipids 20.0 g, dextrin 56.8 g, sucrose 5.0 g and 479 kcal per 100 g powder. MCT oil (Kissei Pharmaceutical Co., Ltd., Tokyo) contains MCT 85 g and long chain fatty acid 15 g per 100 g.

Gene Analysis

The Ethics Committee of Yamagata University School of Medicine approved this study. Peripheral blood specimens were used for genomic DNA extraction with written informed consent from the patient's family. *SLC25A13* was analyzed according to previous reports (Tabata et al. 2008; Dimmock et al. 2009).

Patient 1

The patient was a 10-year-and-4-month-old Japanese boy. He was born at 40 weeks gestation after an uneventful pregnancy. His birth weight was 2,520 g (14th percentile) and length was 48.5 cm (40th percentile). He had unrelated and healthy parents and a healthy elder sister. At the age of 3 months, he presented with jaundice and steatorrhea. On physical examination, he weighed 6,740 g (70th percentile) and measured 61.0 cm (40th percentile) in length. His skin and bulbar conjunctiva were jaundiced. His liver and spleen were palpable 5 cm and 1 cm below the costal margin, respectively. Laboratory findings showed hypoalbuminemia, cholestasis and mildly elevated liver transaminases (Table 1). There was no significant change in the complete blood cell count, coagulation tests, blood ammonia, serum alpha-1 antitrypsin, lipoprotein X or urinary coproporphyrin. Plasma amino acid analysis showed an increase in the concentration of citrulline, threonine, methionine, tyrosine and arginine (Table 1). In addition, there was a mild decrease in the concentration of branched chain amino acids, glutamine and alanine. Hepatobiliary imaging by 99mTc-pyridoxyl-5-methyl-tryptophan showed a delay in hepatic clearance, but there was no obstruction of either the common or intrahepatic bile ducts. Liver biopsy demonstrated macro- and micro-vesicular steatosis, cholestasis, fine sinusoidal fibrosis, small round cell infiltration in the portal region and absence of prominent giant cells. *SLC25A13* analysis demonstrated a compound heterozygous mutation, c.1177+1G>A/c.1750+72_1751-4dup17ins NM_138459.3: 2667 (Tabata et al. 2008). He was treated with MCT formula supplemented with essential fatty acids (Meiji Milk Product Co., Ltd., Tokyo) and arginine supplementation starting at 4 months of age. His condition (cholestasis, jaundice and failure to thrive) and laboratory findings rapidly improved (Fig. 1a). All laboratory findings except for a mild increase in the plasma level of tyrosine had improved to the normal range by 9 months of age. However, the parents changed from the formula containing MCT to ordinary milk for 1 month at the age of 11 months and his condition and laboratory findings worsened. MCT administration was decreased by mixing MCT formula with ordinary milk and finally discontinued at 17 months of age when all abnormal laboratory findings had normalized. At the age of 10 years and 2 months, he weighed 29.3 kg (30th percentile) and was 136.6 cm (50th percentile) tall. He had a preference for protein-and fat-rich food, but he did not have any symptoms or abnormal laboratory findings.

Patient 2

The patient, a younger sister of patient 1, was 2 years and 8 months old. She was born at term after an uneventful pregnancy. Her birth weight was 2,300 g (5th percentile) and length was 48.0 cm (40th percentile). At 2 months of age, she demonstrated cream-colored bulky stools (steatorrhea). On physical examination, she weighed 4,508 g (10th percentile) and had a length of 52.2 cm (below the 1st percentile). There was no apparent jaundice of the skin or bulbar conjunctiva. The liver was palpable 4.5 cm below the costal margin. She showed hypoalbuminemia, cholestasis and mildly elevated liver transaminases. She also had increases in the levels of plasma citrulline, threonine, methionine, tyrosine and arginine, but there was no significant change in the other amino acids, showing a profile consistent with NICCD (Table 1). Blood galactose was elevated to >1.1 mmol/L and was normalized after 2 weeks of treatment with MCT formula supplemented with essential fatty acids (Meiji Milk Product Co., Ltd., Tokyo) and with fat-soluble vitamins and arginine administration. Her condition and laboratory findings were improved and nearly normalized at 7 months of age (Fig. 1b). Fat-soluble vitamins and MCT formula were finally discontinued at 6 months of age and 10 months of age, respectively. She demonstrated the same *SLC25A13* mutation as patient 1. At 2 years and 8 months of age, she weighed 11.4 kg (20th percentile) and measured 85.6 cm (10th percentile) in length.

Table 1 Summary of formula and laboratory data of four patients with NICCD

		Patient 1			Patient 2			Patient 3			Patient 4		
Age at testing (days)		124	155	264	75	104	209	55	76	146	85	110	155
Body weight (g)		6,740	7,600	9,210	4,508	5,410	7,800	3,856	4,786	7,150	5,394	6,520	7,990
Formula													
Protein (g/kg/day)		1.6	2.4	1.9	4.2	4.1	2.6	3.3	2.8	1.8	2.8	2.8	2.2
Carbohydrate (g/kg/day)		10.7	10.4	8.5	20.6	17.6	12.0	16.3	11.8	8.6	18.0	12.3	9.6
Lactose (g/kg/day)		9.5	4.8	6.0	19.7	8.1	9.5	15.3	5.5	8.3	16.1	0	0
Fat (g/kg/day)		5.2	4.6	3.8	9.3	7.8	5.4	7.0	5.2	3.9	8.6	6.5	5.3
MCT (g/kg/day)		0	4.1	3.4	0	7.0	4.8	0	4.7	3.5	0	2.0	1.0
Energy (kcal/kg/day)		96.4	93	75	182	157	106	141	105	76	163	116	85
Laboratory data	Reference ranges												
Citrulline (μmol/L)	3–35	263	24	26	323	43	36	168	32	21	346	29	31
Threonine (μmol/L)	24–174	251	75	124	472	129	82	471	191	132	431	149	85
Methionine (μmol/L)	9–42	45	16	25	606	53	21	32	48	29	87	77	36
Tyrosine (μmol/L)	22–108	120	86	114	102	35	58	27	95	83	151	115	132
Arginine (μmol/L)	12–133	91	29	108	294	65	84	148	133	207[a]	235	73	60
Albumin (g/L)	39-48	32	37	46	35	42	46	31	39	40	30	43	48
T. Bil (μmol/L)	3–12	124	10	2	54	13	6	20	5	2	90	29	6
D. Bil (μmol/L)	<4	91	7	1	34	7	1	8	1	1	36	17	2
AST (IU/L)	25–85	292	164	62	104	109	58	65	50	42	115	51	50
ALT (IU/L)	12–62	78	58	57	80	64	49	58	28	40	31	30	43
LDH (IU/L)	369-817	401	387	377	354	326	299	285	296	266	330	280	226
ALP (IU/L)	334-982	888	476	472	3,322	1,232	859	1,812	796	850	7,289	1,264	873
γ-GTP (IU/L)	10–47	53	167	41	251	343	28	163	58	30	335	100	34
ChE (IU/L)	264–569	137	299	426	234	366	390	183	404	425	225	374	457
TBA (μmol/L)	<36	717	61	8	230	148	3	174	28	5	399	164	5
NH_3 (μmol/L)	18–74	65	29	36	53	N.D.	N.D.	N.D.	27	N.D.	55	N.D.	N.D.
AFP (ng/mL)[b]		8,128	5,790	10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	>10,000	4,033	123
PSTI (ng/mL)	<20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	22	N.D.	N.D.	59	22	19

T. Bil total bilirubin; *D. Bil* direct birirubin; *AST* aspartate transaminase; *ALT* alanine transaminase; *LDH* lactate dehydrogenase; *ALP* alkali phosphatase; *γ-GTP* γ-glutamyltranspeptidase; *ChE* Cholinesterase; *TBA* total bile acid; NH_3 ammonia; *AFP* alpha-fetoprotein; *PSTI* pancreatic secretory trypsin inhibitor; *N.D.* not determined

[a] Specimen was obtained after arginine intake

[b] Normal range of AFP in adults is <6.2 ng/ml. Infants have four or more orders of magnitudes above normal range, decreasing to the adult range after the first 2 years of life

Patient 3

The patient, a younger brother of patients 1 and 2, was 1 year and 4 months old. He was born at 37 weeks gestation, weighing 2,320 g (5th percentile) and measuring 46.0 cm (10th percentile) in length. He was carefully checked because of his familial history. At 48 days old, there were no apparent problems, but deterioration of his laboratory findings were noted. On physical examination, he weighed 3,632 g (below the 3rd percentile) and measured 51.5 cm (below the 3rd percentile) in length. There was no apparent jaundice of the skin or bulbar conjunctiva. The liver was palpable 2.0 cm below the costal margin. He showed hypoalbuminemia, cholestasis and a profile consistent with NICCD (Table 1). He was fed MCT formula supplemented with essential fatty acids (Meiji Milk Product Co., Ltd., Tokyo) as well as fat-soluble vitamins and arginine. He quickly improved and received the therapy from 55 days to 76 days of age (Fig. 1c). *SLC25A13* mutation was confirmed. At the age of 1 year and 4 months, he weighed 9,205 g (20th percentile) and measured 76.2 cm (15th percentile) in length.

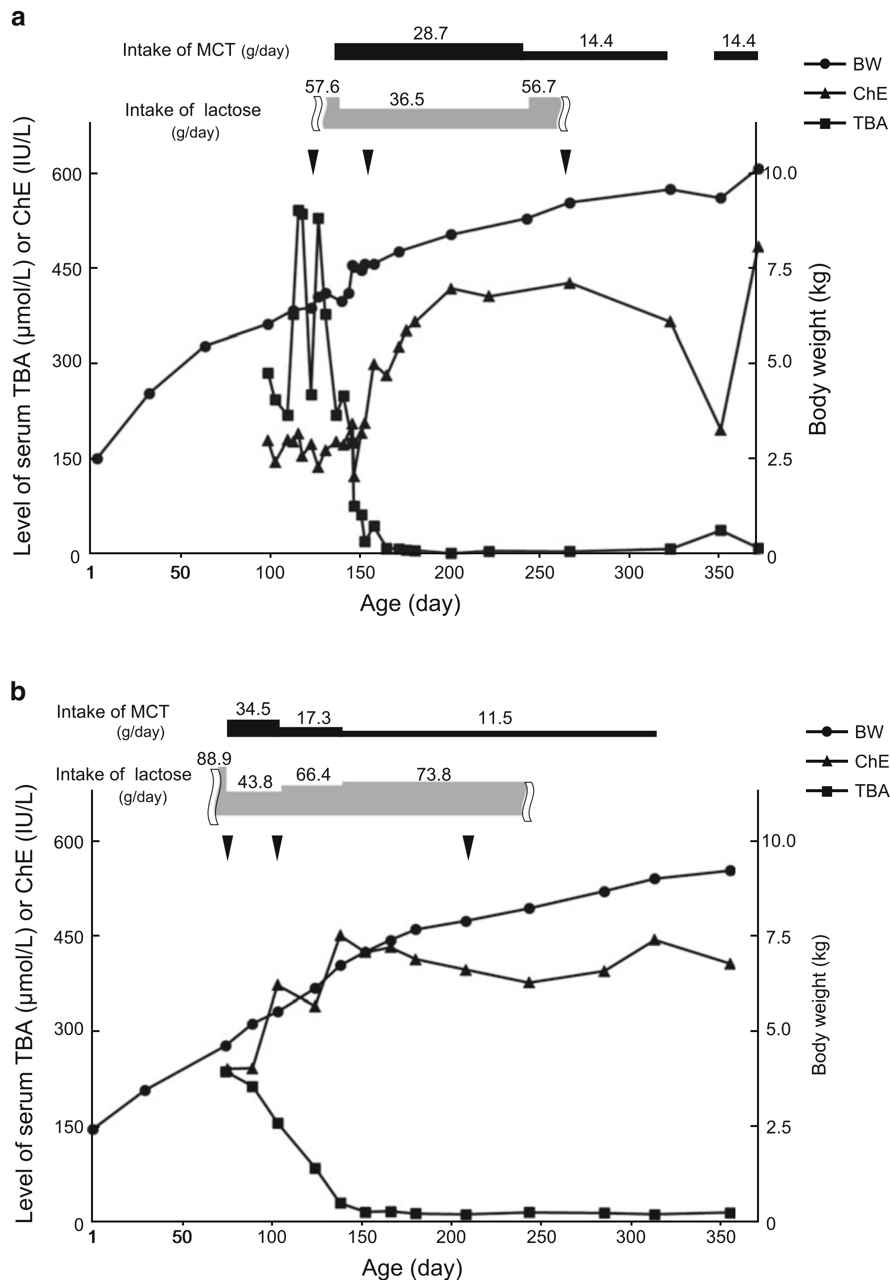

Fig. 1 (continued)

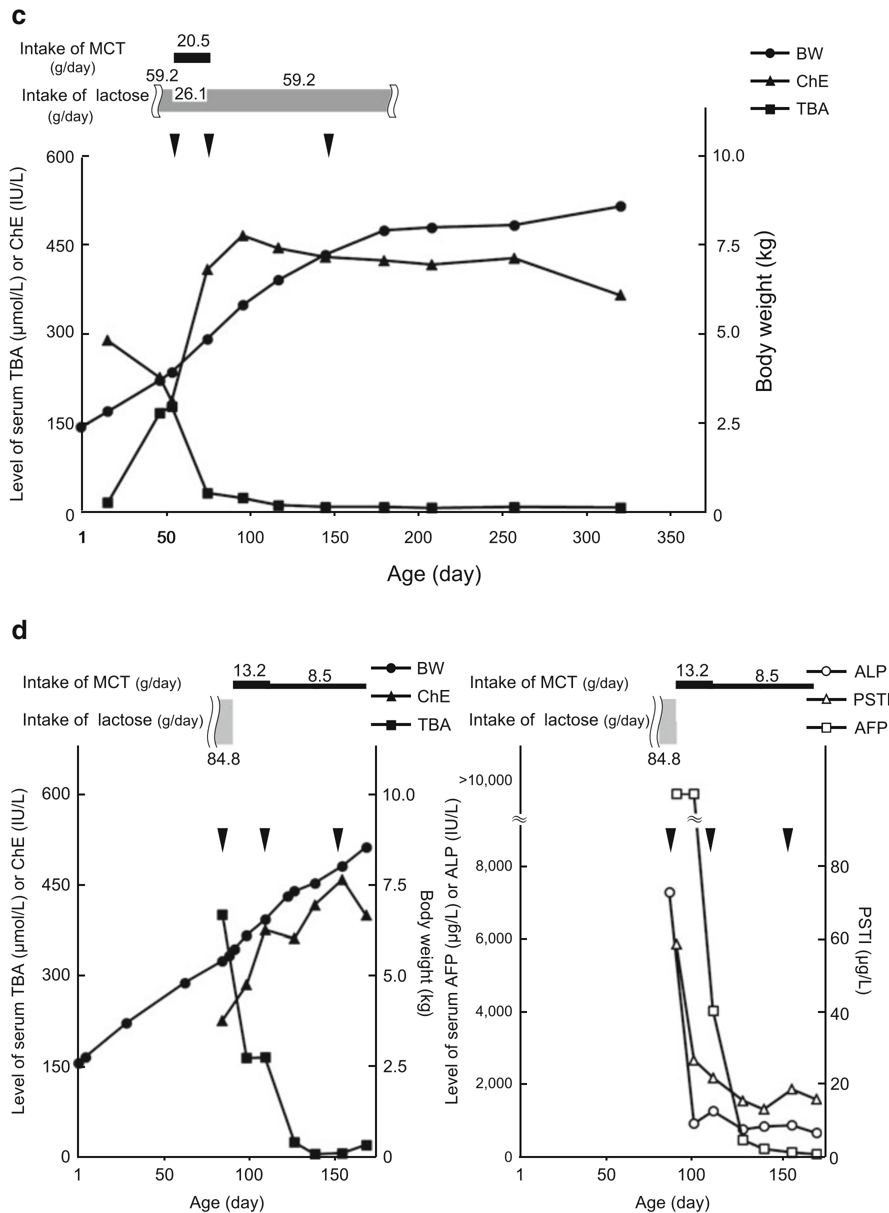

Fig. 1 Clinical courses of patients showing changes in body weight and laboratory findings. (**a**) patient 1, (**b**) patient 2, (**c**): patient 3, and (**d**): patient 4. Each patient received standard daily calorie intake appropriate for his or her age. *Closed circles*, *triangles* and *squares* represent body weight, levels of serum cholinesterase (*ChE*), and levels of serum total bile acids (*TBA*), respectively. *Open circles*, *triangles* and *squares* in Fig. 1d represent levels of serum alkaline phosphatase (*ALP*), pancreatic secretory trypsin inhibitor (*PSTI*), and alpha-fetoprotein (*AFP*), respectively. *Arrowheads* indicate the ages at testing of the patients described in Table 1

Patient 4

The patient was a 5-month-old boy. He was born at 39 weeks gestation and weighed 2,580 g (20th percentile) and measured 49.5 cm (60th percentile) in length. He had unrelated and healthy parents and a healthy older brother. At 2 months of age, he was found to have mild bilateral cataracts and was transferred to our department. He weighed 5,374 g (45th percentile) and measured 58.0 cm (45th percentile) in length. Physical examination disclosed mild jaundice and hepatomegaly, 7 cm below the costal margin. His stool was cream-colored and bulky (steatorrhea). He showed hypoalbuminemia, cholestasis, mildly elevated liver transaminases and typical changes in plasma amino acids, suggesting NICCD (Table 1). He also showed increases in the levels of alpha-fetoprotein, PSTI and blood galactose (4.6 mmol/L). Urine was positive for clinitest (reducing sugars), but negative for glucose. *SLC25A13* analysis demonstrated a compound heterozygous mutation, c.1177+1G>A>A/c.1801G>T. He was diagnosed as hav-

ing NICCD accompanied by cataracts due to galactosemia. MCT oil (13.2 g/day) was administered with a lactose-free formula (Morinaga Milk Industry Co., Ltd., Tokyo) for 2 weeks and then the dosage was decreased to 8.5 g/day. As shown in Table 1 and Fig. 1d, he rapidly responded to therapy, showing an improvement of steatorrhea and laboratory findings.

Results and Discussion

The present series comprised four cases including three siblings with NICCD treated with a lactose (galactose)-restricted and MCT-supplemented formula. Under this formula, all patients except patient 1 proportionally decreased their intake of protein, carbohydrate and fat (Table 1). Patient 1 was breast-fed before treatment and his protein intake increased under the formula. For the three sibling cases (patients 1, 2 and 3), we started the therapy at 4 months of age, 2 months of age and 55 days of age in patients 1, 2 and 3, respectively. The older patients had more severe symptoms, but all patients responded to the therapy and showed steady improvement in both cholestasis and laboratory findings (Fig. 1a–c). Therapy were required until 9 months of age, 7 months of age and 5 months of age in patients 1, 2 and 3, respectively, by which time all laboratory findings were nearly normalized. This suggests that early treatment is highly effective and long-term administration is not required. Patient 4 presented with mild cataracts possibly due to galactosemia. A favorable outcome was achieved similar to those in patients 1, 2 and 3 by lactose-free formula supplemented with MCT oil (Fig. 1d). These treatments quickly and steadily normalized the markers specific for citrin deficiency, plasma citrulline, serum alpha-fetoprotein and PTSI (Kobayashi et al. 1997).

Lactose (galactose)-free formula has been employed for the treatment of NICCD patients complicated by galactosemia. Lactose (galactose)-restricted and MCT-supplemented formula improved galactosemia in patient 2 after 2 weeks of treatment, however, we administered lactose (galactose)-free formula to patient 4 because of an apparent complication by cataracts. Patients with citrin deficiency likely have an impairment of glycolysis due to an increase in the cytosolic NADH/NAD ratio in liver. As shown in Fig. 2, one molecule of galactose will generate one molecule of NADH at the step of UDP-glucose 4-epimerase reaction in the liver and enter the glycolytic pathway. Naito et al. (2002) described that one NICCD patient with galactosemia, who showed an improvement of his clinical condition following treatment with lactose-free formula beginning at the age of 29 days, deteriorated again when challenged with a lactose-containing formula at the age 56 days, and then improved again after a return to the

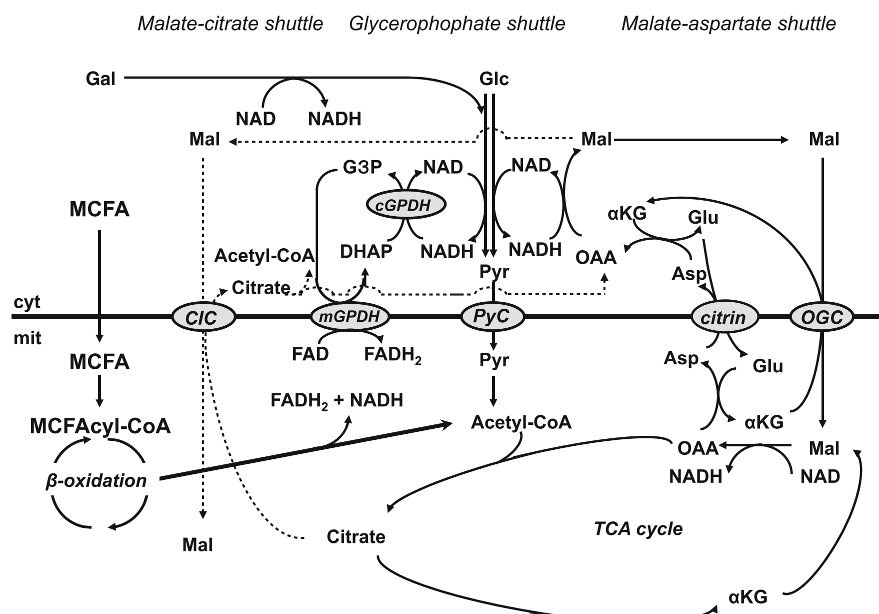

Fig. 2 Three NADH shuttles and metabolism of medium chain free fatty acids. Abbreviations: *αKG* α-ketoglutarate; *Asp* aspartate; *cGPDH* cytosol glycerophosphate dehydrogenase; *CIC* citrate carrier; *cyt* cytosol; *DHAP* dihydroxyacetone phosphate; *$FADH_2$* Reduced flavin adenine dinucleotide; *Gal* galactose; *Glc* glucose; *Glu* glutamate; *G3P* glyceraldehyde 3-phosphate; *Mal* malate; *mit* mitochondria; *MCFA* medium chain free fatty acid; *mGPDH* mitochondrial glycerophosphate dehydrogenase; *NADH* reduced nicotinamide adenine dinucleotide; *OAA* oxaloacetate; *OGC* α-ketoglutarate/malate carrier; *PyC* pyruvate carrier; *Pyr* pyruvate. *Dotted lines* indicate the malate-citrate shuttle

lactose-free formula. Considering galactose metabolism, a lactose (galactose)-restricted formula should be of value for citrin deficiency.

Formulas containing MCT have been administered to NICCD patients with cholestasis (Ohura et al. 2003). An MCT-containing formula induced rapid improvement in our patients as shown in Fig. 1 and its effect seemed to be extremely different from the effects of MCT on patients with cholestasis due to other causes, such as neonatal hepatitis. These findings suggest that MCT administration improves the basic metabolic impairment in the liver with citrin deficiency. The pathophysiology of citrin deficiency may be an energy shortage in the liver caused by an impairment of glycolysis due to an increased NADH/NAD ratio in cytosol. MCT is quickly hydrolyzed and is absorbed mainly as medium chain free fatty acids (MCFA) (Bach and Babayan 1982). MCFA reach the liver via the portal vein and are metabolized to acetyl-CoA by beta-oxidation, enhance TCA cycle activity and increase ATP levels. As shown in Fig. 2, excess acetyl-CoA is also expected to enhance the activity of the malate–citrate shuttle, which transports citrate from mitochondria to the cytosol and transfers reducing equivalents from the cytosol to mitochondria. However, experiments with ^{14}C-labeled MCT administered intravenously to rats demonstrated that the majority of MCT was retained in the liver and about 90% of the MCT was converted to carbon dioxide within 24 h (Bach and Babayan 1982; Johnson et al. 1990). The effect of MCT therapy on citrin deficiency may not occur via enhancement of the malate-citrate shuttle, but via a supply of acetyl-CoA, $FADH_2$ and NADH to hepatic cells as energy sources.

The symptoms of NICCD usually start in the neonatal period and resolve within the first year of life (Ohura et al. 2007). This clinical feature may be associated with changes in the energy source of the liver and or maturation of hepatic cells. It is interesting to note that 15–20% of the fatty acids in cord blood have eight or fewer carbon atoms (Pilz 1964). The energy source of the liver may switch from fatty acids to glucose after birth. Neonates with NICCD likely have an impairment of glycolytic pathways due to NADH accumulation in the cytosol. Furthermore, the major carbohydrate of milk is lactose, which is composed of glucose and galactose. Galactose intake may further increase the cytosolic NADH/NAD ratio in liver (Fig. 2) and act as a precipitating factor. It is also interesting to note that citrin-knockout mice do not show any significant phenotype because the mouse liver contains an active glycerolphosphate shuttle, which compensates for the defect of the malate–aspartate shuttle (Sinasac et al. 2004). In the human liver, the glycerolphosphate shuttle is not as active as that in the mouse, but can be expected to compensate to some extent. The activity of liver cytosol glycerol-3-phosphate dehydrogenase is extremely low in the fetal period and then rises to adult levels during infancy (Sadava et al. 1987). Maturation of hepatic cells and or intake of carbohydrates other than lactose after the weaning period may be associated with a resolution of symptoms within 1 year.

Histological findings in the liver of patients with NICCD showed a combination of mixed macrovesicular and microvesicular steatosis, cholestasis, a necroinflammatory reaction and iron deposition (Kimura et al. 2010). Microvesicular fatty changes are a characteristic feature of Reye syndrome or hepatic mitochondrial DNA depletion syndrome (Becroft 1966; Mandel et al. 2001), suggesting a low energy state of the liver. The pathogenesis of citrin deficiency is likely an energy shortage of the liver due to impaired glycolysis.

Lactose (galactose)-restricted and MCT-supplemented formula can improve energy metabolism of the liver and is recommended for patients with NICCD and possibly for the patients with CTLN2.

Acknowledgements We would like to thank the late Dr. Keiko Kobayashi (Kagoshima University) for analysis of *SLC25A13* in patients 1 and 2.

Take-Home Message

Lactose (galactose)-restricted and medium-chain triglyceride-supplemented formula is a promising therapy for neonatal intrahepatic cholestasis caused by citrin deficiency.

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CASE REPORT

Fatal Myocardial Infarction at 4.5 Years in a Case of Homozygous Familial Hypercholesterolaemia

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Abstract Management of homozygous familial hypercholesterolaemia is notoriously difficult. For these patients, LDL apheresis is considered the treatment of choice. Treatment initiation is advocated generally from the age of seven years onwards (Thompson et al., Atherosclerosis 198:247–255, 2008). Here, we present the case of a young girl from a large inbred family of Turkish descent with homozygous familial hypercholesterolaemia and fatal outcome at the early age of 4½ years.

In conclusion, this case suggests that management of homozygous familial hypercholesterolaemia may require earlier and more aggressive treatment, including LDL apheresis before the age of seven years.

Introduction

Familial hypercholesterolaemia (FH) is the most common autosomal dominant condition. It is due to defective LDL receptor protein giving rise to increased plasma cholesterol and leading to premature cardiovascular morbidity. In order to allow early detection and treatment of the condition, current guidelines recommend lipid screening in children, although no consensus exists regarding the best screening schedule (Daniels et al. 2008; Koletzko et al. 2007).

The homozygous form of FH (HFH) is rare and manifests early in life with severe hypercholesterolaemia and typical skin lesions. If untreated, it leads to early atherosclerosis and cardiovascular death typically in the second to third decade. The disease is notoriously difficult to manage. The medical treatment combining several cholesterol-lowering drugs, usually a statin with the intestinal cholesterol-absorption inhibitor ezetimibe, does not result in satisfactory reductions of either total or LDL-cholesterol levels. Several other therapeutic approaches have been proposed such as liver transplantation and more recently several pharmacological approaches, such as MTP inhibition, as well as gene therapy, all still being experimental. For the last 30 years or so, LDL apheresis has been used and progressively become a mainstay in the management of HFH.

Case Presentation

The patient (born in 1996) was the first child of consanguineous parents (first-degree cousins), both with known hypercholesterolaemia (cholesterol levels not known) from a widespread and repeatedly inbred family, originating from Turkey and with an extensive history of premature cardiovascular morbidity and mortality as early as the third and fourth decades of life. Several family members have homozygous hypercholesterolaemia with pretreatment levels of total cholesterol above 20 mmol/L. A year before our patient's birth, the parents had received genetic counseling regarding the risk of heterozygous and homozygous transmission of FH.

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Pregnancy and birth – at term with normal birth weight – had been uneventful, a right club-foot had been operated successfully at 9 months, otherwise no past history of note.

The girl was referred to our department at the age of two years by her paediatrician for investigation of multiple xanthomas at her wrists, elbows and right knee. At presentation, she had no corneal arcus lipoides and, apart from the xanthomas, clinical examination was normal. Her total cholesterol was 29.2 mmol/L (1,124 mg/dl; normal range: 2.9–5.2), HDL-cholesterol was 0.70 mmol/L (normal: >1.20), the total to HDL cholesterol ratio was 42, and triglycerides were marginally increased at 1.8 mmol/L (normal <1.55).

The clinical and biochemical picture suggested a homozygous genotype of FH and the patient was put on a restrictive diet without red meat, chocolate and butter, instead olive oil and skimmed milk. So far, no medical treatment was prescribed and it was decided to defer initiation of LDL apheresis because of the invasive nature of the treatment and the young age of the patient. While on dietary treatment only, her total cholesterol decreased to levels around 25 mmol/L.

At 3 years of age, she had normal growth and BMI, as well as psychomotor development, and her parents reported no symptoms suggestive of cardiovascular disease (CVD). She had, however, developed an arcus lipoides corneae and the xanthomas had extended further to the areas of both axillae and the left Achilles tendon. While under dietary measures only, her total cholesterol had decreased to 25 mmol/L, HDL cholesterol 0.80 mmol/L and liver enzymes were normal. Lipoptrotein (a) was within the normal range (<300 mg/L). Echocardiography and ECG at rest were normal.

At each follow-up visit at our out-patient clinics, the xanthomas extended progressively over limbs to the hands and calves, buttocks and the head. Total cholesterol was always between 25 and 26 mmol/L, HDL cholesterol $\leq$ 0.8 mmol/L and triglycerides $\geq$ 2.50 mmol/L. A 24-h ECG was normal.

At around 3½ years, a treatment with atorvastatin 5 mg daily (= 0.25 mg/kg/d) was started. This treatment slowed down the apparent progression of the skin lesions and was paralleled by a consistent decrease of total cholesterol from 25 to around 20 mmol/L, as well as HDL cholesterol to 0.6 mmol/L at the following visits. Liver and muscle enzymes remained normal. At age 4.3 years (1½ months before death), a newly appeared systolic heart murmur was found to be due to mitral regurgitation with left heart dilation. On echocardiography, the main trunk of the left coronary artery was well visualized and appeared normal. No abnormality of the aortic valve or root was noted. It was nevertheless decided to start LDL apheresis treatment as soon as possible and the surgical insertion of a dialysis catheter was scheduled, which, for non-medical reasons, was again postponed for some six months. Around that time, the patient began to complain about recurring abdominal pain. Microhaematuria prompted emergency consultation with the nephrologist, who, faced with a child in reduced general condition, with abdominal pain and dyspnoea, suspected (inferior) myocardial infarction. The diagnosis was confirmed by repeated elevation of cardiac enzymes (troponin-I up to 11.5 mg/L [Normal <0.6] and CK-MB up to 20.7 mg/L [<4.0]). The ECG showed left ventricular (LV) hypertrophy and deep Q-waves in derivations III and aVF. The angiography of the coronary arteries showed a severe three-vessel disease with extensive complete and sub-complete stenoses involving the peripheral segments (Fig. 1).

On the following day, the patient developed ventricular flutter that rapidly progressed to refractory asystolic cardiac arrest and exitus lethalis despite immediate intensive care and full medical treatment. The parents declined post-mortem examination. Genetic analysis showed the homozygous mutation c.1849delA (p.Val597Tyrfs45X). The mutation has not been described so far; it presumably leads to reading frame shift creating a premature stop codon and thus to a truncated protein (Laboratoire de génétique moléculaire, CHU de Liège, Belgium).

Discussion

Patients with homozygous hypercholesterolaemia develop clinical CVD considerably earlier than heterozygous patients, with first symptoms generally appearing during second and third decades of life. Children with manifest CVD before 10 years of age are the exceptions, and in one cohort, no abnormalities in either coronary angiography or non-invasive test results were found in children younger than 6 years (Kolansky et al. 2008). Very few cases of fatal cases before age 10 years have been published (see Table 1 for review of the published cases). The patient presented here reminds us of the importance of adapting the general therapeutic approach to individual patients with more severe disease.

Current treatment guidelines consider LDL apheresis as the treatment of choice for HFH. They stipulate treatment initiation at around 7 years of age (Thompson et al. 2008). Our patient showed a very severe course of disease and fatal outcome at the early age of 4½ years, suggesting that in her case, more aggressive management would have been warranted and the AHA recommendation that “treatment should be instituted as soon as possible” should have been followed by the letter (Kavey et al. 2006), including statin and apheresis treatment. Importantly, her total cholesterol level at diagnosis was close to 30 mmol/L and remained

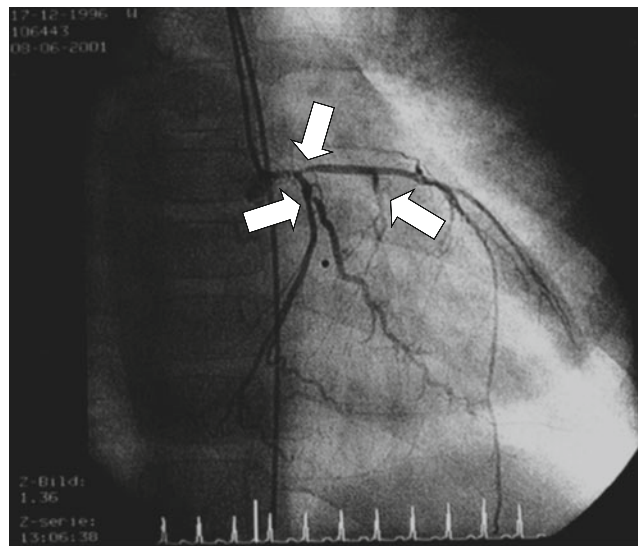

Fig. 1 Coronography showing three-vessel disease (*arrows* point to stenoses)

elevated above 20 mmol/L despite drug treatment, leading to *"fulminant"* coronary artery disease. In the first place, aggressive medical treatment is certainly warranted. In our case, this strategy was hampered by the parents' reluctance to administer an off-label drug to their child.

Other members of the same extended family with clinical homozygous hypercholesterolaemia – presumably carrying the same mutation– had LDL apheresis started at later ages, without clinical signs of CVD, despite pretreatment levels of total cholesterol above 20 mmol/L for some of them. This remarkable clinical heterogeneity within a large inbred family carrying the same mutation has been repeatedly recognized (Ferrières et al. 1995; Heiberg and Slack 1977) and is presumably due to the considerable impact of genetic modifiers (e.g., Abifadel et al. 2009; Koeijvoets et al. 2009) rather than the chronically acting risk factors of the adult patient. In our patient, no additional risk factors were found. There were repeated measures of normal blood pressure, fasting glucose, Lp (a) and renal function (normal plasma creatinine and urea, no proteinuria). Post-mortem analysis revealed a heterozygous carrier state of the thermo-labile C677T-variant of the MTHFR gene. Although a heterozygous state is usually not associated with an increased homocysteine and thrombotic risk, one cannot exclude a contribution to the dismal course in this patient. Unfortunately, homocysteine was never measured.

In the literature, several cases of very early initiation of LDL apheresis have been reported. National registries from the USA (Hudgins et al. 2008), France (Palcoux et al. 2008) and Italy (Stefanutti et al. 2001) list in their cohorts patients as young as 3 years of age at treatment initiation. Coker et al. (2009) from Turkey started apheresis even in a 2 years old patient. Difficulty with acceptance of the invasive nature of the treatment has contributed to postpone apheresis in our patient, as well as in others (Awan et al. 2008). Interestingly, however, in most of the small children mentioned, vascular access was achieved with a peripheral venous cannula, and not with a surgically inserted central line.

Other, equally invasive approaches, such as liver transplantation (Moyle and Tate 2004), or portocaval shunt, have been proposed, but have not found general acceptance.

Less invasive alternatives are not available, yet: Recently, one promising approach among others (for review see Lilly and Rader 2007), inhibition of microsomal triglyceride transfer protein, has been shown to be effective in reducing LDL cholesterol levels, but also leads to serious side effects that preclude its clinical use (Cuchel et al. 2007). Gene therapy is still experimental.

The homozygous mutation c.1849delA (p.Val597-Tyrfs45X), found in our patient, has not been described before. The deletion of one base pair leads to a frameshift. A study investigating genotype–phenotype correlation has found that frameshift mutations were associated with higher levels of LDL-cholesterol than those found in missense mutations (Graham et al. 1999). As the biochemical features and the clinical course make a receptor-negative phenotype in our case most likely, we have not performed functional studies of the mutated LDL receptor.

The considerable clinical heterogeneity has prompted a search for markers that predict severity of cardiovascular involvement. Awan et al. (2008) examined a patient diagnosed with phenotypic homozygous familial hypercholesterolaemia who had similar cholesterol levels as our patient: his pretreatment total cholesterol was 30.2 mmol/L and decreased to 24.0 under medication only treatment without evidence of vascular disease or coronary artery

Table 1 Cases of early death (<10 years) from CVD in hoFH reported in literature

Reference	Type of report	Case documentation	Receptor function	LDL-R gene mutation	Gender	Age-at-death (years)	Tot chol (mmol/L)	Country/ethnic origin	Additional CV risk factors
Widhalm et al. (2011)	Clinical image	A	n.d.	W556R[a]	M	4	21.66	Austria/Turkish	n.k.
Naoumova et al. (2004)	Review	B	neg/null	E387K[a]	F	3	24.4	UK/?	n.d.
Al-Shaikh et al. (2002)	Case series	C, B, G	neg/null	Fr-Canadian I[a]	M	3.1	24.57	Canada/French	n.d.
Rose et al. (1982)	Case report	C, B, A	n.d.	n.d.	M	3	24.65	Canada/?	n.k.
Seftel et al. (1980)	Case series	B	n.d.	n.d.	M	6	18.5	South Africa/ Afrikaner	n.d.
Seftel et al. (1980)	Case series	C, B	n.d.	n.d.	F	6	18.7	South Africa/ Afrikaner	n.d.
Kawahara et al. (1973)	Case report	A	n.d.	n.d.	F	4	24.9	Japan	n.d.
Fredrickson and Levy (1972)	Book Chapter	No details	n.d.	n.d.	?	1.5	?	South Africa/ Caucasian	n.d.
Watanabe et al. (1968)	Case report	A	n.d.	n.d.	M	4	22.8	Japan	n.d.
Bloom et al. (1942)	Case series	C (history)	n.d.	n.d.	F	6.5	n.d.	USA/Syrian	n.k.

n.d. not done/determined, *n.k.* none known (clinical exam), *A* Autopsy, *B* Biochemistry, *C* Clinical work up, *G* Genetic data

[a] Homozygous

disease by the age of 10 years. His cholesterol-year score was estimated at 156 mmol-year/L. This score correlates with calcific atherosclerosis and was proposed by Schmidt et al. (1996) as a simple means to quantify the risk of atherosclerosis. In their cohort, calcific atherosclerosis was not observed until the cholesterol-year score exceeded 260 mmol-year/L. The score of our patient amounts to around 120 mmol-year/L, which is well below the proposed limit of 260. The cholesterol-year-score may be used in heterozygous FH, but does not apply to the homozygous patient, as already stated by Rallidis et al. (1998). They also observed that atherosclerotic lesions almost always involve the aortic root in the homozygote as opposed to heterozygous patients. This specific pathology of homozygous hypercholesterolaemia "appears to be related to the early exposure to high cholesterol levels, and not to the overall burden of cholesterol exposure" (Koh 2005). The relationship between actual levels of plasma cholesterol and progression of coronary artery disease is not clear (see e.g., Sprecher et al. 1985 or Moorjani et al. 1989).

On clinical examination, the high cholesterol levels were mirrored by extensive xanthomatous skin lesions. Increased Achilles tendon thickness and corneal arcus both due to tissue cholesterol deposition are correlated with severity of calcific atherosclerosis (Zech and Hoeg 2008). The appearance of a significant systolic heart murmur due to mitral regurgitation and ventricular dilatation heralded the progressive ischaemia of the myocardium. The murmur was a strong sign of the clinically otherwise silent ongoing myocardial infarction implicating the posterior pillar of the mitral valve. The ensuing mitral regurgitation may well have worsened coronary insufficiency and precipitated the progression of myocardial infarction. Several autopsy studies of hoFH patients who had died in their first or second decade report extensive subendocardial scarring testifying to chronic ischaemia prior to the lethal event (Rose et al. 1982; Sprecher et al. 1985). The diffuse three-vessel type of CAD in our patient therefore may have predisposed her for subendocardial infarction; additional risk factors, namely LV hypertrophy, LV dilation and mitral regurgitation were brought about by papillary muscle infarction (Davies 1977).

Homozygous FH patients typically develop cholesterol deposits on the aortic valve and osteal stenosis of the coronary arteries. In our patient, no aortic valvular or supravalvular abnormality was found on echocardiography, either at the age of 3 or at almost 4½ years. At that latter time, however, the newly appeared mitral regurgitation was prominent. Kolansky and coworkers found poor concordance between evidence of CVD from coronary angiography and results from non-invasive tests. Only the presence of mild-to-moderate aortic valve regurgitation

on echocardiography was significantly associated with angiographic coronary stenosis (Kolansky et al. 2008, see also Kawaguchi et al. 1999). This poses the question of adequate follow-up investigations, as both electro- and echocardiography can miss early signs of CVD, even in experienced hands. Conventional coronary angiography, on the other hand, is an invasive procedure with its own inherent risk, as reported by Marais et al. (1990) in another fatal case of homozygous FH, and should be reserved for the investigation of symptomatic CVD. In our case, death from intractable ventricular fibrillation appeared unrelated to angiography, as it occurred several hours later, after uneventful extubation on the ICU ward. Santos et al. (2008) have suggested CT angiography as a reliable non-invasive method. Even though it is not designed for children below 10 years, it might prove a valid procedure in the future for older patients.

Conclusion

The rapid progression of coronary artery disease and fatal outcome at the early age of 4.5 years in our patient with homozygous familial hypercholesterolaemia suggest that treatment should be initiated as soon as possible after diagnosis. Early initiation of LDL apheresis, in conjunction with high dose medical treatment combining a statin and ezetimibe, is advocated, in order to rapidly achieve the therapeutic goal of total (mean) cholesterol < 9 mmol/L (Thompson et al. 2010). This treatment approach has proved to be safe and highly efficient, including in small children.

Synopsis

Clinical and biochemical diagnosis of homozygous familial hypercholesterolaemia should prompt treatment initiation, including both pharmacological and LDL apheresis therapy, at any age as soon as possible in order to reach rapidly the treatment goals (Total cholesterol < 9 mmol/L).

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RESEARCH REPORT

The Oral Health Needs of Children, Adolescents and Young Adults Affected by a Mucopolysaccharide Disorder

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Abstract *Background*: As one of the 4 United Kingdom national centres for Lysosomal Storage Disorders, Birmingham Children's Hospital (BCH) cares for 49 patients with a mucopolysaccharide (MPS) disorder. Presently, their oral health needs have never been fully investigated. Enzyme therapies are revolutionising medical treatment, and will prolong life expectancy. Therefore, oral health and its impact on quality of life are increasingly important.

Hypothesis: Patients with an MPS disorder have greater oral health needs in comparison with the general population.

Design: Forty-six patients with an MPS disorder were investigated for caries, gingival health and enamel opacities. Questionnaires to establish the utilisation of dental services and assess the perceived difficulties in achieving adequate oral health care were completed.

Results: Patients with an MPS disorder have specific oral health needs depending on the type of MPS experienced. This study found 76% of patients with MPS IV had experienced dental caries and they all showed evidence of a generalised unspecified enamel defect. Subjects with MPS I, II and III did not have an increased caries rate; 50% of the study group had received dental treatment in the past, of which 74% (17, $n = 23$) required a general anaesthetic.

Conclusion: The MPS disorders can have a significant effect on the developing dentition. Subjects with MPS IV do have an increased oral health need in comparison with the general population and the other MPS groups due to their increased caries rate and enamel defects. Further research is required to fully assess their needs and investigate any structural tooth defects.

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Introduction

The mucopolysaccharide (MPS) diseases comprise a set of rare inherited disorders caused by the deficiency of particular lysosomal enzymes, which determine the type of MPS exhibited. A lysosomal enzyme deficiency results in disturbances in the catabolism of glycosaminoglycans (mucopolysaccharides) and leads to increased storage of mucopolysaccharides in the lysosomes of cells in various tissues throughout the body. The clinical effects are wide ranging and are dependent on the type of stored mucopolysaccharide (Wraith 1995). Severe physical and neurological developmental problems can arise including: abnormal upper airways, restrictive lung disease, skeletal abnormalities, cervical spine deformities and behavioural difficulties (Kircher et al. 2007; Kumar and Clark 1998). The MPS diseases include seven different disorders classified into various sub-types each exhibiting specific phenotypes (Table 1) (Wraith 1995; Kircher et al. 2007; Santos and Hoo 2006; Soong et al. 1988). All MPS diseases are caused by a recessive gene inheritance with the exception of MPS II or "Hunters disease", which is X-linked (Wraith et al. 2008).

As a consequence of the multiple effects of each disease, patients with an MPS disorder are often managed in a multidisciplinary setting comprising specialised

Table 1 Classification of mucopolysaccharide diseases

Syndrome (OMIM ref)	Eponym	Enzyme deficiency	Stored material
MPS I H #607014	Hurler syndrome	Alpha-L-iduronidase	Dermatan sulphate Heparan sulphate
MPS I S #607015	Scheie syndrome	Alpha-L-iduronidase	Dermatan sulphate Heparan sulphate
MPS II A +309900	Hunter syndrome (severe)	Iduronate sulfatase	Dermatan sulphate Heparan sulphate
MPS II B +309900	Hunter syndrome (mild)	Iduronate sulfatase	Dermatan sulphate Heparan sulphate
MPS III A #252900	Sanfillipo syndrome A	Sulfamidase	Heparan sulphate
MPS III B #252920	Sanfillipo syndrome B	N-acetyl-alpha-glucosaminidase	Heparan sulphate
MPS III C #252930	Sanfillipo syndrome C	N-acetyl-transferase	Heparan sulphate
MPS III D #252940	Sanfillipo syndrome D	N-acetylglucosamine-6-sulfatase	Heparan sulphate
MPS IV A #253000	Morquio syndrome A	n-acetyl-galactosamine-6-sulfatase	Keratan sulphate
MPS IV B #253010	Morquio syndrome B	Beta-galactosidase	Keratan sulphate
MPS VI A #253200	Maroteaux–Lamy syndrome A	Arylsulfatase B	Dermatan sulphate
MPS VI B #253200	Maroteaux–Lamy syndrome B	Arylsulfatase B	Dermatan sulphate
MPS VII #253220	Sly syndrome	Beta-glucuronidase	Dermatan sulphate Heparan sulphate Chondroitin sulphate
MPS IX #601492	No eponym	Hyaluronidase	
MSD #272200	Multisulphatase deficiency	Multiple sulphatase deficiencies	Numerous materials including: sulfatides, glycosaminoglycans, sphingolipids, steroid sulphates

paediatric teams including cardiology, neurology, psychiatry, orthopaedics, physiotherapy and ophthalmology. Recently, significant advances in enzyme therapies have revolutionised the medical management of a number of MPS disorders with hopes of substantially prolonging life expectancy and the interim quality of life. As a major contributor to quality of life, the management of the oral health of these patients is becoming increasingly important (Sheiham 2005).

A wide range of craniofacial and dental abnormalities have been described in the literature as occurring in patients with an MPS disorder, which may or may not predispose to an increased risk of dental disease. Children with an MPS disorder would also be considered to be a population with various special healthcare needs depending on the severity of their disease. Those with MPS I, II and III will usually have significant cognitive impairment and behavioural difficulties, whereas those with type IV disease will be within the normal range of intelligence (Kircher et al. 2007). Various oral health studies over the world acknowledge that vulnerable populations often have particular oral health needs in comparison with the general population. Many studies show a higher prevalence of caries, malocclusions, and poorer periodontal health. Access to dental care is often reported to being more difficult and therefore there is a larger unmet restorative need. (Lewis 2009; Shaw et al. 1986; Purohit et al. 2010). The literature to date investigating MPS disorders suggests there are alterations in the structure of the enamel and dentine and in particular the enamel/dentine junction in MPS type I (Guven et al. 2008) and type IV presentations (Lustmann 1978) however, the extent and significance of this is unclear. (Table 2). Patients with an MPS disorder represent a challenge for dentists to treat due to the complicated medical and physical disabilities. Many, and in particular those with MPS III (Kircher et al. 2007) also exhibit behavioural problems which are progressive with the disease process. When general anaesthesia is required to deliver, essential health-care surgical units with adequate intensive care facilities and paediatric anaesthetists are required as patients with an MPS disorder present complex anaesthetic difficulties and particularly with intubation (Wraith 1995). The management of dental and

Table 2 Dental anomalies described in the literature to date are described below

Author/date	MPS disease	Number	Reported dental anomalies
McGovern et al. (2008)	MPS I	25 cases	Delayed dental development Spaced dentition Hypodontia Microdontia
Guven et al. (2008)	MPS I	1 case	Thick gingivae Hypoplastic teeth primary teeth Cysts around unerupted teeth Obliterated pulp canals Taurodont molars Hypoplastic mandibular condyles
Thomas and Tandon (2000)	MPS I	1 case	Large tongue Thick gingivae Spaced dentition Delayed eruption of permanent dentition
Keith et al. (1990)	MPS I Hurler-Scheie	2 cases	Broad maxilla and forehead Minor hypertelorism Broad chin Short ramus and deficient condyles Broad dental arches Dentigerous cysts Gingival hyperplasia Macroglossia
Schmidt et al. (1987)	MPS I	2 cases	Short mandibular necks Flattened condyles Hypertelorism Spaced teeth Dentigerous cysts
Gardner (1971)	MPS I and II	12 cases	Radiolucent mandibular lesions (7 cases) Spaced dentition Histologically normal teeth Hyperplastic gingivae (6 cases)
Worth (1966)	MPS I	24 cases	16 had condyle abnormalities All had small teeth, some peg shaped Spacing of the teeth in most cases All had short narrow rami Many had a redruded chin 13 showed mandibular bone destruction
Hopkins et al. (1973)	MPS II	2 cases	Anterior open bite Large protuberant tongue Thickened lips Limited neck extension and mouth opening (1 case) Coronoid hyperplasia Abnormal condyles Increased bone density Low caries incidence Spaced dentition
Webman et al. (1977)	MPS III	1 case	Obliteration of pulp canals in primary and permanent teeth
Oncag et al. (2006)	MPS IV	1 case	Flattened nasal bridge Flared nasal alae Prominent lower face Broad mouth Anterior open bite
Kuratani et al. (2005)	MPS IV	1 case	Spaced dentition Thin enamel
Rolling et al. (1999)	MPS IV	3 siblings	Pointed cusps Spade-shaped incisors Thin enamel Pitted buccal surfaces Condylar resorption

Table 2 (continued)

Author/date	MPS disease	Number	Reported dental anomalies
Smith et al. (1995)	MPS IV	8 cases	Delayed eruption Hyperplastic tooth follicles Unerupted and impacted permanent teeth
Kinirons and Nelson (1990)	MPS IV	9 cases	Thin enamel Microdont and opaque teeth Pointed cusps of molars Concave occlusal surfaces Absence of normal fissure patterns Spade-shaped incisors
Lustmann (1978)	MPS IV	2 cases	Lab examination of teeth only: Partial calcification at the enamel- dentine membrane
Levin et al. (1975)	MPS IV	12 cases	Prominent lower face Flat nasal bridge Broad flat palate DMF index average 4.5 Spaced anterior teeth, yellow colour Tapered teeth and pointed cusps Defective enamel in 10 patients
Sela et al. (1975)	MPS IV	2 siblings	Thin enamel Molar cusps small and pointed Generalised enamel hypoplasia Tongue showing geographic features Flattened condyle
Alpoz et al. (2006)	MPS VI	1 case	Enlarged tongue Delayed eruption of anterior permanent teeth Enlarged alveolar process High arched palate with a deep mid groove Unerupted teeth with dentigerous cysts
Smith et al. (1995)	MPS VI	2 cases	Delayed tooth eruption Thickened dental follicles
Roberts et al. (1984)	MPS VI	1 case	Macroglossia Hyperplastic fibrotic gingivae Dentigerous cysts

periodontal disease in patients with an MPS disorder can be extremely challenging in the conscious patient and as such poor oral health not only impacts on quality of life (Sheiham 2005) but can necessitate high risk anaesthesia. To date the limited literature available regarding the oral health of this group of patients is provided by sporadic case reports and epidemiological studies of small cohorts (Table 2). Therefore, the aim of the current investigation was to systematically assess the oral health of all patients under treatment for MPS disorders in a major metabolic disease referral centre.

Materials and Methods

A cross-sectional investigation was undertaken in the Department of Clinical Inherited Metabolic Disorders at Birmingham Children's Hospital, United Kingdom between December 2008 and July 2009. Ethical approval was obtained through the UK National Research Ethics Service prior to the commencement of the study (REF: 08/H1202/119). All patients diagnosed with an MPS disorder and under care of the metabolic department were invited to participate resulting in 49 invitations. If a patient was too un-cooperative to successfully complete an element of the oral examination, the patient was excluded from this part of the study. Attempts were made to recruit an unaffected sibling control group but unfortunately compliance was poor and therefore the national epidemiological data from the most recent child dental health survey in 2003 (White and Lader 2004; Pitts and Harker 2005) matched to age groups was referred to for control data.

Clinical oral examinations were undertaken by a single Paediatric Dentist trained in epidemiological examinations. A validated screening tool using the criteria of diagnosis of the British Association for the Study of Community Dentistry (BASCD 2005) was employed. Due to practical constraints of the multidisciplinary clinic setting, the oral examination was performed under artificial lighting, either on a hospital trolley or where the child was unable to co-

operate for this, in their wheelchair. Data recorded included a dental charting which detailed the number of, decayed, missing and filled teeth (coded as DMFT for adult dentition and dmft for deciduous dentition). The mouth was subsequently divided into sextants and the gingival health was qualified by observing gingival redness (an indirect measure of gingival inflammation) and visible plaque and calculus representative of the level of oral hygiene maintenance. The presence of any gingival overgrowth, enamel opacities and structural anomalies of the dental hard tissues were also recorded. A simple questionnaire (simplified and modified from a previous audit) was provided to the parents or guardians in order to establish the current awareness and uptake of available primary and secondary dental care services and to assess the perceived difficulties in achieving adequate oral health care for their child. These were completed by the parents/guardians independently in the waiting area before their appointment; any questions were answered by the research nurses.

Results

Of the 49 invited patients with an MPS disorder, 46 agreed to take part in the study. This group comprised of 22 females and 24 males, aged between 2 and 28 years old. The groups included 5 subjects with MPS I, 6 with MPS II, 15 with MPS III, 17 with MPS IV, 2 with MPS VI and 1 patient with multi-sulphatase deficiency. All patients with MPS I, II and VI were currently receiving enzyme replacement therapy.

Experience of Dental Caries

All 46 patients were examined for dental caries. Overall 23 (51%) patients had no decayed, missing or filled teeth, 22 (49%) had decay experience. The level of dental decay was inconsistent between the different groups of MPS disorders with the patients with an MPS type IV disorder having experienced an increased level of dental caries (Table 3).

The MPS I and IV groups did experience considerable dental caries. Group IV ranged from 3 to 28 years old and the number of decayed teeth ranged from 0 to 11. The average dmft (deciduous dentition) was 3.21 (45/14, $n = 14$ with teeth in the primary dentition) and the average DMFT (permanent dentition) was 1.67 (14/12, $n = 12$ with erupted first permanent molar teeth). The total number of dmft and DMFT is over 17 because some subjects are in the mixed dentition and therefore the coding system allows them to have both a dmft and DMFT. Figures 1 and 2 show the level of dental decay experienced by the study group as a whole and the type IV group specifically against the Child Dental Health survey levels of decay in both the West Midlands and the UK as a whole for both the primary and secondary dentition. Both the West Midlands data and the UK data are useful for comparison because the study group has been recruited from a regional centre and therefore not all the recruited patients will reside in the West Midlands.

Gingival Health

As a whole group (40 patients were examined, 6 were poorly co-operative and accurate observations were not possible): 17 (43%) exhibited marginal gingivitis, 24 (60%) had evidence of plaque deposits and 19 (48%) had evidence of calculus. Aged matched comparisons were performed

Table 3 Decay experience stratified according to MPS diagnostic group

Research group	No of patients with caries experience	Total patients in group	% patients experiencing dental caries
MPS I	3	5	60
MPS II	1	6	17
MPS III	4	15	27
MPS IV	13	17	76
MPS VI	0	2	0
MSD	1	1	100

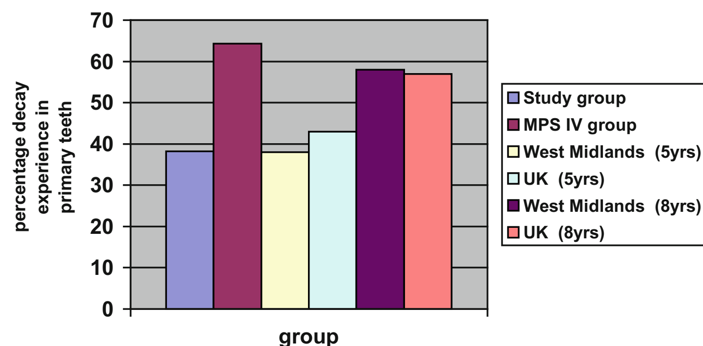

Fig. 1 A graph comparing the level of decay experience in primary teeth of the study group, MPS IV and the general population

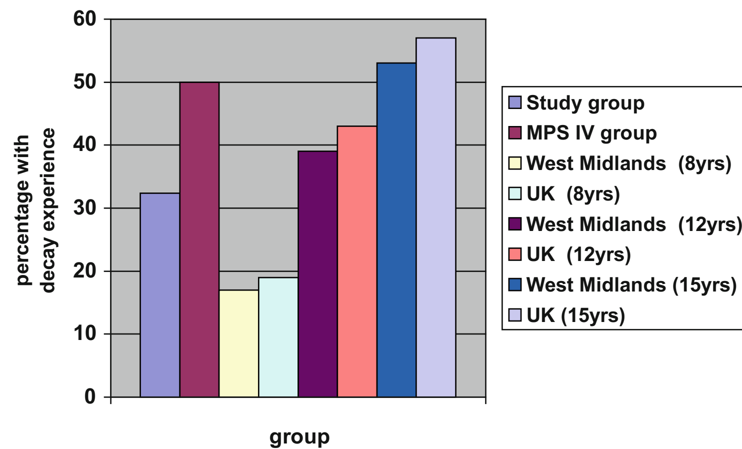

Fig. 2 A graph showing the level of decay experience in the adult dentition of the study group, MPS IV and the general population

Table 4 Child dental health survey 2003 results

Age	Gingivitis %	Plaque %	Calculus %
5	32	50	5
8	63	76	16
12	65	73	20
15	52	63	32
Study group	43	60	48

against National epidemiological data and increased levels of plaque and calculus deposits were observed in the study group but no increase in gingivitis was evident (White and Lader 2004). Table 4 shows the Child dental health survey 2003 results.

Opacities and Enamel Defects

Enamel opacities were recorded in patients with permanent incisor teeth only (19 patients) of which 10 patients exhibited opacities on their teeth. Although not included in the criteria of diagnosis, it was noted that all patients with MPS IV exhibited enamel defects. In both the primary and permanent dentition, the defects gave the appearance of generalised white chalky enamel and the teeth had pronounced sharp pointed cusps.

Oral Health Maintenance

The parents or carers of all patients ($n = 46$) responded to the oral health maintenance questionnaire. Despite being subject to both recall bias and the possibility that some of the answers given were provided to “please the researchers”, it suggested some interesting thoughts worthy of mentioning in this report; 48% of the study population considered themselves to regularly access dental care for their child. Of the 46 patients, 23 had visited a dentist in the preceding 6 months and a further 12 within the past year. Five parents reported only accessing dental care when they believed their child was in pain and of concern, 9 reported never having visited the dentist. Of the reported regular dental attendees, 47% accessed their care through the Children’s Hospital, 44% in a local community setting and 9% visited both services. When questioned whether they had ever received advice on how to maintain their child’s oral hygiene 67% reported they had and 59% reported to have received advice regarding preventing tooth decay. The parent/guardians recall of patient brushing habits were as follows: 2 patients never brushed their teeth, 1 managed once a week, 4 once or twice a week, 24 once a day, 13 twice a day and 2 reported to brush more than twice a day. It was reported that 23 patients had experience of having dental treatment carried out of which 17 patients required a general anaesthetic for this purpose. Parents commented that cleaning their child’s teeth can be extremely difficult and that they would be concerned if their child required dental restorations.

Discussion

The series of 46 patients with an MPS disorder described is the largest cohort to undergo a detailed oral health assessment reported in the medical and dental literature to date. However given the rarity of the diseases and the large number of clinical and patient-based variables, it was accepted that the power of any statistical enquiries to compare with national epidemiological data for unaffected individuals would be too weak for a valid analysis. At the outset, attempts were made to recruit sibling controls to

account for familial dietary and hygiene patterns but unfortunately few siblings, although invited, attended the dental examination. In order to provide the most valid comparison with national epidemiological data, where possible the conditions of examination matched those of the 2003 UK Child Dental Health Survey.

The group of patients described had significant oral health needs as a consequence of the effects of both their MPS disorder and the special measures required providing care for them on a day-to-day basis. The study highlights that access to regular professional dental care is important in order that problems in the form of developmental anomalies or disease are identified and that appropriate preventative interventions can be provided. With the exception of MPS IV patients, there was considerable heterogeneity in the prevalence of predisposing dental anomalies and poor hygiene and in the incidence of decay and gingivitis between and within classes of MPS. Therefore, given the serious challenges in providing dental treatments, and considering the evidence from the literature about increased oral health needs in vulnerable groups of patients (Lewis 2009; Shaw et al. 1986; Purohit et al. 2010) the authors recommend that dental professionals should consider all patients with an MPS disorder as being at high-risk of dental disease. Failure by parents and carers to access dental care needs to be addressed. According to the questionnaire results; 5 parents reported only accessing dental care when they believed their child was in pain and of concern, 9 reported never having visited the dentist showing that despite attending a unit where access to dental care is possible many are not receiving regular oral health care. The identification of oral health problems is often particularly important to rule out/identify issues when behaviour has changed e.g. teething or drooling, this is particularly important in patients with an MPS III disorder as their disease symptoms progress and disruptive behaviour becomes evident.

Within the limitations of the current study, the findings support the observations of Mc Govern et al. (2008) that MPS I patients do not have an increased dental caries rate. The MPS groups II, III and VI also show a low caries rate though firm conclusions are difficult to justify given the small sample size. The MPS IV group did appear to show an increased caries rate in comparison with the other MPS groups and the national epidemiological studies. The group also displayed developmental anomalies of both the permanent and primary dentition. Such defects in the appearance of the enamel in patients with MPS IV have been reported previously (Lustmann 1978) and have been described as being similar in appearance to that of amelogenesis imperfecta (Sela et al. 1975). However, to date no studies have commented on an increased caries rate. Given the structural defects, one could conceive these teeth may be more prone to caries in an unfavourable oral environment; however, further studies would be required to confirm this finding. It is important to note that the development of the dental enamel of the primary dentition and of the several permanent successor teeth occurs largely *in-utero* or soon after birth. Therefore, despite the advances in enzyme replacement therapies, the management of such dental anomalies is likely to remain a concern and problem.

The current investigation demonstrated that despite the special needs and physical difficulties of patients with an MPS disorder, a similar level of oral hygiene to the general population was maintained. Within the study population, only a slight increase in the levels of plaque and calculus was observed. Although this measure of oral health is relatively crude and based only on visual assessment, the observation was surprising and indicates that despite the adversities patients are still managing a reasonable level of oral hygiene indicating the possible increased caries rate is due to other factors. Any gingival hypertrophy was considered the dental assessment but none was noted. In the past, this has been mentioned as a feature of MPS I (Guven et al. 2008; Thomas and Tandon 2000). This finding is possible due to the fact that the MPS I patients in our study were all on enzyme therapy, and this perhaps is prevented or reduced to an undetectable level by the enzyme therapy. As a group the patients with an MPS disease do use the BCH dental services and with so many medical appointments it is often convenient for the families to attend at BCH; 50% of the study group had already received some dental treatment and 43% required this to be carried out under a general anaesthetic. Despite this, a significant number were still not accessing the dental services. It is therefore important that we continue to work with these patients in order to improve the oral health care they receive.

Summary

- MPS disorders do have an effect on the developing dentition and to date the effect of enzyme therapy is largely unknown.
- Maintaining good oral health is challenging; however, prevention of dental decay, periodontal disease and erosion is extremely important.
- Regular visits to the dentist are recommended.
- We need to work together to improve existing services and create a service for the future generation, including adolescent and adult services.

Synopsis

Synopsis: MPS disease does have an impact on oral health and the developing dentition, the extent of which varying between disease type. To date the effect of enzyme therapy on the oral health is largely unknown.

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RESEARCH REPORT

Treatment of Human Fibroblasts Carrying NPC1 Missense Mutations with MG132 Leads to an Improvement of Intracellular Cholesterol Trafficking

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Abstract Niemann Pick type C (NPC) disease is an autosomal recessive disorder characterized by the lysosomal/late endosomal (LE) accumulation of unesterified cholesterol and other lipids due to a defect in the intracellular lipid trafficking. About 95% of patients present mutations in the *NPC1* gene. Among the 290 mutations reported in the *NPC1* gene, about 70% are missense. However, little information is available regarding the impact of missense mutations on NPC1 protein stability and function. In this study, we in vitro characterized the pathogenic effect of 7 *NPC1* missense mutations. In all cases, the basal levels of mutant NPC1 expression were reduced with respect to wild type. Treatment of fibroblasts carrying NPC1 missense mutations in homo or hemizygosity, with the proteasome inhibitor MG132 or glycerol 10%, a chemical chaperone known to stabilize misfolded proteins, resulted in a significant increase of NPC1 protein levels in all cell lines, indicating that these mutants are subjected to proteasomal degradation due to protein misfolding The increment of NPC1 mutant protein induced by the proteasome inhibitor was associated with a localization of NPC1 protein within lysosomal/LE compartment. In cell lines carrying mutations p.N1156S, p.L1191F, p.V1165M, and p.I1061T, the increment of NPC1 mutant protein resulted in an improvement of the intracellular trafficking of cholesterol and GM1. These findings showed that it is possible to correct the NPC cellular phenotype by increasing the amount of endogenous NPC1 mutated protein, suggesting that at least some *NPC1* mutations might be potentially rescued by small molecules-based chaperone therapy.

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Introduction

Niemann Pick type C (NPC) disease (NPC1, MIM 257220; NPC2, MIM 607625) is an autosomal recessive neuro-visceral disorder with an estimated incidence of 1:150,000 live birth and characterized by the accumulation of a broad spectrum of lipids including unesterified cholesterol, glycosphingolipids (GSLs), sphingosine and sphingomyelin within the lysosomes/late endosomes (LE) due to a defect in the intracellular lipid trafficking (Patterson et al. 2001; te Vruchte et al. 2004; Lloyd-Evans et al. 2008).

Clinically, NPC disease presents a highly variable phenotype ranging from fetal to adult age. Although it encompasses a continuous spectrum of phenotypes, it has classically been classified by the age at onset of neurological symptoms in a severe infantile form (onset before 2 years of age), a late infantile form (onset between 3 and 5 years of age), a juvenile form (onset between 5 and 16 years), and an adult form (onset at age > 16 years) (Patterson et al. 2001; Vanier and Millat 2003).

Two disease-causing genes, *NPC1* (MIM#607623) and *NPC2* (MIM#601015), have been identified (Vanier et al. 1996; Carstea et al. 1993; Naureckiene et al. 2000). *NPC1* gene, located on chromosome 18q11-q12, encodes a large membrane glycoprotein of 1,278 aminoacids containing 13 transmembrane domains and located predominantly in late endosomes (Davies and Ioannou 2000). *NPC2* gene is mapped to chromosome 14q24.3 and encodes a small soluble protein present in the lumen of the lysosomes (Naureckiene et al. 2000; Vanier and Millat 2004). Although it is known that both NPC1 and NPC2 bind unesterified cholesterol and both are involved in the egress of cholesterol and other lipids from the lysosomes, the precise mechanisms by which these proteins exert this function is not clear. Recent studies have begun to clarify the roles of NPC1 and NPC2 in the lysosomal export process. It has been demonstrated that a water soluble fragment of NPC1 binds cholesterol in an orientation opposite to NPC2 (Infante et al. 2008). Based on these results, it has been postulated that, after liberation from LDL, cholesterol is bound by NPC2 which carries it to the lysosomal membrane, where it transfers to the N-terminal domain of the membrane bound NPC1 (Kwon et al. 2009).

About 95% of human NPC disease is caused by mutations in the *NPC1* gene, (Carstea et al. 1997), while the other 5% is due to mutations in the *NPC2* gene.

To date, more than 290 mutations of *NPC1* gene have been reported (http://npc.fzk.de/) (Runz et al. 2008), most of them are present in single families. Only three relative frequent mutations have been found within distinct patient groups: the p.G992W, almost exclusively seen in Acadian patients from Nova Scotia (Greer et al. 1998), the p.P1007A found in about 15% of mutated alleles in different European populations (Ribeiro et al. 2001; Fancello et al. 2009) and the p.I1061T that accounts for 15–20% of mutated alleles in Western Europe and USA (Millat et al. 1999; Sun et al. 2001; Park et al. 2003; Millat et al. 2005). However, a study performed in 44 Italian NPC patients showed that the p.I1061T mutation is much less common in Italy, representing only a 4.7% of the *NPC1* alleles (Fancello et al. 2009).

Although the *NPC1* mutational profile is exceedingly heterogeneous, nearly 70% of *NPC1* alleles are due to point mutations distributed throughout the coding region of the *NPC1* gene and resulting in codon replacements that may affect the correct folding of the protein (Runz et al. 2008).

In fact, it has been recently demonstrated that the NPC1-I1061T protein is recognized as misfolded by the endoplasmic reticulum (ER) quality control machinery and targeted for proteasomal degradation. Transient transfection of *npc1*-deficient CHO cells with GFP-tagged NPC1-I1061T led to lysosomal localization of the mutant protein and functional complementation of the NPC mutant phenotype. Therefore, it has been suggested that therapeutic strategies directed to enhance the amount of mutated protein, such as the use of chemical chaperones, may rescue the pathologic phenotype in patients carrying the p.I1061T mutation (Gelsthorpe et al. 2008).

In this study, we have characterized the effect of 7 *NPC1* missense mutations on protein degradation and provided evidences indicating that by increasing the amount of endogenous mutated protein it is possible to correct the cellular phenotype. Our findings suggest that some *NPC1* missense mutations, other than the p.I1061T might be potentially rescued by small molecules-based chaperone therapy.

Materials and Methods

Cell Culture and Treatments

Human fibroblasts were obtained from skin biopsies from seven patients affected with NPC disease and normal controls. All NPC patients presented with the classical biochemical phenotype characterized by massive lysosomal/LE accumulation of unesterified cholesterol in cultured fibroblasts. The diagnosis was confirmed by sequencing both *NPC1* and *NPC2* genes. All patients had mutations in the *NPC1* gene. This study was approved by the ethical committee of the University Hospital "S. Maria della Misericordia," and written consent was obtained from all subjects.

Human fibroblasts were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), and 50 mg/ml penicillin/streptomycin (Gibco, Paisley, UK).

Fibroblasts were treated with vehicle or 15 μM of the protease inhibitor MG132 (Sigma, St Louis, MO, USA) for 24–48 h, with 10–1,000 nM of MG132 for 10 days or with glycerol 10% for 24 h.

Mutational Analysis

Genomic DNA from NPC patients was extracted from cultured skin fibroblasts and amplification of *NPC1* gene was performed as previously described (Tarugi et al. 2002). After purification, the polymerase chain reaction (PCR) fragments were sequenced in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK). In all cases the presence of the mutation has been confirmed in both parents who resulted to be heterozygous carriers.

Extraction of RNA, cDNA Synthesis and PCR Analysis

Total RNA was isolated from cultured fibroblasts using Trizol reagent (EuroClone Gibco, Paisley, UK) according to

the manufacturer's instructions. RNA was quantified spectrophotometrically at 260 nm in a Beckman Coulter DU®730 spectrophotometer (Fullertone, CA, USA). The RNA purity was evaluated by measuring the ratio A260/A280, considering RNA with appropriate purity those showing values between 1.8 and 2.0; its integrity was evaluated by gel electrophoresis. Total RNA (1 μg) was reverse transcribed using iScript^{TM} cDNA Synthesis kit BioRad according to manufacturer's instructions. PCR analysis was performed as previously described (Di Leo et al. 2004) using appropriate sets of primers, which allow the amplification of ten overlapping fragments covering the whole *NPC1* messenger RNA (mRNA) (Tarugi et al. 2002).

Real-Time Quantitative PCR

Real-Time quantitative PCR was performed in i-Cycler IQ; 18S and GAPDH were used as housekeeping genes. All primer pairs were synthesized by Sigma Genosys Ltd. (London Road, UK) and were designed using the software Beacon Designer 7.91 (PREMIER Biosoft International, Palo Alto, CA, USA). Primer sequences and references are specified in Table 1. PCR amplification was carried out in 25 μL reaction volume containing 25 ng of cDNA, 1x iQ SYBR Green Supermix [100 mM KCl; 40 mM Tris–HCl, pH 8.4; 0.4 mM each dNTP; 50 U/mL iTaq DNA polymerase; 6 mM MgCl2; SYBR Green I; 20 nM fluorescein; and stabilizers] and gene-specific sense and anti-sense primers. Standard curves using a "calibrator" cDNA (chosen among the cDNA samples) were prepared for each target and reference gene, and the efficiency was calculated. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Nonspecific products of PCR were not found in any case. The relative quantification was made using the Pfaffl modification of the ΔΔCt equation, taking into account the efficiencies of individual genes. The results were normalized to 18S and GAPDH, the initial amount of the template of each sample (treated) was determined as relative expression versus its reference sample (untreated control) which in each case was considered the 1x sample. At least three different determinations for each gene were performed.

Western Blot Analysis

Twenty micrograms of protein extracts were resolved on 8% SDS PAGE gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). After overnight blocking with 5% nonfat dry milk in PBS-Tween 0.1% (PBS-T), the membranes were probed with anti-NPC1 polyclonal antibody (Novus Biologicals, Littleton, USA) overnight at 4°C. Anti-rabbit HPR conjugated antibody was used as a secondary antibody. Immunoreactive bands were detected by enhanced chemiluminescence ECL (Amersham). The signals were normalized to those obtained for actin using a polyclonal anti-actin antibody (Sigma, St Louis, MO, USA).

Filipin Staining

Filipin staining was performed using the method described by Blanchette–Mackie et al. (Blanchette-Mackie et al. 1988). Briefly, the cells were rinsed with PBS and fixed with 3% paraformaldehyde. After washing them with PBS, the cells were incubated with 1.5 mg of glycine/ml PBS for 10 min, stained with filipin (0.05 mg/ml, in PBS 10% FCS) for 2 h and examined using a Zeiss fluorescence microscope.

Immunolocalization of NPC1 Protein

Immunolocalization of NPC1 was performed in human NPC and normal fibroblasts.

Cells were grown as described above on glass cover slips. After 72 h, the cells were fixed with 4% paraformaldheyde (Sigma, St. Louis, MO, USA) and permeabilized with 0.2% Triton X-100. The cells were then blocked with 2% BSA (Sigma, St. Louis, MO, USA) and immunolabeling was carried out using an anti-NPC1 polyclonal antibody (Novus Biologicals, Littleton, USA). The secondary antibody was a FITC-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark). For colocalization studies, a monoclonal anti-LAMP-1 (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used as a primary antibody, and an alexa fluor-conjugated anti-mouse (Invitrogen, Carlsbad, CA, USA) as a secondary

Table 1 Set of primers used in real-time quantitative PCR

Gene name	Accesion number	Forward	Reverse	Concentration (nM)	Efficiency (%)
NPC1	NM_000271	TTCAGACACTAAAAGACAA	ACAGGTTCAGTAGGTTAT	300	100
RN18S1	NR_003286	TAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	100	95
GAPDH	NM_002046	TCAGCCGCATCTTCTTTTG	TCAGCCGCATCTTCTTTTG	250	96

antibody. The glass cover slips were mounted and analyzed using a Zeiss fluorescence microscope.

GM1 Staining

Cells were grown on glass cover slips and then fixed with 3% paraformaldheyde (Sigma, St. Louis, MO, USA) for 20 min, washed with PBS and permeabilized with 0.1% Triton X-100 in 2% BSA (Sigma, St. Louis, MO, USA) for 5 min. After washing the cells with PBS, they were incubated with 3 μg/ml cholera toxin B subunity FITC conjugated (Sigma, St. Louis, MO, USA) in 0,2% BSA for 30 min at room temperature. For colocalization studies, cells were washed with PBS and stained with filipin working solution (0,05 mg/ml, in PBS 10% FCS) for 2 h at room temperature (Blanchette-Mackie et al. 1988). The glass cover slips were mounted and analyzed using a Zeiss fluorescence microscope.

Results

The genotypes of the 7 NPC patients included in this study are summarized in Table 2. Genotypes of patients 1–6 had been already established and published in a previous study (Fancello et al. 2009). Patients 2–6 presented missense mutations in homozygosity (confirmed by the presence of the mutation in both parents), while patient 1 presented a missense mutation associated with a nonsense mutation in codon 670. The truncated protein that would be translated from this allele was not detected by western blot. Therefore, we considered that all the cell lines derived from patients 1–6 expressed only one NPC1-mutant variant.

Sequencing analysis of the entire coding region and the intronic flanking sequencing of *NPC1* gene in patient 7 showed the presence of the known mutation p.V1165M (c.3493G>A) in one allele. The second allele presented a new intronic mutation c.2795+1G>C located at the 5′ donor splice site of intron 18. RT-PCR analysis of the *NPC1* mRNA extracted from patient's fibroblasts followed by sequencing of the PCR product showed that only the allele carrying the p.V1165M mutation was expressed even after 40 cycles of amplification. Based on this result, we assumed that patient 7 presents on one allele the p.V1165M mutation and on the other a splicing mutation that probably generates an unstable mRNA, which would be rapidly degraded, leading to the lack of the corresponding transcript. Therefore, we considered that the cell line derived from patient 7 also expressed only one NPC1-mutant variant.

To analyze the impact of the missense mutations on protein abundance, we first analyzed the basal levels of NPC1 protein expression in fibroblasts cell lines by western blot. As shown in Fig. 1, the levels of mutant NPC1 expression were reduced in NPC fibroblasts with respect to normal controls. It has been well established, using other disease models, including lysosomal diseases, that some mutated misfolded proteins are retained in the ER, from where they are retro-translocated back to the cytosol to be eliminated by the ubiquitin–proteosomal pathway. This process is known as ER-associated degradation (ERAD) (Kopito 1997). In order to determine whether the reduction of NPC1 mutant protein is due, at least in part, to an increase in the rate of proteasomal degradation, normal and NPC fibroblast cell lines were treated for 24 h- with 15 μM of the proteasome inhibitor MG132 or vehicle and NPC1 protein abundance was determined by western blot (Fig. 2a). MG132 treatment resulted in a 2- to 6-fold increase of NPC1 mutant protein levels, suggesting that all these mutant variants are subjected to proteasomal degradation (Fig. 2b). An increase of wild-type NPC1 protein abundance was also observed after treatment with MG132. However, this effect was not statistically significant.

Table 2 Genotypes of NPC patients included in the study

Patient	Allele 1	Allele 2	Reference
NPC-1	p.T1205K (c.3614C>A)	p.R607X (c.1819C>T)	Fancello et al. (2009) (patient NP10)
NPC-2	p.V1023G (c.3068T>G)	p.V1023G (c.3068T>G)	Fancello et al. (2009) (patient NP4)
NPC-3	p.Y1019C (c.3056A>G)	p.Y1019C (c.3056A>G)	Fancello et al. (2009) (patient NP29)
NPC-4	p.N1156S (c.3467A>G)	p.N1156S (c.3467A>G)	Fancello et al. (2009) (patient NP14)
NPC-5	p.L1191F (c.3571C>T)	p.L1191F (c.3571C>T)	Fancello et al. (2009) (patient NP2)
NPC-6	p.I1061T(c.3182T>C)	p.I1061T (c.3182T>C)	Fancello et al. (2009) (patient NP25)
NPC-7	p.V1165M (c.3493G>A)	r.0 (c.2795+1G>C)	This study

The lysosomal accumulation of unesterified cholesterol was demonstrated by filipin staining. All cells presented a classical biochemical phenotype characterized by massive lysosomal accumulation of unesterified cholesterol

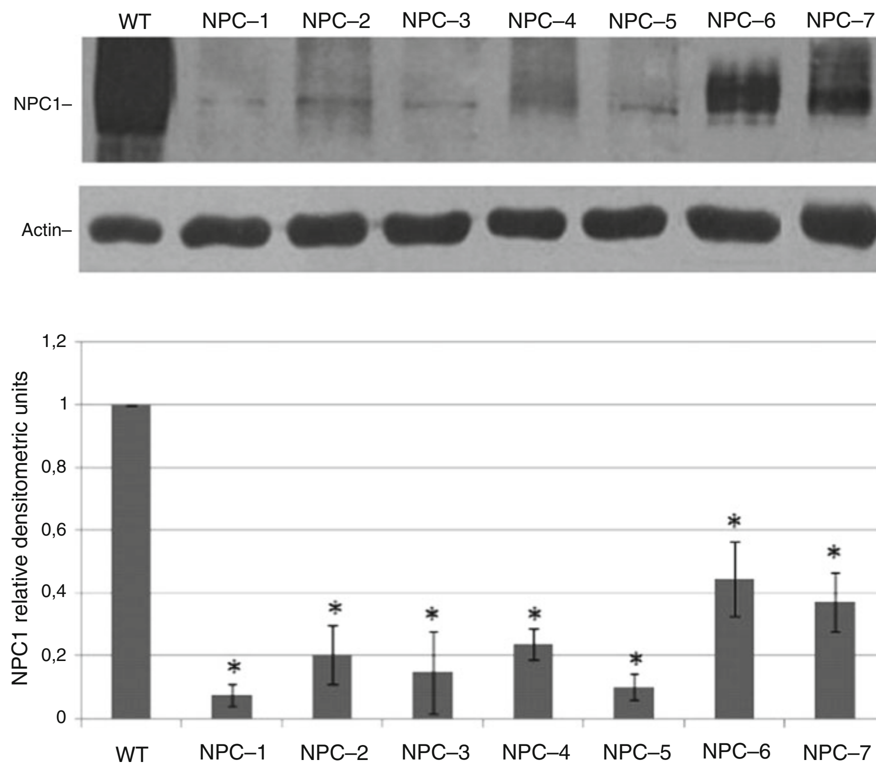

Fig. 1 NPC1 protein abundance in NPC fibroblasts. (**a**) Representative western blot analysis of NPC1 protein expression in NPC and normal fibroblast cell lines. (**b**) The intensity of the NPC1 signals was normalized against actin. The NPC1 protein content in NPC fibroblasts was expressed as a percentage of the NPC1 protein content found in fibroblasts from a normal control. Data are means ± SD of 3 independent experiments (*$p < 0.05$)

In order to verify whether MG132, in addition to its effect as a proteasome inhibitor, exerts also an effect of *NPC1* mRNA expression, a quantitative real-time PCR in normal and NPC fibroblasts treated with vehicle or MG132 15 μM for 24 h was performed. A slight but not statistically significant increase of *NPC1* mRNA expression was observed after treatment (fold of increase with respect to nontreated cells: 2.38 ± 1.49; $p = 0.07$). Taken together, these data demonstrate that the increase of NPC1 protein abundance after 24 h treatment with MG132 was mainly due to an inhibition of its proteasomal degradation.

In addition, NPC cells were treated with glycerol, a chemical chaperon known to stabilize misfolded proteins, for 24 h and the NPC1 protein abundance was determined. As shown in Fig. 2(c and d), glycerol treatment resulted in a significant increase of NPC1 protein levels in all cell lines. These data provide further evidence indicating that these mutants are selected to ERAD due to protein misfolding.

We then wanted to address whether the increment of the intracellular levels of endogenous mutant protein leads to the clearance of accumulated free cholesterol.

Treatment of NPC cell with 15 μM of MG132 for 24 h did not exert any effect on cholesterol accumulation (data not shown). However, experiments performed in our laboratory showed that NPC cells cultured in the absence of serum become negative to the filipin staining only after 3–5 days of starvation. Therefore, we hypothesized that the exposure of NPC cell to the proteasome inhibitor for 24 h may not be enough to cause a sensible reduction of the intracellular cholesterol accumulation. Since treatment of NPC fibroblasts with 15 μM of MG132 for more than 48 h resulted in a massive reduction of cell viability, NPC cells were treated with decreasing concentrations of MG132 (1,000, 500, 100, 50, and 10 nM) or vehicle for 10 days. Treatment of NPC cells with 500–1,000 nM of MG132 still resulted in a significant reduction of cell viability, while the minimum concentration of MG132 still able to cause an increase of NPC1 protein abundance was 50 nM (Fig. 3a, b).

It is worth of note that treatment of cells with MG132 50 nM for 10 days did not exert any effect on *NPC1* mRNA expression, suggesting that the increase in protein abundance observed in cells treated with 50 nM for 10 days, is due to the inhibition of protein degradation.

Under these experimental conditions MG132 treatment resulted in a significant reduction of cholesterol accumulation in fibroblasts cell lines carrying the p.N1156S,

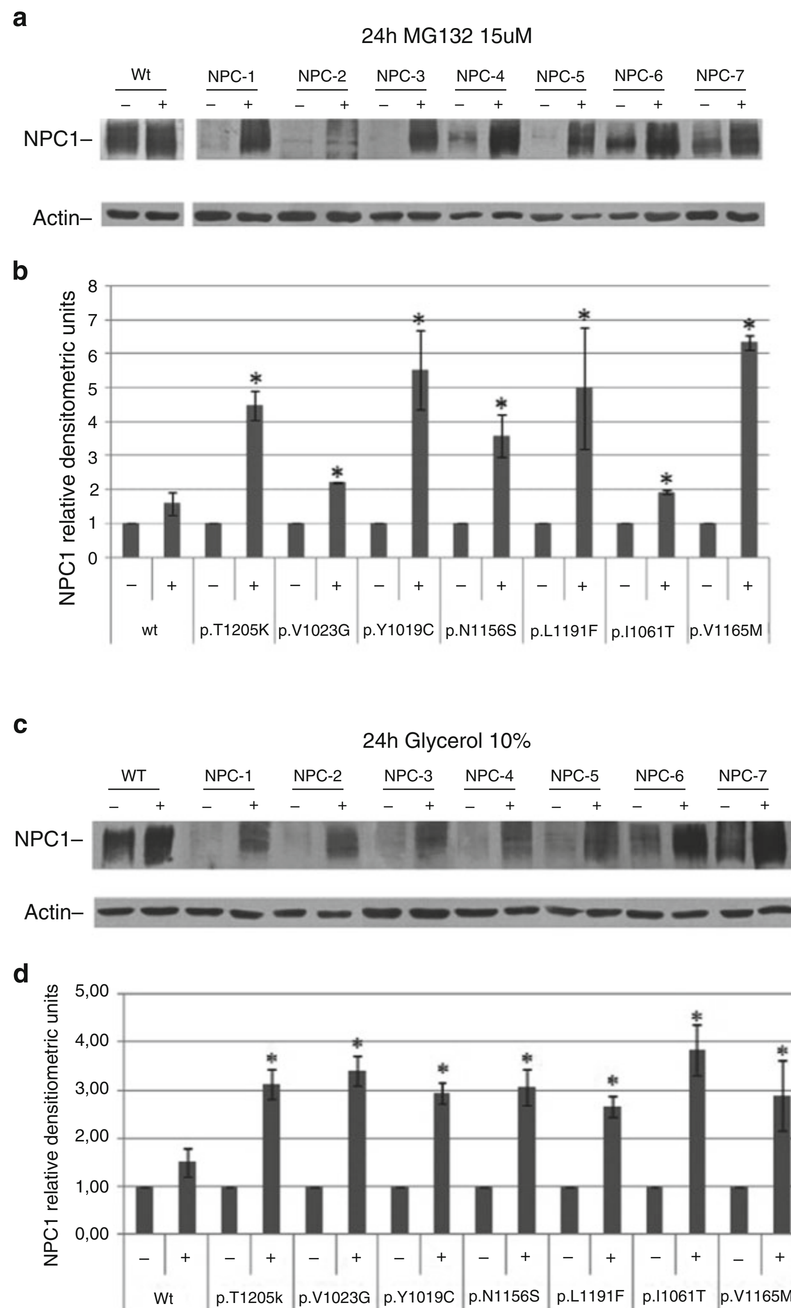

Fig. 2 NPC1 protein abundance in normal and NPC fibroblasts after treatment with MG132 15 μM or glycerol 10% for 24 h. Representative western blot analysis of NPC1 protein expression in normal and NPC fibroblasts cell lines treated with MG132 for 24 h with 15 μM (panel **a**) or glycerol 10% (panel **c**). The intensity of the NPC1 signals were normalized against actin. The NPC1 protein content in NPC1 fibroblasts was expressed as a percentage of the NPC1 protein content found in vehicle treated cells (panels **b** and **d**). Data are means ± SD of 3 independent experiments (*$p < 0.05$). Treatment of NPC cells with MG132 15 μM or glycerol significant increase the content of NPC1 mutant protein

p.L1191F, p.V1165M, and p.I1061T *NPC1* mutations. No effect on cell carrying the p.T1205K, p.V1023G and p. Y1019C was observed (Fig. 4). The same result was obtained even in cell treated with 100 nM of MG132 (data not shown).

To investigate whether the reduction of cholesterol accumulation was associated with an increase of NPC1 protein within the lysosomal/LE compartment, the intracellular localization of the NPC1 mutant variants was assayed by immunofluorescensce. As shown in Fig. 5, under basal

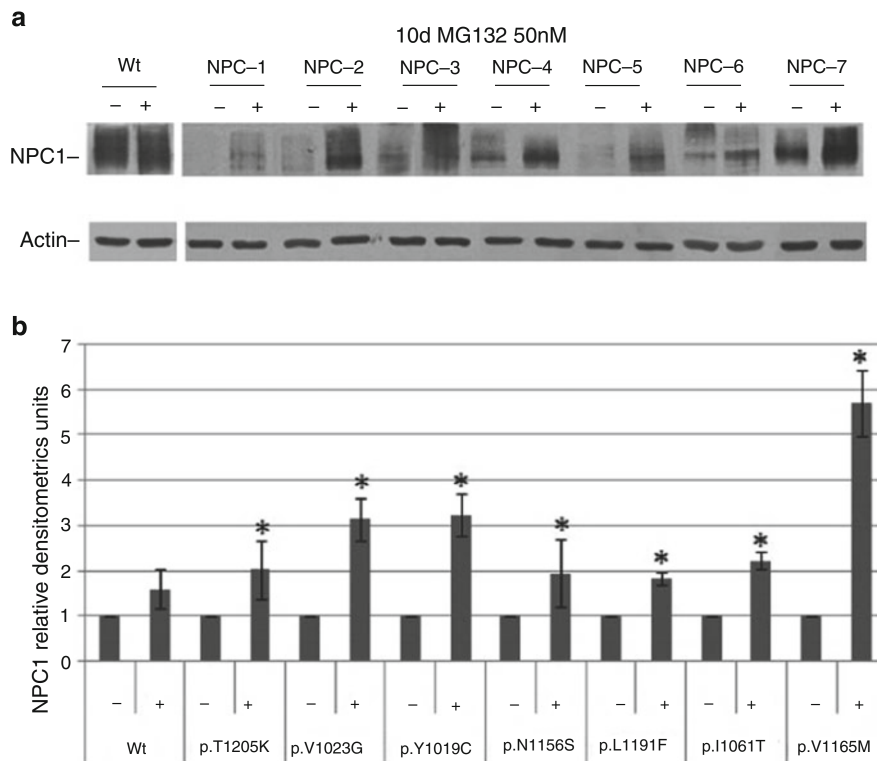

Fig. 3 NPC1 protein abundance in normal and NPC fibroblasts in the absence or presence of MG132 50 nM for 10 days. (**a**) Representative western blot analysis of NPC1 protein expression in normal and NPC fibroblasts cell lines treated with MG132 50 nM for 10 days. (**b**)The intensity of the NPC1 signals were normalized against actin. The NPC1 protein content in NPC1 fibroblasts was expressed as a percentage of the NPC1 protein content found in vehicle treated cells. Data are means ± SD of 3 independent experiments (*$p < 0.05$). Treatment of NPC cells with MG132 50 nM for 10 days significant increase the content of NPC1 mutant protein

conditions the levels of mutant NPC1 protein were extremely low. Treatment of NPC cells for 10 days with low dose of MG132 resulted in a substantial increase of NPC1 mutant protein signal and a staining typical of lysosomal/LE localization, not only in fibroblasts carrying the p.N1156S, p.L1191F, p.V1165M and p.I1061T *NPC1* mutations, but also in all 7 NPC studied cell lines. Furthermore, NPC1 colocalized with the lysosomal protein Lamp-1 providing further evidence that MG132 treatment improved the delivery of the NPC1 mutant to the lysosomal compartment. These data suggest that p.T1205K, p.V1023G, and p.Y1019C mutations may have an impact not only on NPC1 folding, trafficking, and degradation but also on the protein function itself.

Since the storage profile in NPC cells include other lipids such as GSLs, we analyzed whether the increment of NPC1 protein results, not only in a clearance of unesterified cholesterol but also in the reduction of GM1 accumulation. Once again, treatment of NPC cell with 15 μM of MG132 for 24 h did not exert any effect on GM1 accumulation (data not shown). However, a significant reduction of GM1 accumulation was observed in fibroblasts cell lines carrying the p.N1156S, p.L1191F, p.V1165M, and p.I1061T *NPC1* mutations after 10 days treatment with 50 nM of MG132 (Fig. 6).

Discussion

Loss of function diseases are often caused by the inability of a mutated protein to achieve a correct folding within the secretory pathway. In fact, it has been well documented that some mutant variants are recognized as misfolded by ER quality machinery, made up of molecular chaperones, which ensures that only properly folded proteins are secreted from the ER. Thus, these misfolded proteins are retained in the ER, from where they are retro translocated back to the cytosol to be eliminated by the ubiquitin–proteosomal pathway. This process is known as ER-associated degradation (ERAD) (Kopito 1997).

Protein misfolding has been implicated in many lysosomal storage disorders, such as Gaucher disease (Alfonso et al. 2005), Fabry disease (Fan et al. 1999), GM1 gangliosidosis (Tropak et al. 2004), and Pompe disease (Parenti et al. 2007).

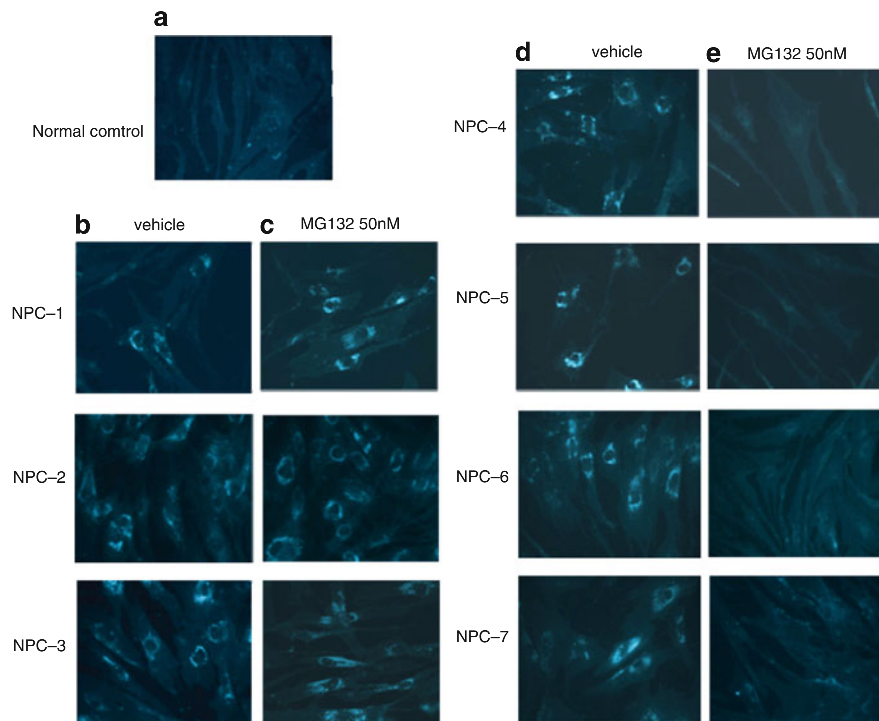

Fig. 4 Filipin staining of intracellular unesterified cholesterol. (**a**) Normal fibroblasts; (**b** and **d**) NPC fibroblasts treated for 10 days with vehicle showed a massive lysosomal accumulation of unesterified cholesterol; (**c** and **e**) NPC fibroblasts treated for 10 days with MG132 50 nM. Only in cells carrying the p.N1156S, p.L1191F, p.V1165M and p.I1061T *NPC1* mutations (NPC-4, NPC-5, NPC-6, NPC-7) showed a reduction of intracellular cholesterol staining

In this paper we demonstrated that, besides the p.I1061T NPC mutant protein, other 6 NPC1 mutant variants are unstable and subjected to an increased rate of proteasomal degradation. In fact, treatment of NPC fibroblasts carrying different missense mutations with the proteasome inhibitor MG132 resulted in a significant increment of mutant NPC1 protein levels and an improvement of the trafficking to the lysosomal/LE compartment. In NPC fibroblasts cell lines carrying mutations p.N1156S, p.L1191F, p.V1165M, and p.I1061T, the increment of NPC1 protein content led to a reduction of lysosomal/LE free cholesterol. Furthermore, we have demonstrated that by enhancing the NPC1 protein content, it is also possible to improve the trafficking of GSLs. Our data suggest that these mutations may render the NPC1 protein unstable and rapidly degraded but they would not completely abolish the NPC1 functionality. These finding are in line with those described by Gelsthorpe et al. (Gelsthorpe et al. 2008) showing that a twofold overexpression of GFP-tagged NPC1 I1061T in transient transfected *npc1*-deficient CHO cells was enough to partially restore the cholesterol trafficking. These authors postulated that a percentage of 2–5% of the nascent NPC1 I1061T mutant protein would be able to fold properly, escape the ER quality control and reach the lysosome/LE compartment. In cells overexpressing the mutant protein even if only a 2–5% of the protein achieves the correct folding, the quantity of correct folded mutant molecules would be enough to complement the mutant phenotype. A similar mechanism could be postulated to explain the effect of MG132 treatment on cholesterol and GSLs trafficking in NPC fibroblasts. Indeed, in this cellular model, the increase of NPC1 protein abundance was associated with a localization of NPC1 within the lysosomal/LE compartment. Under these conditions, the amount of NPC1 protein that reached the lysosomes/LE would be enough to reduce both cholesterol and GM1 accumulation in treated cells.

It has been shown that MG132 also enhances chaperone expression levels (Bush et al. 1997; Liao et al. 2006). Therefore, it is possible to hypothesize that the effect observed in NPC fibroblasts may be due to a direct inhibition of proteasomal degradation of NPC1 protein or due to an induction of endogenous chaperons which in turn stabilize NPC1 protein preventing its proteasomal degradation. The relative contribution of these mechanisms to the effect exerted by MG132 on NPC cells needs to be further investigated.

In 3 NPC1 cells lines (NPC-1, NPC-2, and NPC-3), 10 days treatment with MG132 50 nM resulted in an

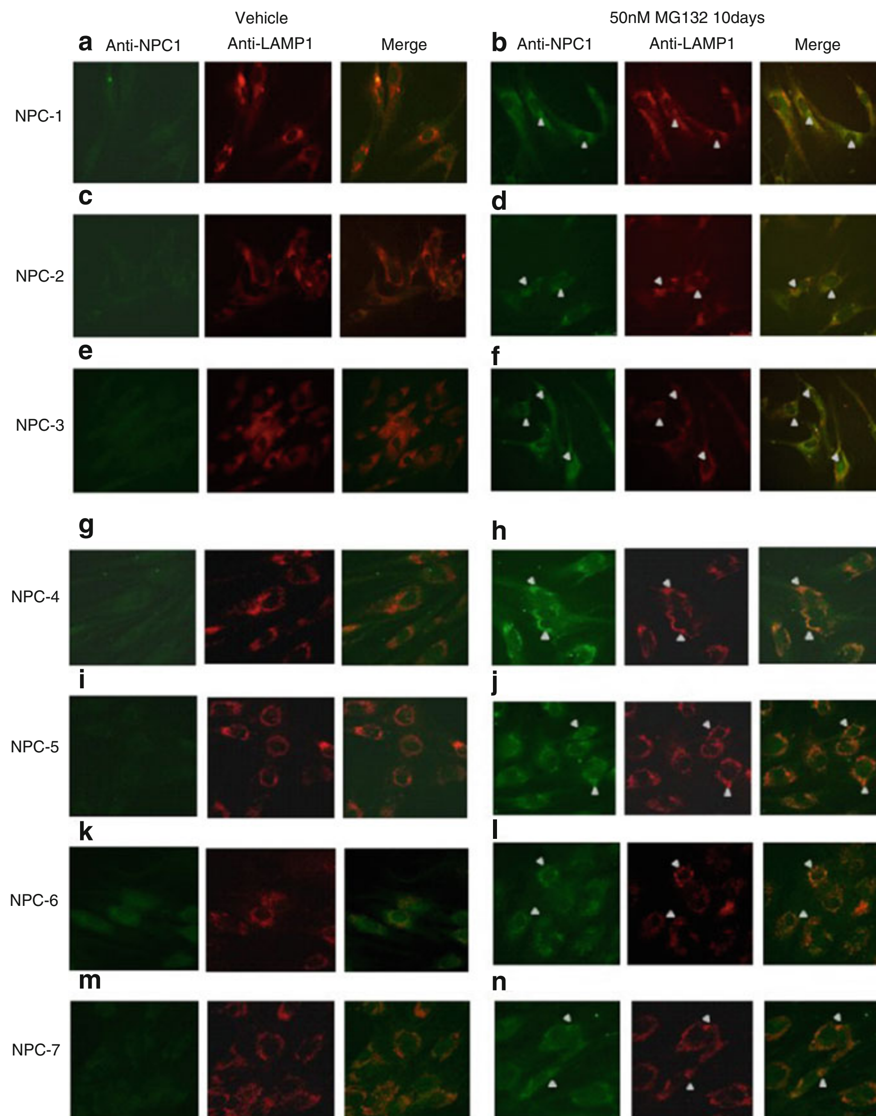

Fig. 5 Immunofluorescence staining of NPC1 protein in NPC fibroblast in the absence or presence of MG132. Under basal conditions negligible immunofluorescent labelling for NPC1 was observed (panels **a**, **c**, **e**, **g**, **I**, **k**, **m**). Treatment of NPC cells for 10 days with MG132 50 nM resulted in an increased immunofluorescence labelling for NPC1 and a colocalization with the lysosomal marker LAMP-1 (panels **b**, **d**, **f**, **h**, **j**, **l**, **n**)

increase of protein content and trafficking to the lysosomal/LE compartment. However, this treatment did not result in a reduction of free cholesterol or GM1 accumulation. These data suggest that these mutations may have an impact not only on NPC1 folding, trafficking and degradation but also on the protein function itself.

In conclusion, our data provide evidence indicating that an increased degradation of mutant NPC1 protein, probably due to protein misfolding, would be implicated in the pathogenesis of NPC1 disease. Furthermore, it has been demonstrated that by enhancing the amount of endogenous mutant NPC1 protein, it would be possible to partially restore both cholesterol and GM1 trafficking in NPC cells.

These are promising results considering that up to date, two different approaches aimed to enhance protein stability and prevent mutant protein degradation, have been successfully assessed in other models of misfolding diseases, such as cystic fibrosis disease and Gaucher: the use of pharmacological chaperons (Alfonso et al. 2005) and the use of molecules that enhance the innate cellular protein homeostasis (Wang et al. 2006; Mu et al. 2008). Although molecules that may function as pharmacological chaperones for NPC1 protein need to be identified and the usefulness of these therapeutic approaches needs to be assessed in NPC cells, our data strongly suggest that, at least some NPC1 patients may benefit from these kind of therapeutic strategies.

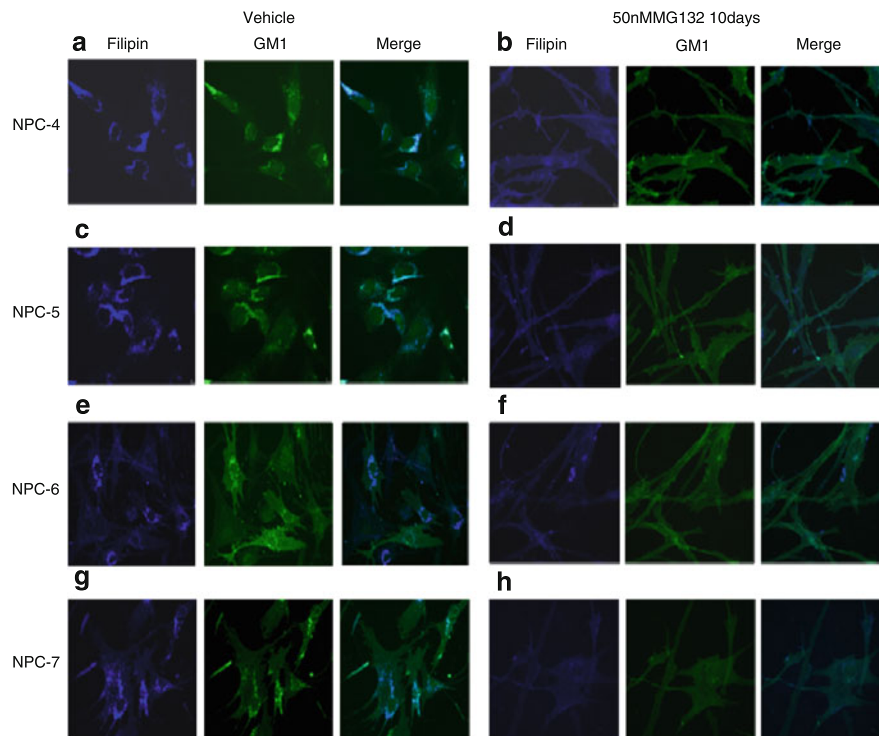

Fig. 6 Immunofluorescence staining of GM1 and filipin staining of intracellular unesterified cholesterol in "responsive" NPC fibroblasts in the absence and presence of MG132. Under basal conditions a massive accumulation of cholesterol and GM1 was observed (panels **a**, **c**, **e**, **g**). Treatment of NPC cells for 10 days with MG132 50 nM resulted in a concomitant reduction of both cholesterol and GM1 accumulation

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Synopsis

Proteasome inhibition results in a significant increase of NPC1 mutant protein levels and an improvement of cholesterol and GM1 trafficking in Niemann Pick C cells carrying different missense mutations.

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CASE REPORT

3-Hydroxyacyl-Coenzyme A Dehydrogenase Deficiency: Identification of a New Mutation Causing Hyperinsulinemic Hypoketotic Hypoglycemia, Altered Organic Acids and Acylcarnitines Concentrations

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Abstract The human *HADH* gene encodes the short-chain-L-3-hydroxyacyl-CoA dehydrogenase, the enzyme which catalyzes the third step of the β-oxidation of the fatty acids in the mitochondrial matrix. Loss-of-function mutations in the *HADH* gene lead to short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency, an autosomal recessive genetic defect of unknown prevalence with a wide spectrum of phenotypic variability. As in other metabolic diseases, the diagnostic relevance of the biochemical evaluations, plasma acylcarnitines, and urinary organic acids, are crucially dependent on the clinical conditions of the patient during specimen collection.

This paper describes the eighth patient carrying a *HADH* gene mutation, a new homozygous deletion c.565delG leading to an early stop codon (p.V116Wfs124X), in an infant with hyperinsulininemic hypoglycemia, displaying abnormal patterns of plasma acylcarnitines and urinary organic acids. We conclude that, when the residual catalytic activity of the mutated enzyme is seriously reduced, the biochemical hallmarks of the disease, namely plasma 3-hydroxybutyrylcarnitine and urinary 3-hydroxyglutaric acid, are invariably present.

FIP and SP equally contributed to this paper and should be considered co-first authors.

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Introduction

The short-chain-L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) is an intramitochondrial homodimer enzyme, catalyzing the third of the four steps of the β-oxidation of the fatty acids, namely the NAD^+-dependent dehydrogenation of the L-3-hydroxyacyl-CoA to the corresponding 3-ketoacyl-CoA. It is encoded by the *HADH* gene, mapped on human chromosome 4q22–q26 (Vredendaal et al. 1996). Loss-of-function mutations in the *HADH* gene lead to short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency (Clayton et al. 2001), an autosomal recessive defect of the β-oxidation of fatty acids, the process providing

energy during time of fasting, severe febrile illness or increased muscular activity (Roe and Ding 2001).

The first reported mutations in the *HADH* gene referred to a compound heterozygous patient affected by short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency who presented at 3 years with fulminant hepatic failure, requiring prompt liver transplant (O'Brien et al. 2000).

Among subjects carrying a mutated *HADH* gene and affected by short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency, a first group of three patients, with residual enzymatic activity less than 10% of controls, was reported as having a common feature of hyperinsulinemic hypoketotic hypoglycemia (Clayton et al. 2001; Molven et al. 2004; Hussain et al. 2005). The increased plasma level of 3-hydroxybutyryl-carnitine and the presence of 3-hydroxyglutaric acid in urine were considered informative biochemical hallmarks of the disease. 3-Hydroxybutyryl-carnitine, belonging stereochemically to the L-configuration, accumulates because of the defective fatty acid metabolism, on the contrary, the D-form would suggest a ketone body utilization defect. The latter could be due to the impairment of a minor pathway of degradation of the aminoacids lysine and tryptophan, in which the short-chain-L-3-hydroxyacyl-CoA dehydrogenase would catalyze the conversion of the 3-hydroxyglutaryl-CoA to 3-ketoglutaryl-CoA (Molven et al. 2004).

In all these patients, improvement of glucose homeostasis was obtained with diazoxide. The molecular action of the diazoxide involves opening of the ATP-sensitive potassium channels (K_{ATP} channels), with decreased insulin secretion. As the matter of fact, the physiological insulin secretion in the pancreatic β-cells is caused by an increase in ATP:ADP ratio, which leads to closure of the K_{ATP} channels, resulting in depolarization of the membrane, influx of calcium and ultimately insulin release (Palladino et al. 2008). The clinical observation of prompt glucose homeostasis improvement after diazoxide administration in these patients has led to the hypothesis that functional K_{ATP} channels were present. Therefore, mutations in the *ABCC8* and *KCNJ11* genes, encoding two subunits of the K_{ATP} channels, responsible of most cases, either recessive or dominant, of congenital hyperinsulinism (Tornovsky et al. 2004), were excluded.

Congenital hyperinsulinism, characterized by the dysregulated secretion of insulin from pancreatic β-cells (Stanley 1997) is a major cause of persistent hypoglycemia in the neonatal and infancy period. The genetic basis involves defects in key genes regulating insulin secretion such as the above-mentioned *ABCC8* and *KCNJ11*. In addition, several other dominant forms are due to activating mutations in *GLUD1* (encoding glutamate dehydrogenase type I) (Stanley et al. 1998), *GCK* (glucokinase) (Glaser et al. 1998), *HNF4A* (hepatocyte nuclear factor 4a) (Kapoor et al. 2008) and *SLC16A1* (monocarboxylate transporter 1) (Otonkoski et al. 2007). Recessive forms are ascribed to mutations in *HADH* (Clayton et al. 2001), with similar prevalence to congenital hyperinsulinism attributable to *HNF4A* or *GLUD1* (Flanagan et al. 2011).

A functional genomics approach (Hardy et al. 2007) and a study of the effects of siRNA-mediated *HADH* silencing (Martens et al. 2007) led to the conclusion that in case of short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency, the mutation in the *HADH* gene is actually the cause of hyperinsulinism.

In a second group of two patients, carrying mutations in the *HADH* gene with residual enzymatic activity in the 35–50% of the control range, the plasma acylcarnitine and the urinary organic acid profiles were either completely normal (Kapoor et al. 2009), or remarkable for increased plasma C4-carnitine and urinary glutaric acid (but not 3-hydroxyglutaric acid) (Bennett et al. 2006). In the former case, the hyperinsulinemic hypoglycemia was triggered by dietary protein load, whereas in the latter case, the patient was never hypoglycemic, but presented at 10 months of age with hepatomegaly and coagulopathy.

One last patient, carrying a homozygous nonsense mutation in the *HADH* gene, first hospitalized at 2 months of age for hypoketotic hyperinsulinemic hypoglycemia, displayed normal plasma acylcarnitine and urinary organic acid profiles, but, upon reevaluation at 7 years of age, he developed elevated levels of urinary 3-hydroxybutyric acid, and slightly increased dicarboxylic aciduria (Di Candia et al. 2009).

Still too few cases are reported in literature to clearly define the clinical and biochemical spectrum of the disease, which is likely influenced both by the residual catalytic activity of the short-chain-L-3-hydroxyacyl-CoA dehydrogenase and the environmental conditions. In particular, the plasma acylcarnitine and the urinary organic acid profiles are informative only in few cases, likely under acute conditions.

In this report, we describe a patient affected by short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency, carrying a severe mutation in the *HADH* gene. The homozygous deletion of one base pair leads to the formation of an early stop codon within exon 3, in the NAD^+-binding domain of the enzyme. The nonsense mutation located at the beginning of the mRNA leads to the prediction of complete absence of the protein. The clinical and biochemical presentation is consistent with hyperinsulinemic hypoketotic hypoglycemia, with informative plasma acylcarnitines and urinary organic acids.

Case Report

The patient was born in Morocco from consanguineous Moroccan parents. In the maternal family, two cases of

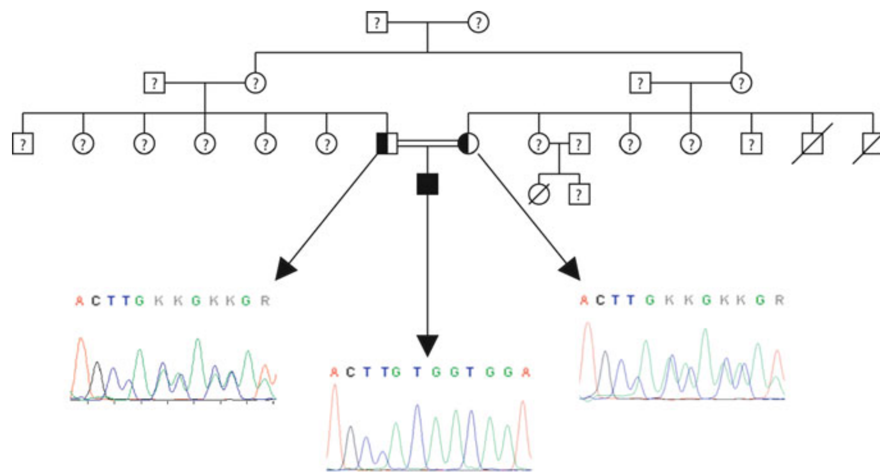

Fig. 1 Pedigree and details of the *HADH* sequences of the patient's family. Half-filled symbols denote heterozygous carrier subjects. Filled symbol represents the homozygous propositus. Automated direct sequencing of PCR amplified genomic DNA of the propositus and his parents evidenced the deletion c.565delG within exon 3 of the NAD^+-binding domain of the enzyme

neonatal death of unknown causes are reported. The pattern of transmission of the disease is consistent with an autosomal recessive inheritance (see Fig. 1).

He was born full-term after an uneventful gestation. Maternal anamnesis was negative for gestational diabetes. He was breast-fed till the last day before hospital admission, when he was also bottle-fed because of breast-milk paucity.

At 3 months of age, he started to show signs of seizures with hypertonia, staring gaze, cyanosis, and frothing at the mouth. The seizures were described by the parents as multiple in a single day, however slight, and spontaneously resolving in a few seconds.

When the baby was brought to the emergency room, he was having seizures and was hypoglycemic (blood glucose 1.3 mmol/L), with no detectable ketonuria. Other biochemical routine tests were in the normal range for age. EEG showed no alteration of electrical activity and cerebral ultrasound scan excluded congenital malformations. The baby received an intravenous glucose infusion, in addition to enteral feed, to maintain normoglycemia (6 mg/kg/min to maintain plasma glucose between 2.6 and 3.0 mmol/L) (Aynsley-Green et al. 2000). During the subsequent days of hospitalization, hypoglycemia occurred whenever a reduction in the glucose infusion rate was attempted: sometimes after a few hour-fast, sometimes in the first 2 h after a meal.

In Table 1, the results of plasma insulin, C-peptide, ammonia, lactate and blood ketone bodies when the patient was hypoglycemic are reported. GH, IGF-1, ACTH, cortisol, and thyroid hormones were all in the reference ranges. After glucagon injection (0.1 mg/kg), an appropriate raise in glucose plasma concentration was obtained.

On the basis of persistent hyperinsulinemic hypoglycemia without evidence of ketosis or metabolic acidosis, we collected capillary blood spots during fasting hypoglycemia to perform acylcarnitine analysis.

Therapy with diazoxide at 5 mg/kg/day was instituted, obtaining prompt clinical response with maintenance of normoglycemia with usual enteral feeding, without the need of intravenous glucose infusion.

Table 1 Patient's biochemical evaluations corresponding to four different specimen collections. Reference values are reported in italics between square brackets (bold types refer to pathological or inappropriate values)

Analyte	Sample 1	Sample 2	Sample 3	Sample 4
Patient's age, months^{+days},(time of withdrawal)	3^{+18}	4^{+10}(9:30 am)	4^{+10}(6:40 pm)	4^{+17}
Plasma glucose (mmol/L) *[3.3–5.6]*	**2.1**	**2.5**	**2.7**	**2.3**
Serum insulin (mU/L) *[4.0–24.0]*	<2.0	4.7	**22**	**16**
Serum C-peptide (ng/mL) *[0.90–7.19]*	0.97	NA	NA	NA
Plasma ammonia (μmol/L) *[13.0–42.0]*	**84**	**44**	**56**	NA
Plasma lactate (mmol/L) *[0.5–2.2]*	1.5	NA	NA	2
Capillary blood D-3-OH-butyrate (mmol/L) *[<0.6]*	0.2	0.2	0.2	0.0

NA not analyzed

The parents denied consent for biopsy and for any further investigation; therefore, no evaluations of transcription and enzymatic activity were carried out.

Materials and Methods

Biochemical Evaluation

For analysis of acylcarnitine and aminoacid profiles, a 3 mm punch of blood spot sample was first processed using a NeoBase Non-derivatized MSMS Kit (PerkinElmer Wallac Oy) and analyzed by multiple reaction monitoring (MRM) in a tandem mass spectrometer TQD Detector (Waters), equipped with an ESI source positively charged.

Unlike the derivatized procedure, the nonderivatized assay cannot distinguish between the two isomers malonylcarnitine (C3DC) and 3-hydroxy-butyrylcarnitine (C4OH), the latter being the hallmark of the short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency. Upon detection of an increase in the corresponding mass transition, in order to evidence which of the two analytes was actually increased (De Jesús et al. 2010), a derivatized procedure was also carried out (Turgeon et al. 2008).

Organic acid profile in urine was obtained according to the conventional method as previously reported (Rinaldo 2008).

HADH Sequencing

Genomic DNA was extracted from peripheral leukocytes using QIAamp DNA Mini Kit (QIAGEN). The eight exons of the *HADH* gene were amplified individually by PCR using intronic primers (Clayton et al. 2001). The amplicons were purified using the Exosap-IT clean up Kit (USB) and then submitted to sequence in both directions (GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit, Beckman Coulter). The sequencing reactions were analyzed on a CEQ™ 8800 capillary sequencer (Beckman Coulter) and results were compared with the NCBI Reference Sequences NC_000004.11 and NM_001184705.2.

Results

Biochemical Findings

During hypoglycemia, two blood spots were collected and acylcarnitines' analyses were undertaken (see Table 2), displaying persistently elevated levels of 3-hydroxybutyryl-carnitine (>0.94 μmol/L). Slightly abnormal values of some aminoacids were detected as well, but they are likely due to the metabolic decompensation and hence without diagnostic relevance.

Urine organic acid analysis performed for diagnostic confirmation at time of fasting, evidenced increased quantities of 3-hydroxyglutaric acid (see Fig. 2).

Mutational Analysis

Direct sequencing of the patient's *HADH* gene revealed a new homozygous deletion, c.565delG (reference sequence NM_001184705.2), located within exon 3 in the NAD^+-binding domain of the enzyme (Fig. 1). The deletion leads to an inserted frame-shift sequence of eight aminoacids (WWKPSWRI), followed by the formation of a stop at codon 124.

The early upstream location along the gene of a stop codon leads to the reasonable conclusion that a complete nonfunctional enzyme is produced. The possible presence of the transcript was not investigated due to the parent refusal to biopsy and to any further investigation.

Table 2 Patient's blood spot acylcarnitines' analysis. Two blood spots, collected during hypoglycemia, were tested with two procedures (derivatized and nonderivatized) in different laboratories

Analyte	Procedure	Sample 1	Sample 2	Sample 3
Patient's age, months$^{+\ days}$,(time of withdrawal)		3^{+18}(9:00 am)	3^{+18}(6:45 pm)	4^{+17}
Plasma glucose (mmol/L) *[3.3–5.6]*		2.1	2.5	2.3
C4-OH (μmol/L)	Derivatized	0.94	NA	1.72
	Nonderivatized (isomers: C4-OH + C3DC)[a]	2.39	1.99	NA
C0 (μmol/L)	Derivatized	43	NA	51
	Nonderivatized	37	43	NA

C4-OH 3-hydroxybutyrylcarnitine, *C3DC* malonylcarnitine, *C0* free carnitine, *NA* not analyzed

[a] When the patient's blood spots were assayed, the internal standard for the determination of the isomers (C4-OH + C3DC) of the NeoBase Non-derivatized MSMS kit by PerkinElmer was the $^2H_6$-C5DC. A more recent version of the kit uses for the determination of the same analytes the internal standard $^2H_3$-C4

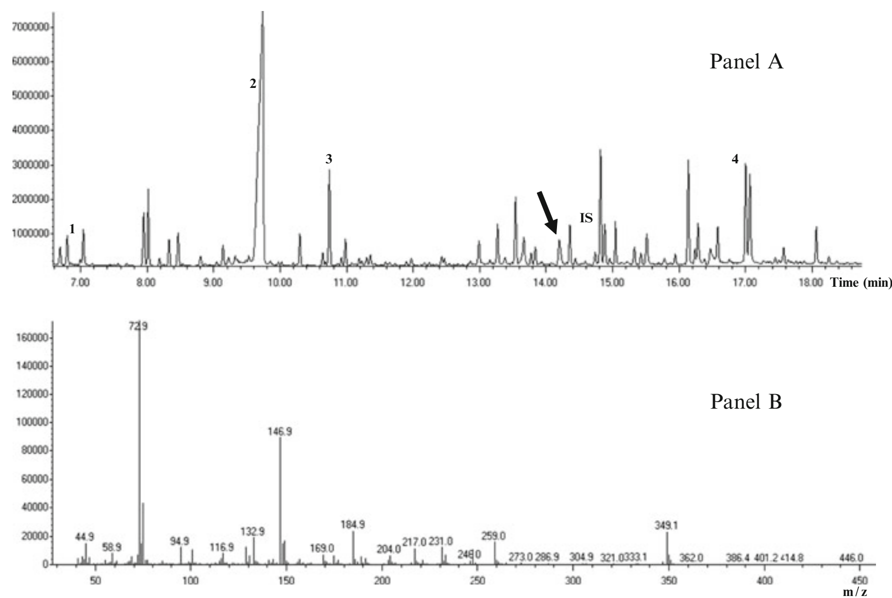

Fig. 2 Total-ion chromatogram of the trimethylsilyl derivatives of organic acids extracted from urine of the patient. Panel **a**: Diagnostic peak of 3-hydroxyglutaric acid is indicated with an *arrow*. For orientation, additional peaks without diagnostic significance are labeled as follows: (*1*) lactic acid; (*2*) urea; (*3*) succinic acid; (*4*) hippuric acid; (*IS*) tropic acid (internal standard). Panel **b**: Mass spectra of 3-hydroxyglutaric acid

Both the parents are heterozygous carriers of the same deletion (Fig. 1).

Discussion

In this paper, we present the eighth case reported in literature of short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency with mutation in the *HADH* gene. At 3 months of age, the patient required hospitalization for severe hypoglycemia, with the need of a glucose infusion rate of at least 6 mg/kg/min to maintain normoglycemia. Hypoglycemia always occurred in the absence of ketonemia or acidosis. Plasma insulin concentration, measured several times during hypoglycemia, was often inappropriately elevated for the glycemia. GH, IGF-1, ACTH, cortisol, and thyroid hormones concentration were in the normal ranges, excluding congenital pituitary hormones deficiency. The mobilization of hepatic glycogen deposits was not impaired, given the normal glycemic response to glucagon injection. During hypoglycemia, ammonia plasma concentration was slightly above the normal range, but never reached levels suggestive of hyperinsulinemia-hyperammonemia syndrome (Stanley et al. 1998). Accordingly, a leucine-sensitivity test was not performed.

The blood spot acylcarnitine profile showed consistently increased concentration of 3-hydroxybutyryl-carnitine, urinary excretion of 3-hydroxyglutaric acid was also elevated. Given the suggestive combination of results, we resolved to investigate the *HADH* gene of the patient, and a new homozygous deletion, c.565delG, was identified in exon 3. The mutation leads to the formation of an early stop codon, within the NAD^+-binding domain of the enzyme. The actual presence of the mRNA and the residual catalytic activity were not assessed due to parent refusal to perform a skin biopsy or any further evaluation as soon as the diagnosis was established. Nevertheless, on the basis of the early location along the gene of the stop codon, it is reasonable to conclude that the corresponding enzyme is virtually absent.

The molecular mechanism of how defects in *HADH* gene lead to unregulated insulin secretion remains unclear. A recent study identified a high expression of *HADH* and a low expression of other β-oxidation enzymes in rat pancreatic β-cells (Martens et al. 2007). In addition, in the same cells of the mouse, the short-chain-L-3-hydroxyacyl-CoA dehydrogenase proved to be a negative regulator of insulin secretion through a mechanism independent of K_{ATP} channel, suggesting a molecular explanation for the diazoxide responsiveness of the hyperinsulinemic hypoglycemia in patients affected by short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency (Hardy et al. 2007). Particularly relevant in this view is the in vitro observation that in human liver mitochondrial extracts, the short-chain-L-3-hydroxyacyl-CoA dehydrogenase specifically interacts with glutamate dehydrogenase type I (*GLUD1*), known to play an important role in the amino acid induced insulin secretion (Filling et al. 2008). Further studies on the molecular interaction between the two proteins in short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency

affected patients could partially explain the unpredictable and intermittent nature of the hyperinsulinemia (Clayton et al. 2001; Hussain et al. 2005), sometimes triggered by a protein dietary load (Kapoor et al. 2009), sometimes even absent (Bennett et al. 2006). Notably, in the *hadh* knockout mouse, an animal model of the hyperinsulinemic hypoglycemia reported in children with recessive inactivating mutation of the *HADH* gene, the insulin secretion dysregulation is due to the loss of an inhibitory protein–protein interaction of the short-chain-L-3-hydroxyacyl-CoA dehydrogenase upon glutamate dehydrogenase (Li et al. 2010).

The diagnostic relevance of the biochemical evaluations, plasma acylcarnitines, and urinary organic acids is known to be crucially dependent on the clinical conditions of the patient during specimen collection. However, when the residual catalytic activity of the mutated enzyme is seriously reduced, the hallmarks of the disease, namely plasma 3-hydroxybutyrylcarnitine and urinary 3-hydroxyglutaric acid, are present in all reported cases.

In conclusion, we have described the eighth case of short-chain-L-3-hydroxyacyl-CoA dehydrogenase deficiency, with a homozygous deletion in the *HADH* gene leading to an early stop codon, characterized by hyperinsulinemic hypoketotic hypoglycemia with enhanced 3-hydroxybutyryl-carnitine in plasma and 3-hydroxyglutaric acid in urine.

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Synopsis

Particularly reduced catalytic activity of the short-chain-L-3-hydroxyacyl-CoA-dehydrogenase leads to hyperinsulinemic hypoketotic hypoglycemia with enhanced plasma 3-hydroxybutyryl-carnitine and urine hydroxyglutaric acid.

Details of the Contributions of Individual Authors

– Florina Ion Popa performed the molecular analysis of HADH gene.
– Silvia Perlini followed the clinical case since patient hospitalization.
– Francesca Teofoli followed the clinical case and was responsible for diagnostic confirmation.
– Daniela Degani followed the clinical case since patient hospitalization.
– Silvia Funghini carried out the urine organic acids' analysis.
– Giancarlo La Marca performed the derivatized tandem mass spectrometry analysis.
– Piero Rinaldo performed the derivatized tandem mass spectrometry analysis and reviewed the paper.
– Monica Vincenzi carried out the nonderivatized tandem mass spectrometry analysis.
– Franco Antoniazzi is responsible for clinical follow up.
– Attilio Boner coordinated the whole diagnostic/therapeutic pathway, which consisted in connecting and keeping in touch different laboratories and clinics.
– Marta Camilot planned and conducted the work described in the article. She wrote the paper.

All Authors edited and approved the final version of the manuscript.

Marta Camilot is the guarantor for the article. She accepts full responsibility for the work, has access to the data and controlled the decision to publish.

All authors have nothing to declare about competing interests.

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Ethics approval was not required. The patient's parents signed an informed consent that is available upon request.

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RESEARCH REPORT

Prevalence of Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency in Estonia

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Abstract The aim of our study was to evaluate the prevalence of long chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) in the general Estonian population and among patients with symptoms suggestive of fatty acid oxidation (FAO) defects. We collected DNA from a cohort of 1,040 anonymous newborn blood spot samples. We screened these samples for the presence of the common c.1528G>C mutation in the *HADHA* gene. Based on the clinical suspicion of FAO defects, we screened suspected individuals since 2004 for the common c.1528G>C mutation in the *HADHA* gene and since 2008 in addition by tandem mass spectrometric analysis of plasma acylcarnitines. Our results showed that the carrier frequency of the c.1528G>C mutation in the Estonian population is high – 1:173. During the screening of symptomatic patients, we identified five LCHADD patients in four families. Three patients were retrospectively identified by molecular screening of the *HADHA* gene. One patient was homozygous for the c.1528G>C mutation in the *HADHA* gene, and two siblings were compound heterozygotes with *HADHA* genotype c.[1528G>C]+[1690-2A>G]. Among patients tested using acylcarnitine profiling, we identified two cases with an abnormal acylcarnitine profile typical to LCHADD. Molecular analysis showed homozygosity for c.1528G>C mutation. Based on a carrier frequency of 1:173 (95% Confidence Interval 1:76–1:454) and taking into account that the c.1528G>C mutation makes up 87.5% of disease alleles in Estonian LCHADD patients, the estimated prevalence of LCHADD in Estonia would be 1: 91,700.

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Abbreviations

LCHADD	Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency
FAO	Fatty acid oxidation

Introduction

Fatty acids are the main source of metabolic energy in humans. Beta-oxidation, a major energy producing pathway in metabolically active tissues such as skeletal muscles and myocardium, also plays an essential role during periods of fasting and metabolic stress (Carpenter et al. 1992a; Tyni and Pihko 1999).

Mitochondrial inner membrane protein complex, the trifunctional protein (TFP) consists of 4 α-subunits harboring

long chain enoyl-CoA hydratase, long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) activity, and 4 β-subunits carrying long chain 3-ketothiolase activity (Kamijo et al. 1994; Carpenter et al. 1992a; Carpenter et al. 1992b). The TFP α-subunit is encoded by the *HADHA* gene (OMIM*600890) and the β-subunit by the *HADHB* gene (OMIM 143450), both of which lie on chromosome 2p23. In rare cases, all three functions of TFP are deficient, but isolated LCHAD deficiency in which hydratase activity is still normal and thiolase activity has decreased moderately is more common (Jlst et al. 1994).

LCHAD deficiency (LCHADD) is caused by mutations in the *HADHA* gene. More than 30 *HADHA* gene variations have been described to date (Gregersen and Olsen 2010), among them c.1528G>C (p.E510Q; NM_000182.4), which is by far the most common mutation, accounting for 87% of all LCHAD alleles. This missense mutation results in the substitution of glutamate to glutamine at amino acid position 510. As this change is located in the catalytically active region, it deteriorates the dehydrogenase activity of the α-subunit protein (Sims et al. 1995; Ijlst et al. 1996).

Isolated LCHADD is an autosomal recessive disease first described in 1989 by Wanders et al. Clinical manifestations of LCHADD can vary from early-onset cardiomyopathy, hypoglycemia, hepatopathy, coma, and sudden infant death syndrome (SIDS) to later onset myopathy, neuropathy, and pigmentary retinopathy (den Boer et al. 2002; Saudubray et al. 1999). Due to the variable clinical spectrum of the disease, it is not easy to differentiate isolated LCHADD from total TFP insufficiency or from other long chain fatty acid oxidation (FAO) disorders. The diagnosis of LCHAD or TFP deficiency is suggested by the finding of characteristic organic acid urine profiles or by an abnormal acylcarnitine profile (Matern 2008). There is generally high morbidity and mortality, and therefore prompt diagnosis at an early age is critical (den Boer et al. 2002). Early recognition and correct treatment are essential in order to lower the mortality rate and alleviate the metabolic crisis.

Although it is acknowledged that LCHADD is a rare but serious life-threatening disease, prevalence of LCHADD has been published for only a few countries, e.g., Sweden 1: 50,000 (Hagenfeldt et al. 1995) and Germany 1: 170,000 (Sander et al. 2005). The incidence of LCHADD by the combined data of the newborn screening programs of Australia, Germany, and USA is 1:250,000 (Lindner et al. 2010). The carrier frequency of the common LCHAD mutation, c.1528G>C, in the overall population is known in only a few populations, and the data show great differences, with 1:217 in Poland (Piekutowska-Abramczuk et al. 2010), 1:240 in Finland (Tyni and Pihko 1999) and zero in the Chinese population (of 1,200 individuals screened) (Zhu et al. 2005).

The main aim of this study was to evaluate the prevalence of LCHADD in the general Estonian population using readily available blood samples collected from newborns and among patients suspected of suffering from FAO defects. The clinical course and outcome in the first reported Estonian patients is described.

Material and Methods

Definition of Study Groups

Group of Anonymous Newborns (N = 1,040)

We collected DNA from a cohort of anonymous newborn samples. These neonates were consecutively born in Estonia in January 2005. We screened these samples for the presence of the c.1528G>C mutation in the *HADHA* gene. DNA was obtained from samples of dried blood spotted on Guthrie cards that had been collected by the Estonian newborn screening program for the detection of cases of phenylketonuria and congenital hypothyreosis. About 90% of newborns born during this month in the whole Estonia were covered.

Study Group of Symptomatic Patients with Suspicion of FAO Defects from the Period 2004–2007 (N = 102)

The blood samples from all individuals were sent to molecular laboratory for both LCHAD and MCAD deficiency from different hospitals in Estonia during 2004–2007. All individuals had clinical suspicion of FAO, but detailed clinical descriptions were not available for us. All were molecularly tested for the common c.1528G>C *HADHA* mutation that causes LCHADD. In some cases, in addition to the molecular analysis (including heterozygotes for c.1528G>C mutation found among general population), acylcarnitine profiling through tandem mass spectrometric (tandem MS) analysis was performed using dried blood spots collected on a Guthrie card in the Charité-Virchow Klinikum (Berlin, Germany).

Study Group of Symptomatic Patients with Clinical Suspicion of FAO Defects from the Period 2008–2010 (N = 425)

Since 2008, all patients with clinical symptoms of FAO defects were first investigated by tandem MS analysis of acylcarnitines from plasma. The indication list for studying plasma acylcarnitine profiles was developed and adapted by Duran (2003). If a child had at least one feature from

the indication list, plasma acylcarnitine analysis was performed. All investigated patients were selected from two regional hospitals (Tartu University Hospital and Tallinn Children's Hospital), which serve as reference centers for the whole of Estonia (which has a population of about 1.34 million). Patients with abnormal acylcarnitine profile were secondly molecularly tested for the common c.1528G>C *HADHA* mutation that causes LCHADD.

This study was approved by the Ethics Committee on Human Research of the University of Tartu.

Molecular Analysis

DNA Extraction

Patients' DNA was extracted from peripheral blood by the standard salting out method or from newborn screening test cards. DNA was extracted from 3 mm disc of the test card, which was soaked in 1 ml of distilled water for at least 2 h, with constant vigorous swirling of the tubes. Then the supernatant was discarded, 100 μl of methanol was added under a ventilation hood, and samples were incubated for 15 min at room temperature. In the next step, the methanol was discarded and 100 μl of freshly prepared 5 mM NaOH and 20–50 μl of mineral oil was added. Then samples were incubated at 100°C for 15 min and immediately placed on ice. The obtained DNA solution can be stored briefly at +4°C or at −20°C for years. This extraction method is cost-effective and also quite robust, as the DNA can easily be used for other PCR-based applications for at least 5 years (Teek et al. 2010; Laugesaar et al. 2010).

Mutation Detection in the HADHA Gene

For c.1528G>C detection, a slight modification of the PCR–RFLP method and primers described by Den Boer (den Boer et al. 2002) were used. PCR was carried out in 96-well plates with a total volume of 10 μl. We used a higher Mg_2Cl concentration (2.5 mM) than that described in the article by den Boer et al., i.e., 1.5 μl of DNA solution and 0.65U HOT FIREPol® DNA Polymerase (Solis BioDyne, Estonia) per reaction. In addition, for test card DNAs the number of PCR cycles was increased from 30 to 35 cycles. The amplified 224 bp PCR products were directly digested in the 96-well plate after adding 1.2 μl of Buffer O and 8U of restriction enzyme *Pst*I (Fermentas; Lithuania). The restriction fragments of 175 bp and 49 bp in wild-type alleles and 117 bp, 58 bp and 49 bp in mutant alleles were analyzed on a 2% (wt/vol) agarose gel with ethidium bromide staining.

HADHA Gene Sequencing

The 20 exons that make up the *HADHA* gene and nearby intronic elements were sequenced as described by Olpin et al. (2005).

Frequency of the HADHA c.1690-2A>G Mutant Allele

Genomic DNA isolated from 59 anonymized dried blood-spots collected from the general Danish population was used to determine the frequency of the *HADHA* c.1690-2A>G mutant allele by sequence analysis of a 355 bp PCR fragment covering exon 17 and nearby intronic elements. The study was performed with the approval of the Central Denmark Regional Committee on Biomedical Research Ethics.

Acylcarnitine Profiling of Plasma Samples

Acylcarnitines were analyzed as butylesters. Sample preparation was performed using standard methods (Matern 2008). About 20 μl of plasma was extracted, with 100 μl of methanol containing the deuterated internal standards and 300 μl acetonitrile was used to sediment plasma proteins. Samples were centrifuged at 4°C for 3 min, and the supernatants were removed. After evaporation, the extracts were butylated using 60 μl butanol/HCl at 65°C for 15 min. The samples were dried again on SpeedVac and finally dissolved in 100 μl acetonitrile/H_2O/formic acid (80:20:0,025, respectively). About 25 μl of the samples was injected into tandem MS (3200 Qtrap, Applied Biosystems MDS Sciex, Canada). Acylcarnitines were measured as positive precursor ion scan for the 85 Da fragment. Quantification was performed using labeled carnitine standards set B from Cambridge Isotopes Inc (MA, USA).

Clinical Data

Clinical data of diagnosed LCHADD cases were obtained from case histories.

Cases 1 and 2 (Family 1)

The first child in this family (a girl, Case 1) died in 1987 at the age of 3.5 months at the local hospital due to cardiomyopathy (no metabolic investigations or autopsy were performed).

The fourth child (a boy, Case 2) was born in 1995. He had his first acute attack of unconsciousness and muscular hypotonia at the age of 9 months. The child had delayed motor development and he needed frequent meals. He was

repeatedly hospitalized due to lethargy and hypoglycemia, additionally liver dysfunction and dilatative cardiomyopathy were observed. At the age of 2.5 years, he referred due to severe hypoglycemia (0.6 mmol/l), lactic acidemia (up to 5.0 mmol/l), and coma. Despite treatment, the child died 8 h after hospitalization. The autopsy showed cardiomyopathy and liver steatosis.

Case 3 (Family 2)

The boy was born in 2007. The mother's obstetric history was complicated: severe hepatic and renal dysfunction had been observed during the first pregnancy. The family's first child died during labor. The course of this pregnancy was also complicated: severe HELLP syndrome was diagnosed during the 28th week of pregnancy. Due to complications in the pregnancy, a caesarean section was performed on the 28th week of gestation. At the age of 6 months, the child was hospitalized. He was pale and weak, clinically presenting with symptoms characteristic of an upper respiratory illness. His blood glucose was slightly over the reference range of 6.3 mmol/l (reference 3.3–5.5 mmol/l) and the infusion with 0.9% NaCl solution was initiated. Six hours after hospitalization and hypoglycemia (0.2 mmol/l) was observed. The child died 10 h after hospitalization.

Case 4 (Family 3)

At the age of 13 months, a girl (born in 2009) became lethargic during an acute viral infection. Hypoglycemia (glucose 2.4 mmol/l), hepatic dysfunction, and increased serum lactic acid (5.8 mmol/l; ref. <2.2) were observed. A diagnosis of LCHADD was later confirmed. A low fat and high carbohydrate treatment was initiated.

Case 5 (Family 4)

The boy (born in 2010) had muscular hypotonia and frequent vomiting since newborn age. At the age of 5 months, he was hospitalized due to somnolence, mild diarrhea, and vomiting. In the hospital generalized muscular hypotonia, hepatomegaly and liver dysfunction was observed. Diagnosis of LCHADD was later confirmed. Dietary treatment with carnitine supplementation (dose 50 mg/kg/die) was initiated, and the child's condition rapidly improved.

Results

Frequency of the c.1528G>C *HADHA* Mutation in the General Estonian Population

We tested 1,040 anonymous Estonian neonates from the general population who were consecutively born in 2005 for the presence of the c.1528G>C mutation in the *HADHA* gene. We detected six heterozygotes for the c.1528G>C mutation, and no c.1528G>C homozygotes. The acylcarnitine profile of all heterozygous individuals was measured from a newborn Guthrie card in the Charité-Virchow Klinikum (Berlin, Germany). None had biochemical abnormalities suggestive of LCHADD. This indicates a carrier frequency of 1 in 173 (95% Confidence Interval 1:76–1:454; Table 1).

Results of Selective Screening of Symptomatic Patients with the Suspicion of FAO Defects

During the screening of symptomatic patients, we identified four patients in three families with LCHADD. Among 102 individuals who were molecularly tested for the c.1528G>C *HADHA* mutation between 2005 and 2007, we found one patient to be homozygous for the c.1528G>C mutation (case 3) and one father who was heterozygous for the same mutation (family 1). His two children had died at an early age (Case 1 and 2). *HADHA* gene sequencing revealed that his wife was heterozygous for a c.1690-2A>G mutation in intron 16 of the *HADHA* gene. The c.1690-2A>G mutation was not identified in 59 DNA samples collected from the general population. The

Table 1 The carrier frequency of the c.1528G>C mutation in different countries

Population	No. of investigated individuals/carriers	Carrier frequency of c.1528G>C mutation	References
Finland	1200/5	1:240	Tyni and Pihko (1999)
The Netherlands	2047/3	1:680	den Boer et al. (2000)
Poland (whole country)	4137/22	1:189	Piekutowska-Abramczuk et al. (2010)
Poland (Pomerania)	2976/41	1:73	Piekutowska-Abramczuk et al. (2010)
China	1200/0	0	Zhu et al. (2005)
Estonia	1040/6	1:173	This study

c.1690-2A>G mutation most likely causes abnormal *HADHA* splicing. According to the splice site prediction program (http://www.fruitfly.org/seq_tools/splice.html), the c.1690-2A>G mutation changes the splice score of the IVS16 3' splice site from 0.97 to 0.0 (maximum splice score is 1.0). Unfortunately the in vivo consequences of the splice site mutation could not be investigated, as no cDNA was available from the mother and the two deceased children. In addition, no DNA was available from those children.

Of the 425 patients who were tested using acylcarnitine profiling in 2008–2010, we identified two patients (Cases 4 and 5) who had an abnormal acylcarnitine profile typical to LCHADD. Molecular analysis showed that both patients were homozygous for the c.1528G>C mutation in the *HADHA* gene.

In our relatively small patient group, we identified eight independent LCHADD alleles from four families. Seven of them (87.5%) carried the c.1528G>C mutation, and in one allele the c.1690-2A>G mutation was found after *HADHA* gene sequencing. Based on a c.1528G>C heterozygote frequency of 1:173 among the general Estonian population and taking into account that this mutation is present on 87.5% of disease alleles in our patients, we calculated that the estimated prevalence of LCHADD in Estonia is 1:91,700.

Discussion

No proven LCHADD cases were reported in Estonia until 2005. We decided to establish whether this was due to the poor clinical recognition of the affected patients or to the low incidence of genetic changes causing LCHADD. In the first step, we investigated the carrier frequency of the common c.1528G>C *HADHA* mutation in the general Estonian population, in order to obtain an estimated prevalence of LCHADD in Estonia. We assumed that the c.1528G>C mutation is the most common LCHADD mutation in Estonia, as is the case in other European (neighboring) countries (Finland, Poland, The Netherlands) (Piekutowska-Abramczuk et al. 2010; Tyni and Pihko 1999; den Boer et al. 2000). Our study results showed that the frequency of heterozygotes for the c.1528G>C mutation in Estonia is high – 1:173 similar to neighboring counties – Finland and Poland (Tyni and Pihko 1999; Piekutowska-Abramczuk et al. 2010) (Table 1). Based on the heterozygote frequency and taking into account that the common mutation is present on 87.5% of disease alleles in our small cohort of patients, we calculated that the prevalence of LCHADD in Estonia is 1:91,700. As the annual birth rate in Estonia in recent years is about 16,000, we can expect one LCHADD patient every 5–6 years. During the last 3 years, however, we have diagnosed one LCHADD case every year. Therefore, the final prevalence may be even higher after the commencement of the nationwide mass screening program for FAO in Estonia. Our results show that the prevalence of LCHADD is comparable to Poland, where the prevalence is 1:118,336 (Piekutowska-Abramczuk et al. 2010), and higher than 1:250,000 observed in Australia, Germany, and the USA (Lindner et al. 2010).

In all of our patients, clinical symptoms appeared at around the age of 1 year (5.5–14 months). At least three patients had hypoglycemia in initial presentation, and in at least two cases hypoglycemic episodes prior to diagnosis were fatal (cases 2 and 3). Retrospective analysis of the disease histories of those two cases revealed that both patients had symptoms indicating LCHADD prior the fatal episode of the disease. These children might not have died if early specific diagnostic possibilities and treatment had been available. Acylcarnitine profiling by tandem MS is considered to be the "cornerstone" in the diagnostics of FAO defects. This method has been available in Estonia since 2008 for symptomatic patients only. Although mutational screening was available a few years earlier, the introduction of tandem MS-based acylcarnitine analysis allowed much wider recruitment of patients with symptoms suggestive of FAO disorders. According to our experience, the indications given by Duran (2003) are very practical and suitable for the selective screening of FAO defects.

In summary, our main goal for the future is to introduce acylcarnitine analysis to all newborns as a part of our national newborn screening program.

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Concise One-Sentence Take-Home Message

The carrier frequency of the c.1528G>C mutation in the *HADHA* gene in the Estonian population is 1:173.

Abbreviated Title

LCHADD in Estonia

References to Electronic Databases

OMIM #609016

Details of the Contributions Made by Individual Authors

K. Joost – development of method and performance of tandem MS analysis of acylcarnitines of all investigated individuals, diagnosis of suspected and confirmed cases, compilation of manuscript, treatment of a patient with LCHAD deficiency.

K. Õunap – planning of study, selection of suspected cases, moderation of cooperation between different centers, diagnosis of suspected patients, compilation of manuscript

R. Žordania – selection of suspected cases, diagnosis and evaluation of a family with LCHAD deficiency

M.-L. Uudelepp – evaluation and treatment of a patient with LCHAD deficiency

R.K. Olsen – sequencing of *HADHA* gene

K. Kall – performance of organic acid GC/MS analyses during the confirmation/exclusion of suspected LCHADD cases

K. Kilk – development of tandem MS analysis of acylcarnitines

U. Soomets – planning of strategy for biochemical diagnosis using tandem MS

T. Kahre – performance of molecular analysis for main mutation in *HADHA* gene in all investigated individuals, compilation of manuscript

Guarantor of Article

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Statement of Competing Interest

All of the authors confirm that they have no competing interests to declare. There is no financial or nonfinancial interest in publishing this article.

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Details of the Ethics Approval

The Ethics Review Committee on Human Research of the University of Tartu approved the study.

Patient Consent Statement

Informed consent was obtained from the parents of the children involved in the research.

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CASE REPORT

Primary Carnitine Deficiency Presents Atypically with Long QT Syndrome: A Case Report

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Abstract Primary carnitine deficiency (PCD) is an autosomal recessive disorder of fatty acid oxidation caused by mutations in the *SLC22A5* gene encoding for the carnitine transporter OCTN2. Carnitine uptake deficiency results in renal carnitine wasting and low plasma levels. PCD usually presents early in life either with acute metabolic crisis or as progressive cardiomyopathy that responds to carnitine supplementation. PCD inclusion in the newborn screening (NBS) programs has led to the identification of asymptomatic adult patients ascertained because of a positive NBS in their offspring. We extensively reviewed the literature and found that 15 of 42 adult published cases (35.7%) were symptomatic. Cardiac arrhythmias were present in five patients (12%). Here, we report the ascertainment and long-term follow-up of the first case of PCD presenting with long QT syndrome. The patient presented in her early twenties with a syncopal episode caused by ventricular tachycardia, and a prolonged QT interval. Arrhythmias were poorly controlled by pharmacologic therapy and a defibrillator was installed. Syncopal episodes escalated during her first pregnancy. A positive NBS in the patient's child suggested a carnitine uptake deficiency, which was confirmed by reduced carnitine transporter activity and by molecular testing. After starting carnitine supplementation, no further syncopal episodes have occurred and the QT interval returned to normal. As precaution, a low-dose metoprolol therapy and the defibrillator are still in place. Although rare, PCD should be ruled out as a cause of cardiac arrhythmias since oral carnitine supplementation is readily available and efficient.

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Introduction

Systemic primary carnitine deficiency (PCD; OMIM# 212140) is an autosomal recessive disorder of the carnitine cycle impairing fatty acid oxidation. PCD is caused by mutations in the *SLC22A5* gene that encodes for the high-affinity carnitine transporter OCTN2 (Nezu et al. 1999). Carnitine transporter defects result in renal wasting of carnitine, low plasma carnitine levels (free carnitine <5 μM), and intracellular carnitine deficiency. Carnitine allows the entry of long-chain fatty acids into mitochondria for subsequent β-oxidation. Carnitine deficiency results in defective energy production from fat during fasting or stress. The diagnosis is suspected because of low plasma carnitine levels with normal renal function and acylcarnitine profile. The diagnosis is confirmed by reduced carnitine transporter activity in fibroblasts or mutational analysis of the *SLC22A5* gene.

The incidence of PCD is about 1:40,000 with a carrier rate of approximately 1% (Wilcken et al. 2003). Patients can present early in life with acute metabolic crisis, hepatic encephalopathy, hypoketotic hypoglycemia, Reye syndrome, or sudden death. Some patients have the insidious onset of progressive cardiomyopathy with or without muscle weakness and hypotonia. Left untreated, the

Table 1 Primary carnitine deficiency: clinical presentation in adult patients

Clinical presentation	n	Age (gender)	Plasma free carnitine (μM)	*SLC22A5* gene sequencing	
				Allele 1	Allele 2
No symptoms[a]	27	21–43 y (25 F) (2 M)	0.9–12 (2.8 ± 2.5; *n* = 19)	See specific references	
Fatigability[b]	7	19–32 y (6 F) (1 M)	0–3.5 (1.37 ± 1.2; *n* = 6)	See specific references	
Fatigability during pregnancy[c] (normal ECG)	1	37 y (F)	4	p.P46S	p.T23M
Easy fatigability, fasting intolerance[d] (normal ECG)	1	28 y (F)	2	p.P46S	p.R28X
Decreased stamina, nonspecific T wave abnormality[d]	1	34 y (F)	4	p.R24X	p.S467C
Ventricular arrhythmias, prolonged QT interval[d, PR]	1	20 y (F)	1.9	p.N32S	p.P46S
Aborted sudden death due to ventricular fibrillation, short QT interval with increased T waves[e]	1	28 y (F)	NA	Confirmed by molecular testing[f]	
Easy fatigability, palpitations, sinus tachycardia[c]	1	28 y (F)	4	p.R83L	p.A214V
Cardiomyopathy, ventricular premature beats (VPCs)[g]	1	34 y (F)	2.17	p.S467C	p.S467C and p.R282Q
Exercise intolerance, diabetes[h]	1	30 y (F)	2	p.P46S	p.P398L

NA not available

[a] Spiekerkoetter et al. (2003), Vijay et al. (2006), Schimmenti et al. (2007), El-Hattab et al. (2010), Lee et al. (2010), Li et al. (2010), Sarafoglou et al. (2010)

[b] Vijay et al. (2006), Lund et al. (2007), Lee et al. (2010)

[c] El-Hattab et al. (2010), Li et al. (2010)

[d] Schimmenti et al. (2007) and present report (PR)

[e] Labarthe et al. (2009)

[f] Confirmed by molecular testing but the specific mutations were not indicated

[g] Lee et al. (2010)

[h] Li et al. (2010)

disease is severe and may be fatal (Stanley et al. 1991). Carnitine supplementation prevents symptoms and reverses cardiomyopathy, supporting PCD inclusion in expanded NBS programs. Wide-scale NBS has led to the identification of asymptomatic affected mothers whose newborns screen positive for low carnitine, as well as other asymptomatic family members (Spiekerkoetter et al. 2003; Vijay et al. 2006; Schimmenti et al. 2007; El-Hattab et al. 2010; Lee et al. 2010; Li et al. 2010; Sarafoglou et al. 2010). However, the disorder is unlikely a benign condition in adults. Fifteen of the forty-two published cases ascertained in adulthood (35.7%) because of a positive NBS in the offspring or through family history were symptomatic (Table 1). Cardiac arrhythmias were described in five patients (12%) (Schimmenti et al. 2007; El-Hattab et al. 2010; Lee et al. 2010; Li et al. 2010), as well as in heterozygous carriers of *SLC22A5* mutations (Sarafoglou et al. 2010). The impact of carnitine supplementation on cardiac abnormalities is still unclear in these patients. Here, we report the long-term follow-up of a case of PCD presenting atypically with long QT syndrome.

Case Report

A 20-year-old Caucasian woman presented with a syncopal episode caused by ventricular tachycardia. Family history was negative for long QT interval or significant cardiovascular events. Prior to the episode, she lived an active lifestyle with no episodes of hypoglycemia, liver problems, or muscle weakness. No signs of cardiomyopathy were present and echocardiogram was completely normal. Upon hospitalization, therapy with metoprolol (50 mg/day) was started and a defibrillator was implanted. Over the following 5 years, the cardiac symptoms were difficult to control with pharmacologic therapy, and the patient experienced three more syncopal events. An ECG showed prolonged QT interval (QT 355 ms; QTc 441 ms, normal range <440 ms). At age 25, she had multiple syncopal episodes after becoming pregnant. At 14 weeks of gestation, metoprolol was increased (75 mg/day) and mexiletine (450 mg/day) was added. No more episodes occurred during the remainder of her pregnancy.

PCD was eventually diagnosed after her newborn daughter screened positive for low free carnitine. The

patient's plasma carnitine levels were extremely low (total carnitine: 3.0 μM, normal range 26–69 μM; free: 1.9 μM, normal range 16–60 μM). Carnitine uptake in fibroblasts was reduced to 4.8% of normal controls, and molecular analysis of the *SLC22A5* gene showed two changes, c.95 A>G (p.N32S) and c.136 C>G (p.P46S) (Schimmenti et al. 2007). Alternative genetic causes of long QT syndrome were not excluded in our patient. Treatment with high doses of carnitine (3,960 mg/day) was initiated immediately, and has been continued to date. Since the beginning of carnitine treatment, plasma carnitine levels have been within normal limits (at the last visit, total carnitine 47.95 μM, normal range 26–69 μM, free carnitine 36.05 μM, normal range 16–60 μM), no further syncopal episodes have occurred and the QT interval has returned to normal (QT 360 ms, QTc 408 ms, normal range <440 ms). Treatment with carnitine allowed lowering of the metoprolol dose (25 mg/day) and discontinuation of other drugs. However, as a precaution, our patient still carries a defibrillator. At the last cardiologic evaluation, the patient's echocardiogram remains normal. A second pregnancy was uneventful.

Discussion

Systemic PCD is a rare disorder that usually presents in childhood with metabolic crisis or with progressive cardiomyopathy. Cardiomyopathy, which is frequent in infantile patients, is not commonly seen in adults. Among reported cases, dilated cardiomyopathy was present only in one patient (Lee et al. 2010) and excluded in most of the others. Moreover, PCD mutations are no more frequent in adult patients with cardiomyopathy than in the general population (Amat di San Filippo et al. 2008). Cardiac arrhythmias were present in five PCD patients (12%) ascertained in adulthood (Schimmenti et al. 2007; El-Hattab et al. 2010; Lee et al. 2010; Li et al. 2010), suggesting that carnitine uptake deficiency should be considered as a potential cause of unexplained cardiac arrhythmias. Our case also shows that a long QT interval may be present in PCD.

We did not rule out mutations in the 12 different genes associated with long QT syndromes. To our knowledge, a cardiac calcium channel mutation has been found only in one case of adult-onset PCD presenting with short QT interval (Labarthe et al. 2009). A causal relationship between cardiac arrhythmias and PCD has not yet been established, although in our patient carnitine supplementation prevented further syncopal episodes. Long-term data are essential to establish the relationship between arrhythmias and carnitine deficiency. Recently, heterozygosity for PCD mutations has been associated with cardiac arrhythmias that resolved after carnitine treatment (Sarafoglou et al. 2010). PCD may have a much higher incidence than originally described as has been noted in the Faroe Islands (Lund et al. 2007). Additionally, asymptomatic males are less likely to be identified through NBS. Although rare, PCD should be routinely investigated as a cause of cardiac arrhythmias since episodes could be prevented by oral carnitine supplementation.

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Synopsis

The ascertainment and the 5-year follow up are described for an adult case of PCD presenting atypically with long QT syndrome successfully treated with carnitine supplementation.

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RESEARCH REPORT

Long-Term Pharmacological Management of Phenylketonuria, Including Patients Below the Age of 4 Years

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Abstract BH4 therapy is an advancement in the treatment of phenylketonuria, reducing blood phenylalanine (phe) levels and increasing tolerance to natural proteins of responding patients. We report the results of 16 patients undergoing long-term BH4 treatment. Responding patients to BH4 was usually based on 24-h loading tests; a $\geq$30% decrease in blood phe was considered a positive response. Weekly loading made it possible to identify an additional "slow responder." The 16 responders constitute 24.6% of patients who completed the trial (87.5% of responders in mild hyperphenylalaninemia, 38.1% in mild PKU, and 2.8% in classical PKU).

Mean dose of BH4 used was 9.75 $\pm$ 0.9 mg/kg per day, during a mean of 62 months. Age at treatment start was below 4 years in seven patients; five of which begun treatment during their first month since birth. All but one patient showed good treatment compliance; six continue on BH4 monotherapy without dietary phe restriction; six showed an increase in phe tolerance of 24–55%; and in the five patients who received treatment since the neonatal period an increase in phe tolerance following the phase of maximum growth has persisted. None of the patients showed side effects except one whom vomiting at the beginning of the treatment.

Testing at the time of diagnosis in the neonatal period is very appropriate, and if there is a positive response, the patient can be treated with BH4 from onset with the advantage of being able to continue breast-feeding.

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Introduction

Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism resulting from a deficiency of phenylalanine hydroxylase (PAH:EC 1.14.16.1), the liver enzyme that catalyzes the hydroxylation of phenylalanine (Phe) to tyrosine. This enzyme deficiency leads to elevated levels of Phe in the blood and to other tissues, as well as to corresponding neurotoxic effects. Untreated patients are characterized by mental retardation, microcephaly, delayed speech, seizures, eczema, and behavior abnormalities.

There are different phenotypes of PKU resulting from a mutated PAH: mild hyperphenylalaninemia (HPA), mild PKU, and classical PKU, which are classified on the basis of plasma Phe levels at the time of diagnosis as well as protein tolerance.

Adherence to a low-Phe diet from birth is effective for preventing mental retardation; however, it is very restrictive and limits the quality of life of patients and their families. Since Kure's study in 1999 (Kure et al. 1999), a number of reports have demonstrated the existence of a subset of patients with mutations in the PAH gene who show a positive response to tetrahydrobiopterin (BH4), the natural cofactor of PAH, thus reducing their plasma Phe levels.

This has facilitated important new treatment possibilities for a significant proportion of HPA and PKU patients; in many cases, BH4 treatment (with a dose of 5–20 mg/kg per day) allows these patients to follow a near normal diet, or at least, a less rigid low-protein diet.

A tablet formulation of BH4 (dihydrochloride) has been available for three decades. Although this formulation has been used extensively in experimental studies, it has not been evaluated in formal clinical trials and was not recorded. A newer formulation of BH4 (sapropterin dihydrochloride, Kuvan®) for the treatment of PKU that is more stable at room temperature has been available in the USA and Europe (Burnett 2007) since 2008; in Spain, the Ministry of Health authorized this product for commercial use in 2009. Clinical studies suggest that treatment with sapropterin provides better Phe control and increases dietary Phe tolerance (Belanger-Quintana et al. 2005; Burlina and Blau 2009; Burton et al. 2011; Blau et al. 2010; Vernon et al. 2010; Trefz et al. 2010). No serious adverse events with BH4 treatment were reported in medical literature (Belanger-Quintana et al. 2005; Nielsen et al. 2010); headache, upper respiratory tract infections, and rhinorrhea were the side effects observed in sapropterin-treated patients with PKU in clinical trials (Kuvan® 2008; Trefz et al. 2009a).

In a previous work (Bóveda et al. 2007), we reported a study of response to oral BH4 loading in a group of 36 patients with PKU, and subsequent treatment and follow-up of patients who responded positively in this test. Here, we describe the evolution of patients in the PKU group, with different phenotypes, who responded to BH4 and are currently being treated with sapropterin (Kuvan®) in the Metabolic Disorders Unit of the University Clinical Hospital of Santiago de Compostela (Galicia, Spain).

Patients and Methods

Our Metabolic Disorders Unit is monitoring a total of 107 patients diagnosed with HPA and with different phenotypes: 36 classical PKU (Phe $>$ 1,200 μmol/L, tolerance $\leq$ 400 mg/day), 21 mild PKU (Phe between 600 and 1,200 μmol/L, tolerance $\leq$ 500 mg/day), 8 mild HPA (Phe between 360 and 600 μmol/L, tolerance up to 650 mg/day), and finally 42 benign HPA (Phe $<$ 360 μmol/L, free diet); the latter are subjected to Phe level monitoring, but are not subjected to dieting and, therefore, are not the object of this paper.

Of these patients, 16 responders are being treated with BH4: one classical PKU, eight mild PKU, and seven mild HPA. Table 1 lists the characteristics of the 16 patients who are currently being treated with sapropterin: phenotype, plasma Phe concentration at diagnosis, type of diagnosis (by newborn or late screening), genotype, 24-h BH4 loading (age at testing, % reduction at 24 h).

The screening of the group of patients who responded to BH4 was based on loading with cofactor: 24-h testing with 20 mg/kg of BH4 with previous loading of a dose of 100 mg/kg of phe and baseline Phe endpoints at 4, 8, 15, and 24 h. This screening was started in 2002, since then, BH4 loading is carried out in the neonatal period when the level of Phe is over 400 μmol/L, and there is no previous loading with this amino acid.

A decrease of $\geq$ 30% in blood Phe is considered to be a positive result.

Patients who showed a 25–30% response in the 24-h underwent to a weekly BH4 loading test. We carried out the following protocol: dose of 20 mg/kg per day of BH4, administered for 1 week, with daily Phe-level measurement. A 30% or higher reduction in blood Phe levels by the end of the period was considered to be a positive response.

Responsive patients were treated with BH4 since testing, including those under the age of 4 years who were administered the drug after requesting compassionate treatment. Informed consent was requested from all parents or patients of legal age. Response to BH4 treatment was measured, assessing dietary tolerance to phe and blood Phe levels in treated patients; this response was then correlated with its genotype. Side effects were assessed by asking about headache, vomiting, abdominal pain, rhinorrhea, or other symptoms after BH4 treatment.

Results

A total of 24.6% (16/65) of patients with HPA (excluding patients with benign HPA) who were treated in the Diagnosis and Treatment Unit at the University Clinical Hospital of Santiago de Compostela received BH4 pharmacological treatment (6R-BH4 from Dr. Schircks Laboratories, Jona, Switzerland) and since 2009, Sapropterin dihydrochloridre (Kuvan®) as the single treatment in addition to diet. Age range at the beginning of treatment varied from 1 month to 24 years since birth.

Phe levels decreased by $\geq$ 30% in these 16 patients after 24-h or weekly BH4 loading, except in patient no. 4, who is a sibling of responsive patient no. 3 with similar mutations and phenotypes and whom was administered treatment directly, without loading tests. Patient no. 14, who at 2 years and 5 months underwent combined Phe/BH4 loading test (Bóveda et al. 2007) with a 28% decrease of Phe level after 24 h, continued dietary treatment and at 8 years 6 months underwent a weekly BH4 loading test, with a 34% decrease in phe levels, since then, pharmacological treatment was added to dietary treatment.

Table 2 shows the evolution of the group of patients with PKU treated with sapropterin; the table shows, tolerance and mean Phe levels before and after treatment with sapropterin, cofactor dose in each case, and time to treatment evolution.

All of them began BH4 treatment at a dose of 5–10 mg/kg per day increasing to a maximum of 20 mg/kg per day; this dose

Table 1 Characteristics of patients treated with sapropterin: phenotype, type of diagnosis (newborn or late screening), genotype, 24-h BH4 loading (age at testing, % reduction at 24 h), current age, and psychomotor development (PDI/IQ)

Patient	Phenotype	Diagnosis	Genotype	Age at BH4 testing	% Phe reduction 24 h	Actual age	Current PDI/IQ
1	Mild HPA	NBS	p.R243Q(c.728 G>A) / p.E390G (c.1169 A>G)	5 y 3 m	64.8	12 y 6 m	74
2	Mild HPA	NBS	p.P211T(c.631 C>A) / IVS10nt-11 G>A(c.1066-11 G>A)	1st month	64.2	4 y 11 m	88
3	Mild HPA	late (4 y)	p.S303A(c.907 T>G) / p.G46S (c.136 G>A)	9 y 2 m	90.2	16 y 5 m	99
4	Mild HPA	NBS	p.S303A(c.907 T>G) / p.G46S (c.136 G>A)	n/a	n/a	12 y 7 m	105
5	Mild HPA	NBS	n/a	1st month	64.2	1 y 3 m	110
6	Mild HPA	NBS	p.R176L(c.527 G>T) / –	3 y 9 m	76	11 y	97
7	Mild HPA	NBS	p.Y277D(c.829 T>G) / p.L48S (c.143 T>C)	1st month	60	4 y 5 m	103
8	Mild PKU	NBS	p.R68S(c.204 A>T) / IVS10nt-11 G>A (c.1066-11 G>A)	1st month	60	2 y	93
9	Mild PKU	NBS	p.Y277D(c.829 T>G) / p.L48S (c.143 T>C)	1st month	57.1	4 y 5 m	97
10	Mild PKU	NBS	p.R158Q(c.473 G>A) / p.L48S (c.143 T>C)	6 y 10 m	43	14 y 4 m	106
11	Mild PKU	late (6 y)	p.Q304Q (c.912 G>A) / p.R176L (c.527 G>T)	16 y 5 m	80.7	22 y 4 m	120
12	Mild PKU	NBS	p.V388M(c.1162 G>A) / p.L48S (c.143 T>C)	18 y 5 m	33.6	24 y 9 m	115
13	Mild PKU	NBS	p.V388M(c.1162 G>A) / p.R243Q (c.728 G>A)	13 y 10 m	37.1	18 y 8 m	118
14	Mild PKU	NBS	p.I65T(c.194 T>C) / p.R243X (c.727 C>T)	2 y 5 m	28	9 y 6 m	120
15	Mild PKU	NBS	p.P244L(c.731 C>T) / p.R261Q (c.782 G>A)	9 y 7 m	31.9	16 y 6 m	122
16	Classical PKU	NBS	p.G46S(c.136 G>A) / p.R243Q (c.728 G>A)	16 y 4 m	40.3	22 y 11 m	97

n/a not analyzed; *y* years; *m* months

was adjusted based on later dietary tolerance and phe levels, thus a mean dose of 9.75 ± 0.9 mg/kg per day of sapropterin (range 8–12.5) was maintained in our patients. A few days after confirming positive response to BH4 loading, all patients began treatment; 31.2% (5/16) began treatment during their first month since birth. Mean treatment duration was 5 years 2 months; duration in each of the three patients who have been receiving treatment the longest was 7 years and 3 months.

The five patients who began treatment during the neonatal period (numbers 2, 5, 7, 8, and 9), three mild HPA and two mild PKU, with phe levels at the time of treatment between 403 and 1,066 μmol/L, showed a decrease of more than 30% after 8 h and more than 50% after 24 h since the 24-h BH4 loading test. Treatment with BH4 enabled tolerance to 550 mg/day of phe, and all patients maintained optimal levels of phe (mean 171 ± 98, medium 150, range 135–195 μmol/L). As far as their genotype, except for one patient who was not studied, the remaining patients are compound heterozygous with at least one BH4-responsive allele (Zurflüh et al. 2008). Anthropometric parameters remain between p50 and p90 in all of them with a normal nutritional status.

The 11 patients who began treatment after the neonatal period showed at least a 24% increase in phe tolerance, except for patient no. 12 who was not taking the drug regularly. This was clearly apparent in the four patients with mild HPA since they all moved to a near-normal diet with recommended maximal Phe intake of 100 mg/kg per day (i.e. “controlled free diet”), presenting a tolerance of Phe from 2,000 to 3,000 mg/day by age. In the seven patients with mild PKU and classical PKU, the increase in tolerance was more fluctuating, from 24% to more than 50%, one patient was even able to relax dietary restrictions with a 3,000 mg/day tolerance of Phe. In addition, phe levels in these patients remained slightly higher before BH4 (mean 412 ± 154, medium 303 μmol/L) than after BH4 (mean 403 ± 136, medium 394 μmol/L) and stayed within the appropriate range for their age, except in one patient with classical PKU whose levels remained within the highest

Table 2 Evolution of the 16 patients with different phenotypes treated with sapropterin

Patient	Phenotype	Phe level (μmol/L) and tolerance (mg/day) pre-BH4			Phe level (μmol/L) and tolerance (mg/day) post-BH4			BH4 dose mg/kg per day	Treatment duration
		Mean ± SD	Medium	Phe tolerance	Mean ± SD	Medium	Phe tolerance		
1	Mild HPA	254 ± 104	242	640	273 ± 65	279	2,000[a]	10	7 y
2	Mild HPA	n/a	n/a	n/a	192 ± 84	182	880	8.9	4 y 10 m
3	Mild HPA	208 ± 80	188	580	182 ± 35	182	3,000[a]	8	7 y 3 m
4	Mild HPA	222 ± 84	212	600	233 ± 63	212	2,200[a]	10	7 y 3 m
5	Mild HPA	n/a	n/a	n/a	135 ± 35	127	1,100[a]	10	1 y 2 m
6	Mild HPA	230 ± 127	194	650	164 ± 42	151	2,000[a]	9.4	7 y 3 m
7	Mild HPA	n/a	n/a	n/a	170 ± 111	139	800	10.4	4 y 4 m
8	Mild PKU	n/a	n/a	n/a	195 ± 142	179	565	10	1 y 11 m
9	Mild PKU	n/a	n/a	n/a	164 ± 118	127	750	10	4 y 4 m
10	Mild PKU	290 ± 145	273	430	310 ± 158	324	739	9.5	6 y 6 m
11	Mild PKU	418 ± 127	394	500	396 ± 127	376	3,000[a]	9.7	6 y
12	Mild PKU	356 ± 151	303	400	559 ± 64	539	500	9	6 y 9 m
13	Mild PKU	497 ± 139	509	350	409 ± 212	427	480	9.4	5 y 1 m
14	Mild PKU	241 ± 139	212	390	216 ± 81	194	513	12.5	1 y
15	Mild PKU	288 ± 105	285	470	269 ± 88	254	750	9.3	6 y 11 m
16	Classical PKU	794 ± 273	891	350	666 ± 224	648	780	10	6 y 7 m

n/a not analyzed; *y* years; *m* months

[a] Controlled free diet

recommended limit; however, in this patient mean values decreased after BH4 treatment. The genotype is quite varied; patients no. 10, 12, 13, and 15 have two different BH4-responsive alleles, the remaining patients have at least one BH4-responsive mutation. In our opinion, p.G46S mutation may be classified as responsive in light of the findings by Wang et al. (2007).

Patient no. 14, who was not responsive to the 24-h test but was responsive to the weekly test, with one year of evolution and BH4 treatment at 11.5 mg/kg per day, showed tolerance to 513 mg/day (31% more) with 10% lower mean phe endpoint values for that year. This patient has a BH4 responsive mutation (p.I65T) in one allele and a mutation that is classified as nonresponsive in the other (p.R243X).

One additional late-diagnosed adult with a severe phenotype and an E280K mutation in homozygosis was tested with a week-long protocol due to difficulties with dietary compliance. He experimented a reduction of 34% in Phe levels and has been treated with a low BH4 dose, showing mild improvement in his Phe tolerance but much greater dietary compliance and refers less mood disorders after a period of one year in treatment. As the long-term responsiveness of this patient is still under evaluation, we have not included his data on the tables.

None of the patients showed BH4 treatment-related secondary effects, except patient no. 12 whom vomiting at the beginning of the treatment.

Discussion

At this time, there is no single protocol for considering a patient to be BH4 responsive (Blau et al. 2010). The 24-h protocol with 20 mg/kg of BH4 with or without combination phe 100 mg/kg is most commonly used (Blau 2008); this is also the protocol we used in most of our patients; since 2003 we applied this test without Phe overload in the neonatal period, as many authors advise (Feillet et al. 2008). We know that BH4 only demonstrates a pharmacological chaperone effect at high Phe concentrations (Gersting et al. 2010), however, BH4 test conducted in our neonates with values of phe not very high (between 403 and 636 μmol/L) showed significant results.

We administered a weekly test with 20 mg/kg of BH4 in one patient with a response that was very close to positive (between 25% and 30% decrease in phe levels) and in this patient phe levels decreased by 34%; therefore, the patient was deemed responsive.

There are recent recommendations for carrying out baseline 48-h loading tests (Blau et al. 2009), but we are currently, in general, administering the 24-h test to all patients during the neonatal period with no previous phe loading; in our opinion, it is very important to adjust timepoints in this particular stage of life in order to prevent excessive treatment delays when there is no response.

However, if they are not administered during this period, their administration is, in our opinion, a good option.

One must take into account that patients with an early diagnosis of PKU (<15 days since birth) can show no decrease in phe levels with BH4, while at the same time being responsive at a later age. That is why, in the event of a negative response in the neonatal period and an indicative phenotype/genotype, repeating the study at later ages through a 7-day test with 20 mg/kg per day of BH4 is recommendable; this option also helps identify "slow responders."

The 16 patients in our study with a positive response to BH4 constitute 24.6% of patients who completed the trial (87.5% of responders in mild HPA, 38.1% in mild PKU, and 2.8% in classical PKU). This percentage is similar to that of other studies (Trefz et al. 2010). Other authors obtained a far superior rate of response, 62%, (Vernon et al. 2010); this may be because the loading test experiments are performed under dietary supplementation with a low-phe food.

All patients who underwent BH4 treatment showed good treatment compliance except patient no. 12, who also suffered from vomiting at some points when administration began, although it was transitory. In the 15 remaining patients, after a slight decrease in phe levels following 9.75 ± 0.9 mg/kg per day of BH4 for a mean time of 5 years, phe tolerance increased significantly. We did not use higher BH4 medium doses because we did not observe better protein tolerance. Five of the seven patients with mild HPA received BH4 monotherapy without dietary Phe restriction; in patients with mild PKU and classical PKU, one patient with mild PKU is receiving BH4 monotherapy and the other patients (who began treatment after the neonatal period) phe tolerance increased 24–55%; however, patients no.12–14 had a moderate improvement of the diet, which meant less burdensome for these patients and their families, although the cost benefit ratio, due to the high price of the drug, is an issue to take into account.

On the other hand, patients with a higher percentage of response to BH4 showed higher tolerance to phe; as a result, patient no. 11 with mild PKU and a decrease in phe levels of 51% and 80.7% at 8 and 24 h since BH4 loading, respectively, receives only monotherpy with BH4 without dietary restrictions.

Seven of the 16 patients who received BH4 treatment were below 4 years old at start of treatment, five of them had already begun treatment on their first month of life. With a mean evolution time of 53 months (12–87 months), phe tolerance increases have persisted following the phase of maximum growth after six months since birth, which constitutes a "honeymoon period," and protein requirement, including Phe intake, is easily assimilated (Burlina and Blau 2009) without any secondary effects resulting from the medication. Medical literature includes few published studies that use sapropterin at those ages (Spaapen et al. 2001; Shintaku et al. 2004; Hennermann et al. 2005; Burton et al. 2011), like us, all of them found the drug to be safe and no significant side effects were observed. Classical dietary treatment of PKU may cause several micronutrient deficiencies (Acosta and Yannicelli 1999); children treated with BH4 since neonatal period had a good somatic growth development and an adequate nutritional status, including selenium which was deficient in many patients treated with dietary treatment. However, comparative studies with more patients and longer period of study are needed.

Although this is not always the case, similarly to other studies, we usually found a correlation between genotype and response to BH4; some studies establish a 76% correlation, others deem there to be good correlations, particularly in homozygotes, for nonresponsive mutations (Daniele et al. 2009; Desviat et al. 2004; Trefz et al. 2009b; Zurflüh et al. 2008).

In our 5-year experience with 6-methyltetrahydrobiopterin and one year and a half with sapropterin, we assessed no difference between each during phe level decrease (unpublished data), and patients prefer to take sapropterin.

As a result, in light of our results we can conclude that the 24-h BH4 loading test is most commonly used and that tests carried out once a week or every three weeks can identify additional responders. The level of response to BH4 with BH4 loading is very important for later, long-term treatment. Test administration at the time of diagnosis is more practical, if detection is neonatal, Phe tolerance with BH4 treatment between the ages of 6 to 12 months will be reevaluated for false positives. To date we have not assessed any side effects with BH4 treatment, including children below the age of 4 years, so we consider that BH4 test should be made in the neonatal period and start treatment in those with positive response.

Synopsis

This study presents long-term evolution of 16 PKU patients undergoing BH4 treatment showing its efficacy. Furthermore, it highlights the usefulness of BH4 test at the time of diagnosis in the neonatal period and the early treatment if there is a positive response.

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CASE REPORT

Short-Term Outcome of Propionic Aciduria Treated at Presentation with N-Carbamylglutamate: A Retrospective Review of Four Patients

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Abstract N-carbamylglutamate (NCG) has been reported to decrease ammonia levels in patients with propionic aciduria (PA) and methylmalonic aciduria (MMA), but reports on clinical outcomes remain scant. Here, we report a retrospective series of four patients with neonatal PA treated with NCG at presentation. Patients presented between 2 and 9 days of age and peak plasma ammonia ranged from 524 to 1,572 μM. Patients received bolus (30–200 mg/kg) and sustaining (115–300 mg/kg per day) doses of NCG in addition to a standard treatment regimen that included ammonia scavenger drugs. Ammonia levels decreased significantly in three of the four cases within 2 h after administration of NCG and fell below 100 μM in all within 12–29 h. Two patients received NCG (bolus 200 mg/kg) while ammonia was above 500 μM (740 and 1,572) and their levels fell below 500 μM by 4 and 8 h post-treatment, respectively. Outcomes of these NGC-treated patients were not improved over previously reported PA patients who did not receive NCG: two died during the initial episode and one after his third metabolic decompensation at 46 days. The survivor is now 3 years old and has a well-controlled seizure disorder and a mild developmental delay mostly in language. We conclude that despite a trial of NCG and a rapid fall in plasma ammonia, the short-term outcome of these patients was not improved.

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Introduction

Propionic aciduria (PA) is a disorder of branched-chain amino acids caused by a deficiency of propionyl-CoA carboxylase, a hetero-oligomeric enzyme complex consisting of α- and β-subunits encoded by the *PCCA* and *PCCB* genes, respectively. Patients with neonatal onset of PA present with encephalopathy, ketoacidosis, and hyperammonemia. They may be symptomatic before the result of newborn screening is available. Mortality is significant in cases presenting in the neonatal period, with 30–36% dying at the initial decompensation or before 2 years of age (Dionisi-Vici et al. 2006; Surtees et al. 1992). Predictors of neurological outcomes include coma duration, and frequency and severity of metabolic decompensations. Various mechanisms have been proposed to explain the poor outcomes including inhibition of mitochondrial energy metabolism and direct ammonia toxicity (Picca et al. 2001; Schwab et al. 2006).

Hyperammonemia in PA is thought to be related to inhibition of N-acetylglutamate synthase (NAGS) enzyme by intramitochondrial accumulation of propionic acid (Coude et al. 1979), which results in decreased allosteric activation of the urea cycle enzyme, carbamoylphosphate synthetase I (CPSI). In fact, N-carbamylglutamate (NCG),

an analog of N-acetylglutamate, can activate CPSI, and NCG treatment has been shown to reduce hyperammonemia in initial, as well as subsequent, PA decompensation episodes (Filippi et al. 2010; Gebhardt et al. 2003, 2005; Jones et al. 2008; Levrat et al. 2008; Schwahn et al. 2010). Moreover, increased ureagenesis has been demonstrated in PA patients after administration of NCG (Ah Mew et al. 2010). Other mechanisms have been proposed to explain hyperammonemia in organic aciduria including depletion of Krebs cycle intermediates, resulting in reduced alpha-ketoglutarate for glutamate and glutamine production, leading to altered synthesis of N-acetylglutamate. Indeed, glutamate and glutamine levels are reduced in PA (Al-Hassnan et al. 2003; Filipowicz et al. 2006). If the ammonia toxicity has a significant impact on the neurological outcomes of PA patients, then decreasing the hyperammonemia by NCG supplementation could reduce associated morbidity. However, reports on clinical outcomes of infants with PA treated with NCG are scant. Here, we report four neonatal cases of PA treated by NCG at presentation and their short-term clinical outcomes.

Case Reports

Between 2006 and 2009, in two tertiary pediatric hospitals in Montreal, Province of Quebec, Canada, a total of four cases of symptomatic neonatal-onset PA were treated by NCG. In Quebec, newborn screening for organic acidurias by tandem mass spectrometry is not yet available. NCG was obtained from Orphan Europe through a special program of Health Canada. Clinical characteristics, laboratory results, and management at presentation are shown in Table 1.

For all patients, pregnancy and delivery were unremarkable. Patients presented between 2 and 9 days of age and were from different ethnic backgrounds: Indian, Hispanic, Nigerian-Dominican, and Greek. Two patients (A and D) showed a more severe clinical picture with severe metabolic acidosis (pH < 7.1) and hypothermia. Three patients (A, C, and D) were transferred from a local hospital to the tertiary pediatric center where diagnostic investigations and metabolic treatment were promptly instigated. All patients showed characteristic metabolites on urine organic acid profiles by GC-MS, and diagnosis was confirmed by identification of mutations in either *PCCA* or *PCCB* genes (Table 1). Plasma glutamine levels were elevated only in patient D. Serial measurements of blood ammonia after admission to the tertiary hospital are shown in Fig. 1. Delay between reported first symptoms and initiation of treatment ranged from 8 to 23 h (Table 2). Upon admission to tertiary center, calories were provided in the form of intravenous dextrose and lipids. In addition, the good neurological status of patient B permitted the early introduction of enteral nutrition with protein-free formula. Protein was reintroduced in parenteral nutrition for patients B, C, and D at, respectively 48 h (0.6 g/kg per day), 48 h (0.5 g/kg per day) and 62 h (0.9 g/kg per day) after admission. The total caloric intake for each patient during the course of the admission is shown in Fig. 1. Relevant medications provided are listed in Table 1 and include L-carnitine, ammonia scavenger drugs (priming and sustaining infusion), and NCG (priming and sustaining oral doses by nasogastric tube). Sustaining doses of NCG and ammonia scavengers were provided until ammonemia normalized. Timing of ammonia scavenger drugs, NCG and dialysis, if performed, is indicated in Fig. 1.

Administration of NCG was concomitant to the scavenger drugs, except for patient B where NCG was given 12 h after starting the scavenger drugs. Plasma ammonia levels decreased significantly in three of the four patients within 2 h after administration of NCG. The nonresponder (patient A) had a transient elevation of ammonia lasting ~10 h. The ammonia level in Patient B showed a downward trend just prior to NCG administration. Blood ammonia levels fell below 100 μM in all patients within 12–29 h of treatment initiation. For patients C and D, whose blood ammonia was above 500 μM prior to NCG bolus (C: 740 μM, D: 1,572 μM), levels fell below 500 μM in 4 and 8 h post-treatment, respectively. Among the four patients, hemodialysis was performed in patient D only, and was begun after a dramatic decrease of blood ammonia had occurred.

The outcomes of our four patients are shown in Table 2. Two patients (A and D) died at the initial metabolic decompensation. Patient C survived the initial episode and was subsequently treated with a protein-restricted diet and carnitine supplementation. Axial hypotonia was noted at 1 month of age and brain MRI showed delayed myelination. He was re-admitted to the ICU for two additional metabolic decompensations; on the second, he presented with lethargy, pH 6.92 and died during the course of the hospitalization at 46 days of life. Patient B, the only survivor, is now 3 years of age. She receives protein restriction and L-carnitine supplementation, without further use of NCG. At 6 months of age, overnight gavage was introduced to improve metabolic control. The patient was admitted once to the ICU in the context of a rotavirus gastroenteritis and dehydration. Mild hepatomegaly was observed early in the course and remained stable without evidence of liver dysfunction. A seizure disorder was diagnosed at age 10 months and remains well controlled with anticonvulsant monotherapy. Head computed tomography and brain MRI were normal. Delayed gross motor milestones were apparent by age 6 months. At 3 years old, she has a mild developmental delay mainly in language. She plays interactive games, copy circles, combines 3–4

Table 1 Clinical presentation, diagnostic laboratory values, and management of propionic aciduria patients in this study

	Patients	A	B	C	D
Initial presentation	Age at presentation	2 days	9 days	2 days	3 days
	Symptoms	Respiratory distress Lethargy Hypothermia	Vomiting Lethargy	Respiratory distress Feeding difficulties	Feeding difficulties Lethargy Hypothermia
	pH/Bicarbonate (mM)	7.06/4	7.38/22	7.29/8	7.0/3
	Anion gap (mM)	34	9	21	42
	Ammonia (µM)	404	188	781	782
Diagnostic labs	Urine organic acids (µmol/mmol creatinine)				
	3-OH-propioniate(normal $<$ 41)	4,712	14,253	15,632	11,704
	Propionylglycine(normal $<$ 3)	42	301	38	14
	Methylcitrate(normal $<$ 13)	1,189	1,227	744	93
	Plasma amino acids (µM)				
	Glutamine(normal 474–736)	450	457	504	1,085
	Glutamate(normal 31–113)	46	197	55	62
	Glycine(normal 138–276)	517	429	1,280	507
	Molecular analysis	*PCCB*:	*PCCB*:	*PCCA*:	*PCCB*:
	Allele 1	c.990dupT (p.E331X)	c.517_518delTT (p.L173GfsX56)	c.878A>G (p.Q293R)	c.331 C>T (p.R11X)
	Allele 2	c.942 C>A (p.T314X)	c.183 + 3 G>C	c.878A>G (p.Q293R)	c.415 C>T (p.Q139X)
Medication	Carnitine (mg/kg per day)	100	100	100	100
	Ammonia scavengers[a]				
	Arginine (mg/kg)	600	250	600	600
	Benzoate (mg/kg)	250	250	250	250
	Phenylacetate (mg/kg)	–	250	250	–
	NCG (nasogastric tube)				
	Priming dose (mg/kg)	75	30	200	200
	Sustaining dose (mg/kg per day)	300 (divided TID)	115 (divided QID)	200 (divided TID)	200 (divided TID)
Dialysis		No	No	No	Yes

[a] Dosage for priming infusion provided. Sustaining infusion use same dosage over 24 h

words and walk up steps. Although our small sample size prevents a formal data analysis to identify prognosis markers, it is noteworthy that the survivor (patient B) had the lowest peak ammonia level, the shortest duration of hyperammonemia, and was never in coma. Furthermore, this patient had a splice site mutation on one allele, which was not studied but could lead to residual wild type transcript and protein.

Discussion

We report the outcomes of four patients with neonatal PA that were treated with NCG at the initial metabolic decompensation. Previous reports have suggested a role for NCG in the treatment of hyperammonemia in PA (Filippi et al. 2010; Gebhardt et al. 2003, 2005; Jones et al. 2008; Levrat et al. 2008; Schwahn et al. 2010). Since inhibition of N-acetylglutamate synthase may be a contributing factor to the hyperammonemia in PA, the use of NCG, a compound analogous to N-acetylglutamate, could stimulate CPSI in lieu of N-acetylglutamate (Hall et al. 1958; Rubio and Grisolía 1981). Recently, other investigators provided support for this idea by showing increased ureagenesis and decreased ammonia levels following NCG administration in PA patients (Ah Mew et al. 2010; Tuchman et al. 2008). However in all reports, including ours, evaluation of NCG efficacy remains limited by concomitant dialysis and /or the co-administration of ammonia scavenger drugs. The efficacy of ammonia

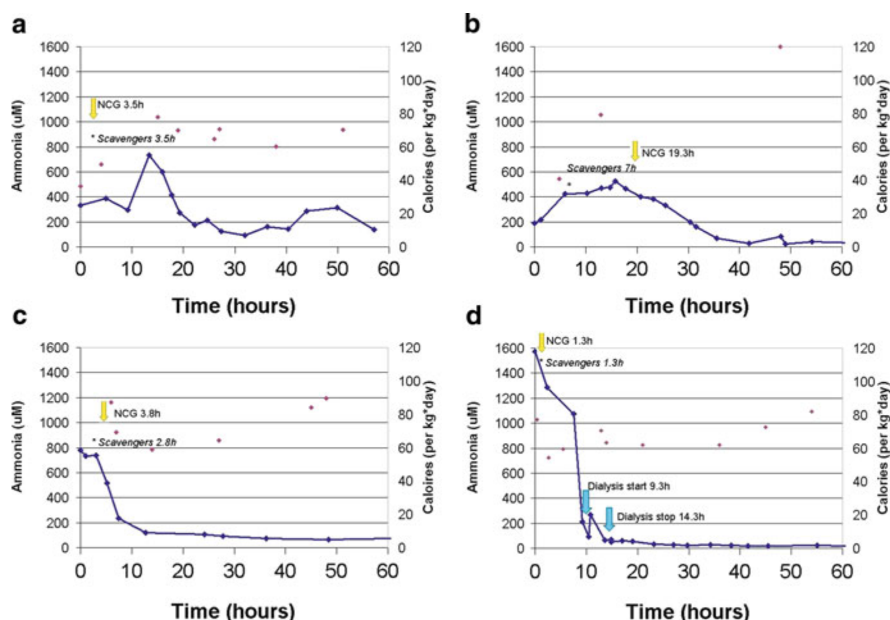

Fig. 1 Decrease in ammonia blood level in propionic aciduria patient treated with N-Carbamylglutamate (NCG). *Solid lines* represent ammonia blood level and single points, the total amount of calories provided during the time course. NCG administration is indicated by *yellow arrow*, *blue arrow* represents dialysis, and *asterisks* indicates the start of ammonia scavengers

Table 2 Patients outcomes

Patient	Initial decompensation					Follow up	
	Onset treatment (h)	Hyperammonemia		Coma (days)	ICU days	Survival	Outcomes (age)
		Peak (μM)	Duration > 500 μM (h)				
A	23	735	6	2	–	Deceased 6 days	–
B	13	524	2	0	1	Alive	Seizure, mild hepatomegaly, mild developmental delay (3 years)
C	8	781	5	1	2	Deceased 46 days	Hypotonia, delayed myelination on MRI(1 month)
D	18	1,572	23	4	–	Deceased 8 days	–

Coma defined as Glasgow score less than 8. *ICU* intensive care unit

scavengers for controlling hyperammonemia in organic acidurias also remains controversial, given the low or normal glutamine plasma levels typically found in PA patients (Al-Hassnan et al. 2003; Filipowicz et al. 2006). Indeed, glutamine was not elevated in three of our four patients. Thus, the contribution of ammonia scavengers to the decrease in ammonia observed is expected to be less robust than that observed in urea cycle defects.

In our series, three out of four patients showed decrease in ammonemia concomitant with NCG administration. Patient B received NCG 12 h after ammonia scavengers alone had not shown a definitive decrease in ammonia levels, thus arguing for a direct response to NCG. Although reversal of catabolism could have caused the decrease in ammonia, calories were increased from only 44 Kcal/kg per day to 80 Kcal/kg per day just 6 h prior the first NCG dose. In patients C and D, the ammonia decreased more rapidly with NCG, but this was concomitant to the administration of the scavenger drugs. Again, reversal of catabolism as the only factor contributing to the reduction in ammonia is unlikely, given the relatively low total calories provided. We suggest that the combination of NCG and scavengers, or possibly NCG alone, was sufficient to lower ammonia below 500 μM within 6 h, which is a time frame required to

Table 3 Previously published outcomes of propionic aciduria patients treated with N-Carbamylglutamate (NCG)

Onset of symptoms (days)	NGC priming dose	Dialysis	Outcome (age)	Reference
2	250 mg/kg	Yes (before NCG)	Microcephaly, sits, polysyllabic (14 months)	Jones et al. (2008)
11	250 mg/kg	Yes (after NCG)	Sits, few words (3.5 years old)	Jones et al. (2008)
3	400 mg	No	Global developmental delay (age?)	Schwahn et al. (2010)
3	400 mg	No	Motor delay, feeding difficulties (age?)	Schwahn et al. (2010)
2	150 mg/kg	No	Diffuse cerebral atrophy, axial hypotonia (2 months old)	Filippi et al. (2010)

start dialysis. Thus, NCG treatment may avoid dialysis in PA when the only indication is hyperammonemia.

The response rate to NCG is unknown in PA, as there is no systematic study of its administration. In this study, one out of four PA patients (patient A) did not initially respond to NCG. An insufficient NCG dose (75 mg/kg) may explain this finding, although one patient with organic aciduria responded to an initial dose of 70 mg/kg (Gebhardt et al. 2003). Others reported initial doses ranging from 100 mg/kg to 250 mg/kg, all associated with reduction in ammonemia (Filippi et al. 2010; Gebhardt et al. 2003, 2005; Jones et al. 2008; Levrat et al. 2008; Schwahn et al. 2010). One reported patient had no response to a dose of 25 mg/kg (Jones et al. 2008).

Outcomes observed in our series of NCG-treated patients were not improved over those with classical treatment without NCG (Dionisi-Vici et al. 2006; Surtees et al. 1992). Mortality of 30–36% has been reported before 2 years of age and development delay is commonly observed. In our series, three patients (A, C, D) out of four were deceased before 2 years of age and the survivor (B) has mild developmental delay. In the latter, it is uncertain whether NCG had a significant role in controlling hyperammonemia and influencing the outcome. Compared to other patients, the lower peak ammonia level, the shorter duration of hyperammonemia and absence of coma could be related possibly to higher residual enzyme activity given presence of a splice site mutation.

There are only five other cases reported in the literature on the outcome of neonatal PA treated by NCG, which are summarized in Table 3. Other reports of use of NCG in PA are limited to the description of the trend of ammonia levels (Gebhardt et al. 2003, 2005; Levrat et al. 2008). Publication bias for successful NCG trials, various NCG doses, and concomitant dialysis and/or ammonia scavengers obscure the ability to draw accurate conclusions. The long-term benefits of NCG in treatment of PA, thus remain unknown. It should be noted that NCG targets only one potential mechanism of neurotoxicity, which is hyperammonemia. Currently, there is no strong evidence that actively treating hyperammonemia improves outcomes in PA, and other mechanisms, such as inhibition of the mitochondrial respiratory chain, may play a more determining role. Better assessment of long-term outcomes of patients treated with NCG is currently needed. This could be facilitated by longitudinal multicenter studies involving a registry and a standard administration protocol.

Conclusion

In summary, we report the use of NCG in the treatment of neonatal PA in four patients. As previously reported, interpretation of NCG efficacy was limited by concomitant use of ammonia scavengers. Nevertheless, a rapid and sustained decrease in ammonia level was observed in three out of four cases. However, short-term outcome was not improved compared to that reported in cases who did not receive NCG. More data is needed to evaluate the long-term benefit of NCG in treatment of hyperammonemia in PA.

Synopsis

While a trial of NCG may be useful in controlling hyperammonemia in PA, the evidence for improved long-term outcomes are still lacking.

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CASE REPORT

Combined Enzyme Replacement Therapy and Hematopoietic Stem Cell Transplantation in Mucopolysacharidosis Type VI

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Abstract Mucopolysaccharidosis type VI, Maroteaux–Lamy syndrome is a lysosomal storage disorder with progressive, multisystem involvement caused by deficiency of the lysosomal enzyme *N*-acetylgalactosamine-4-sulfatase leading to accumulation of the glycosaminoglycan, keratan sulfate. Enzyme replacement therapy (ERT) has been shown to clinically benefit affected individuals. A combined treatment regime of ERT and hemopoietic stem cell transplantation (HSCT) has led to reduced morbidity and mortality in patients with MPS I. We have demonstrated that a treatment regime of ERT combined with HSCT in a 3-year-old girl with MPS VI provided similar benefit. This treatment regimen should be considered in the management of selected patients with MPS VI. Neither HSCT nor ERT can correct or completely prevent progression of the musculoskeletal complications. Long-term follow-up and regular assessments for these complications is necessary.

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Abbreviations

CPAP	Continuous positive airway pressure
ERT	Enzyme replacement therapy
GAGS	Glycosaminoglycans
HSCT	Hematopoietic stem cell transplantation
MPS	Mucopolysaccharidosis

Introduction

Mucopolysaccharidosis type VI, Maroteaux–Lamy syndrome (MIM #253200) is a lysosomal storage disorder resulting from deficient activity of the enzyme *N*-acetylgalactosamine 4-sulfatase (Arylsulfatase B EC # 3.1.6.12), which impairs the stepwise degradation of the glycosaminoglycan, dermatan sulfate. Partially degraded dermatan sulfate accumulates in the lysosomes in a wide range of tissues, causing a chronic progressive disorder with shortened life span. Until recently, treatment has been symptomatic and supportive. Specific therapies are now available to provide deficient enzyme, including hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). To date, a combined treatment regimen of ERT and HSCT has not been previously reported for this disorder.

Case Report

A 2-year 6-month-old girl was referred for evaluation of a possible lysosomal storage disorder. The patient was born in Saudi Arabia at term by cesarean section after a pregnancy complicated by gestational diabetes. There were no problems in the perinatal period; however in her first year, she suffered recurrent otitis media and upper respiratory tract infections. At 1 year of age, she was found to have an abnormal mitral valve.

Her early development was normal. She sat unsupported at 7 months, crawled at 8 months and learned to walk at 11 months. Her expressive and receptive speech development was normal and she developed a pincer grip at 12 months. When evaluated at our center, the patient was mildly developmentally delayed in her locomotor skills when scored using the Peabody two motor scales.

The patient's height was just below the 50th percentile, weight was on the 50th percentile and head circumference was on the 75th percentile. She had coarsening of her facial features, macroglossia, prominent forehead, and had bilateral corneal opacification. She had upper airway obstruction, a small umbilical hernia and moderate hepatosplenomegaly. There was clawing of the distal and interphalangeal joints. In addition, she had restricted wrist flexion, radial deviation, and elbow extension bilaterally.

Initial screening suggested the possibility of upper airway obstruction with minimum oxygen saturation of 85% and evidence of hypercapnia on a morning (arterial) blood gas with pCO_2 value of 54 mmHg. However, an overnight sleep study just 1 week later demonstrated severe obstructive sleep apnea with a total index of respiratory events of 19.5/h of sleep time, minimum oxygen saturation of 80% and a high baseline CO_2 that was further increased by around 10 mmHg during periods of REM sleep when the index of obstructive respiratory events was 77.5/h. As a result of this study, nasal mask CPAP therapy was recommended to alleviate her upper-airway obstruction and although tolerated in hospital at pressures of 6 cm H_2O to alleviate her upper airway obstruction, her parents had trouble maintaining this at home. Following commencement of enzyme therapy and before BMT, a follow-up sleep study showed considerable resolution of her airway obstruction with the total index of respiratory events falling to 8.2 events per hour of sleep time, minimum oxygen saturation 89% normal CO_2 baseline values (39.2 mmHg) and the index of events in REM sleep had falling to 13.3 with only 3–4 mmHg increase in CO_2 during REM sleep.

An echocardiogram showed mitral valve prolapse, moderate mitral valve regurgitation, dilated left ventricle and left atrium with normal ventricular function. She had moderately severe conductive hearing loss. A skeletal survey showed dysostosis multiplex with thickened calvarium, generalized osteopenia, broad long bones, flared ribs, bilateral coxa valga, and shallow acetabulae.

The diagnosis of MPS VI was established at age 2 years and 6 months by the finding of elevated urinary glycosaminoglycans, 76.9 mg/mM (normal range 5.4–26.6 mg/mM creatinine) and a reduced level of the lysosomal enzyme *N*-acetylgalactosamine-4-sulfate sulfatase activity, 0.05 pmol/min per mg protein (normal range 1.5–21.3 pmol/min per mg protein).

The patient was subsequently found to be homozygous for the p.Y251X mutation in the *ARSB* gene.

Combined Enzyme Replacement Therapy: Hematopoietic Progenitor Stem Cell Transplantation

ERT was commenced at 2 years and 8 months of age, 16 weeks prior to HSCT, providing weekly intravenous infusions of recombinant human enzyme Galsulfase at a dose of 1 mg/kg per dose. This was continued for 12 weeks after HSCT, providing a total of 28 weekly infusions. HSCT was performed using matched, T-cell depleted unrelated donor peripheral blood stem cells following conditioning with fludarabine, busulfan, and cyclophosphamide.

Combined ERT/HSCT was well tolerated by the patient. The post HSCT course was complicated by mild skin graft versus host disease, treated with steroids.

She required some physiotherapy treatment whilst an inpatient to maintain joint range of movement, muscle strength, and maintenance of developmental milestones in keeping with HSCT and graft versus host disease rather than a result of the MPS.

Follow-Up 1 Year Post HSCT

The response to ERT was rapid with softening of cutaneous features, improved breathing, regression of tonsillar and adenoidal hypertrophy, resolution of obstructive sleep apnea and hepatosplenomegaly. The umbilical hernia was surgically repaired 8 months after the transplantation.

One year after transplantation, she was growing and developing well. There was an improvement in her joint range of movement, gross motor abilities and independence, although remains delayed for her age. She had only mild progression of metatarsus adductus and did not display calf tightness despite growth.

Marrow engraftment was achieved with 100% donor cell line by chimerism studies at 10 months after transplantation.

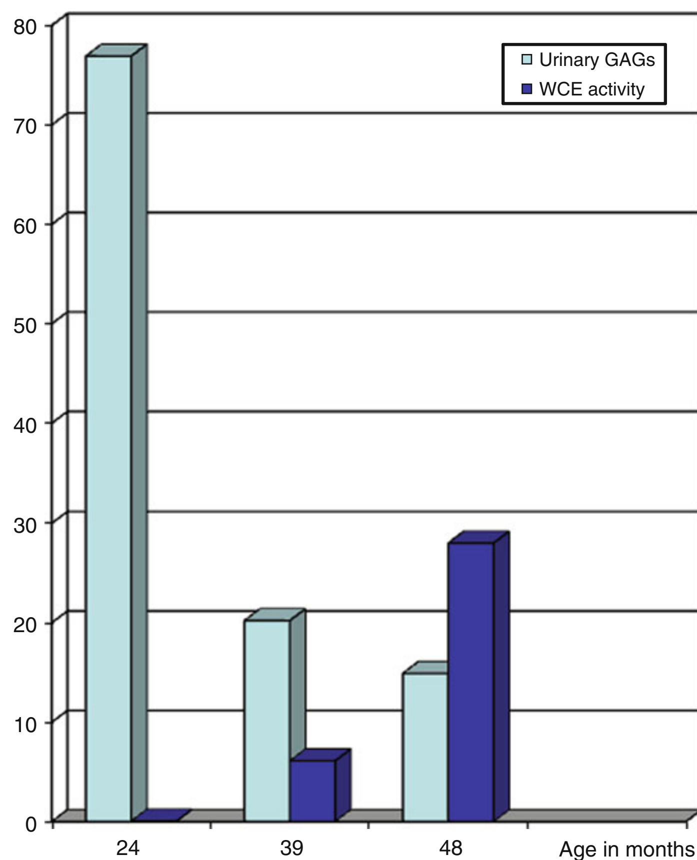

Fig. 1 Urinary GAGS and leucocyte *N*-acetylgalactosamine-4-sulfate sulfatase activity

A follow-up sleep study (10 months after the HSCT) showed that her respiratory indices had now fallen into the normal range, with a total index of respiratory events of 3.3/h, but obstructive events were 0.9/h. Minimum oxygen saturation was 84.0% (after a central apnea). However, she had tachypnea with respiratory rates in the 30s and continued to have mild CO_2 retention (4 mmHg) during REM sleep periods.

A skeletal survey showed that the severity of the gibbus deformity had only minimally progressed since commencing therapy. There was some improvement of her hearing. An echocardiogram showed mitral valve prolapse and moderate mitral regurgitation, dilated left ventricle with normal ventricular function.

Urinary glycosaminoglycans fell to within the normal range. The level of *N*-acetylgalactosamine-4-sulfate sulfatase activity increased to be within the normal range. See Fig. 1.

Discussion

Maroteaux–Lamy syndrome (MPS VI) is an autosomal recessive disorder caused by the deficiency of the lysosomal enzyme *N*-acetylgalactosamine-4-sulfatase (arylsulfatase B). It is a progressive multisystem disorder with a wide phenotypic spectrum and normal intelligence. Pathogenic mutations in the arylsulfatase B (*ARSB*) gene lead to incomplete degradation, cellular accumulation, and increased urinary excretion of the glycosaminoglycan, dermatan sulfate. The estimated incidence of MPS VI is approximately 1:300,000 (Giugliani et al. 2007).

The disorder shows a wide spectrum of clinical manifestations with slowly to rapidly progressive forms. The characteristic skeletal dysplasia includes short stature, dysostosis multiplex and degenerative joint disease. Rapidly progressive forms may have onset from birth, severe dysotosis multiplex and death before the second or third decades. A more slowly progressive form has been described having a later onset with milder musculoskeletal complications and death in the fourth or fifth decades. Other clinical features include cardiac valve disease, hepatosplenomegaly, hearing loss, recurrent otitis media, obstructive sleep apnea, corneal clouding, carpal tunnel disease, umbilical and/or inguinal hernia. Central nervous system manifestations include slowly progressive cervical cord compression caused by, accumulation of T2 negative on MRI fibrogelatinous material, circumferential meningeal

thickening and/or bony stenosis, rarely cervical spine instability, communicating hydrocephalus, optic nerve atrophy, and blindness. Most MPS VI patients have relatively normal intellectual development (Valayannopoulos et al. 2010).

Until recently, supportive care and bone marrow transplantation were the only therapies available for MPS VI patients. HSCT restores endogenous production of inherently deficient enzyme. The morbidity and mortality associated with HSCT in patients with MPS has improved since HSCT was introduced but the general results have been poor compared to the reduction in morbidity and mortality seen in patients receiving HSCT for hemopoietic malignancies (Turbeville et al. 2011). ERT with Galsulfase®, which became available with the first trials commencing in 2002, resulted in significant improvement in endurance, respiratory, cardiovascular, and musculoskeletal function in patients with MPS VI (Hamartz et al. 2008; Giugliani et al. 2007).

A number of recent publications have demonstrated reduced HSCT morbidity and mortality from preconditioning with ERT and support during the first 120 days post-transplant in infants with MPS I (Hurler). Supplementary ERT before HSCT rapidly reduces upper airway obstruction, and improves cardiorespiratory capacity thereby improving patient fitness for HSCT (Grewal et al. 2005; Cox-Brinkman et al. 2006; Muenzner et al. 2009; Wynn et al. 2009; Tolar et al. 2008; Bijarnia et al. 2009; Clarke et al. 2009). We have demonstrated that a treatment regimen of ERT combined with HSCT in our patient with MPS VI provided similar benefit. This treatment regimen should be considered in the management of selected patients with MPS VI. Long-term follow-up studies comparing the morbidity and mortality of HSCT and ERT combined with HSCT are necessary to evaluate the overall benefits of each treatment regime.

Neither HSCT nor ERT can correct or completely prevent progression of the musculoskeletal complications of mucopolysaccharidosis, carpal tunnel syndrome, or corneal changes. Moreover, progressive kyphosis and scoliosis are common complications. Regular, at least annual assessment for these complications is necessary and surgical interventions are indicated and usually successful.

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RESEARCH REPORT

Further Delineation of the Phenotype of Congenital Disorder of Glycosylation DPAGT1-CDG (CDG-Ij) Identified by Homozygosity Mapping

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Abstract Congenital disorders of glycosylation (CDG) are an expanding group of genetic diseases affecting protein and lipid glycosylation. These disorders have a variable presentation and are individually rare. DPAGT1-CDG is a protein N-glycosylation disorder with epilepsy, development delay, severe hypotonia, and dysmorphy, reported in a single patient. Here we present the second family with DPAGT1-CDG identified through homozygosity mapping in a large consanguineous family with 18 affected infants. The patients had severe hypotonia, global developmental delay, seizures, and microcephaly but no dysmorphy. In the index case, the brain MRI revealed delayed myelination, and there was fiber type disproportion on muscle biopsy. Homozygosity mapping identified a large block of homozygosity on chromosome 11p15.5-q25 where two known CDG-I causing genes, *ALG9* and *DPAGT1*, are located. Sequencing *ALG9* did not reveal any mutations while analysis of *DPAGT1* identified a novel homozygous mutation c.902G>A (p.R301H) in two affected infants. The disorder was fatal in all affected cases and mostly in early infancy.

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Introduction

Congenital disorders of glycosylation (CDG) are an expanding group of genetic diseases due to defects in the synthesis of the glycan moiety of glycoproteins or glycolipids and in the attachment of these glycans to proteins and lipids (Freeze 2006; Jaeken 2010). More than 45 CDG have been reported. DPAGT1-CDG is a protein N-glycosylation disorder with only one patient reported showing epilepsy, developmental delay, severe hypotonia, and dysmorphy (Wu et al. 2003; OMIM # 608093).

In a highly consanguineous Saudi family with several affected infants, we identified DPAGT1-CDG through homozygosity mapping. We describe detailed clinical, neuroradiological, and muscle morphology data in the index case. Our report provides a more comprehensive delineation of the phenotype of a very rare type of CDG.

Patients and Methods

Patients

The index case was the product of full term Cesarean section (C-section) due to two previous C-sections. His Apgar scores were 7, 7, and 8 at 1, 5, and 10 min, respectively. He was admitted to the neonatal intensive care unit for 4 days due to respiratory distress that resolved without the need for mechanical ventilation. He was presented to our Medical Genetics clinic at the age of 8 months with a history of developmental delay and hypotonia. He was not able to roll over, sit, or raise his head on prone position. In addition, he started to have episodes of cyanotic spells with brief cessation of breathing. Physical examination revealed a head circumference of

41.5 cm (1 SD below the 5th percentile), weight of 8.2 kg (at the 25th percentile), and length of 76 cm (at the 95th percentile). There was no dysmorphy. He was not able to fix and follow properly but fundoscopy was normal. There was no tongue fasciculation. He had generalized hypotonia, head lag, and depressed deep tendon reflexes. Cardiovascular, respiratory, and abdominal examination was unremarkable.

Investigations revealed the following: complete blood count, renal profile, serum ammonia and lactate, tandem mass spectrometry for acylcarnitines, plasma very long chain fatty acids, urine organic acids, and cerebrospinal fluid glucose, lactate and amino acids were all normal. Liver function test showed elevated alanine aminotransferase at 74 U/L (normal 10–45). Creatine kinase was elevated at 354 U/L (normal 24–195). Ultrasound abdomen and echocardiogram were normal. Brain magnetic resonance imaging (MRI) showed normal appearance of the posterior fossa, of the midline, and of the ventricular system. There was diffuse paucity of the myelin. The cerebral white matter exhibited a hypointensive appearance with respect to the cortex in the temporal polar area which suggested a delay in myelination. No apparent diffusion abnormality was identified. The MRI spectra showed a slight increase of the choline peak and a decrease in N-acetylaspartate and creatinine peaks with normal lactate suggesting increased myelin turnover. Electroencephalography revealed multiple spikes, polyspikes, and waves in multiple regions involving right and left temporal lobes. Visual evoked potentials were normal. Muscle biopsy showed two populations of muscle fibers: uniformly smaller type-1 and normal-sized type-2 fibers. Gomori stain revealed no evidence of mitochondrial abnormalities or abnormal cytoplasmic inclusions. There was no evidence of glycogen or lipid storage. Cytochrome c oxidase was present in all fibers. Acid and alkaline phosphatases were unremarkable. Nonspecific esterase revealed scattered highly atrophic angulated fibers consistent with denervation. Immunohistochemistry revealed normal dystrophins 1, 2 and 3, sarcoglycans (α, β, γ, and δ), β dystroglycan, merosin, and spectrin. Isoelectric focusing of serum transferrin showed a type-I pattern. Phosphomannomutase and phosphomannose isomerase activities in fibroblasts were normal.

The patient was started on phenobarbitone which controlled convulsions. He continued to show severe global developmental delay with frequent episodes of aspiration. He succumbed during one of these episodes at the age of 5 years.

His brother (individual V12, Fig. 1) was presented to our clinic at the age of 2 months with a similar presentation. His mother reported decreased fetal movement. He was noticed to have hypotonia with poor feeding and recurrent choking since birth. His head circumference was 1 SD below the 5th percentile, while weight was at the 10th percentile and length at the 50th percentile. He was not dysmorphic. His pupils were equal and reactive to light. Extraocular movements were intact. He had head lag, generalized hypotonia, and depressed deep tendon reflexes. Cardiovascular, respiratory, and abdominal examination was unremarkable. Relevant investigations revealed elevated alanine aminotransferase at 101 U/L (normal 10–45). Abdominal ultrasound was unremarkable. Brain MRI at the age of 3 months showed no visible myelination of the splenium and anterior limb of the internal capsule but discrete myelination of the white matter in the centrum semiovale. The cerebellum was normally myelinated. There were no focal changes and the ventricular system was normal in size with no evidence for atrophy. The posterior

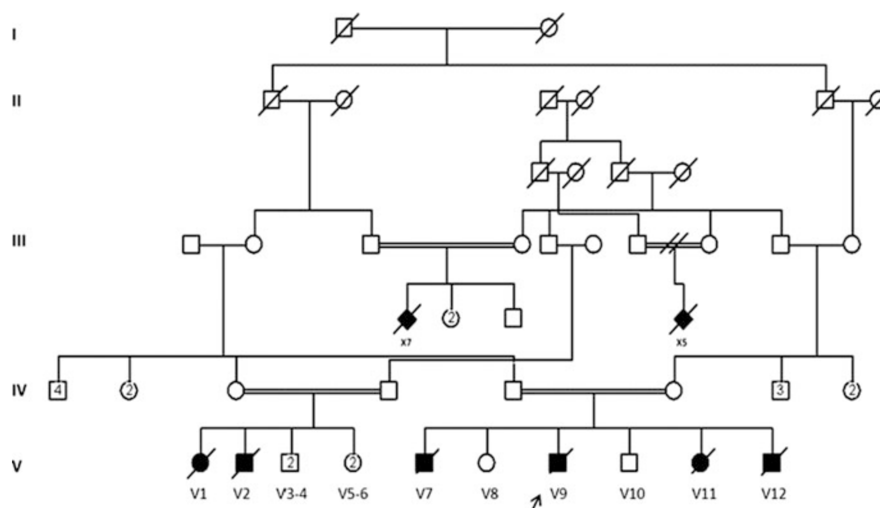

Fig. 1 The family pedigree showing high degree of consanguinity and multiple affected family members. *Arrow* indicates index patient

fossa was unremarkable. Mildly delayed myelination could not be excluded due to the age of the patient. Immunonephelometry assay for transferrin was abnormal at 24.5% (normal ≤ 2.47).

The patient died at the age of 7 months with aspiration pneumonia. His consanguineous parents lost two other children (individuals V7 and V11, Fig. 1) with similar presentations. They had frequent aspirations requiring intensive care unit admission in the local hospital and both died at the age of 8 months before being able to refer them to our hospital for evaluation. The family pedigree was remarkable for 14 cousins in three sibships who died in infancy with severe hypotonia and developmental delay of unknown etiology.

Methods

Sample Collection and DNA Extraction

Whole blood samples were obtained from the two affected patients and their parents and genomic DNA was extracted for each sample by standard salt-precipitation methods (Miller et al. 1988).

Homozygosity Mapping

SNP-based genotyping was performed on the index case and his parents using the Affymetrix® GeneChip Human Mapping 6.0 Array (Affymetrix, Santa Clara, CA, USA). The genotypes of SNPs were called using Affymetrix GCOS 1.4 software, which generated an overall average SNP call rate of 97% and was further analyzed to detect regions of homozygosity using the GTConsole (Affymetrix, Santa Clara, CA, USA) software package. Conventionally, regions or blocks of homozygosity are defined as fragments where SNPs are homozygous for a stretch of consecutive alleles, in this study defined as 2 Mb or longer in length, in affected individuals and heterozygous or homozygous for the other allele in unaffected members of the same family. This genome-wide homozygosity mapping analysis approach was used in this family as it assumes that individuals affected with an autosomal recessive disease, born from parents of a consanguineous marriage, are very likely to be homozygous for the pathogenic mutation and for a substantial number of SNPs surrounding it (Woods et al. 2006).

Mutation Screening in *ALG9* and *DPAGT1*

Genomic DNA of the proband and his parents was amplified by PCR using intronic primers that were designed to flank (50–100 bp) the coding exons (as defined by Ensembl Genome Browser; http://www.ensembl.org/index.html) of *ALG9* (NM_001077690.1) and *DPAGT1* (NM_001382.3). PCR was performed in a final volume of 20 μL containing approximately 10 ng of genomic DNA, using standard conditions (primer sequences and conditions are available on request).

Automated Sequencing

Purified PCR amplicons covering the entire coding region of each gene were directly sequenced with the dideoxy chain-termination method using an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit following the manufacturer's instructions and processed on a MegaBACE 1000 DNA Analysis System (Molecular Dynamics; Sunnyvale, CA, USA). Sequence analysis was performed using the SeqMan 6.1 module of the Lasergene (DNA Star Inc., WI, USA) software package, then compared to the reference GenBank sequence. Numbering commenced with the A of the ATG initiation codon as +1.

Results

Homozygosity Mapping

Analysis of the SNP-based genotyping identified a large block of homozygosity (~85 Mb) on chromosome 11p15.5-q25 that was unique to the index case and not shared by his parents (Fig. 2). The known CDG-I genes, *ALG9* and *DPAGT1*, were directly sequenced in this family as they were located in the homozygous block on chromosome 11p15.5-q25. No other CDG-I genes that have been reported previously were found in any other regions of homozygosity that fit the aforementioned criteria.

Mutation Detection in *ALG9* and *DPAGT1*

Direct sequencing in both the forward and reverse directions for *ALG9* did not show any variation from the reference sequence. However, subsequent analysis of the *DPAGT1* gene in the proband revealed the presence of a homozygous c.902G>A mutation, resulting in the substitution of arginine to histidine at position 301 (p.R301H). This novel missense mutation segregated with the disease phenotype; both parents were heterozygous carriers, and the individual V11 was homozygous for the mutation. The mutation was not found in 200 ethnically matched normal controls, which indicated that this variant was pathogenic. Protein sequence alignment of *DPAGT1* orthologs demonstrated that the arginine residue is highly conserved across 42 different species from human to zebrafish (UCSC Genome Browser Vertebrate Multiz Alignment and Conservation Tool; http://genome.ucsc.edu/). In

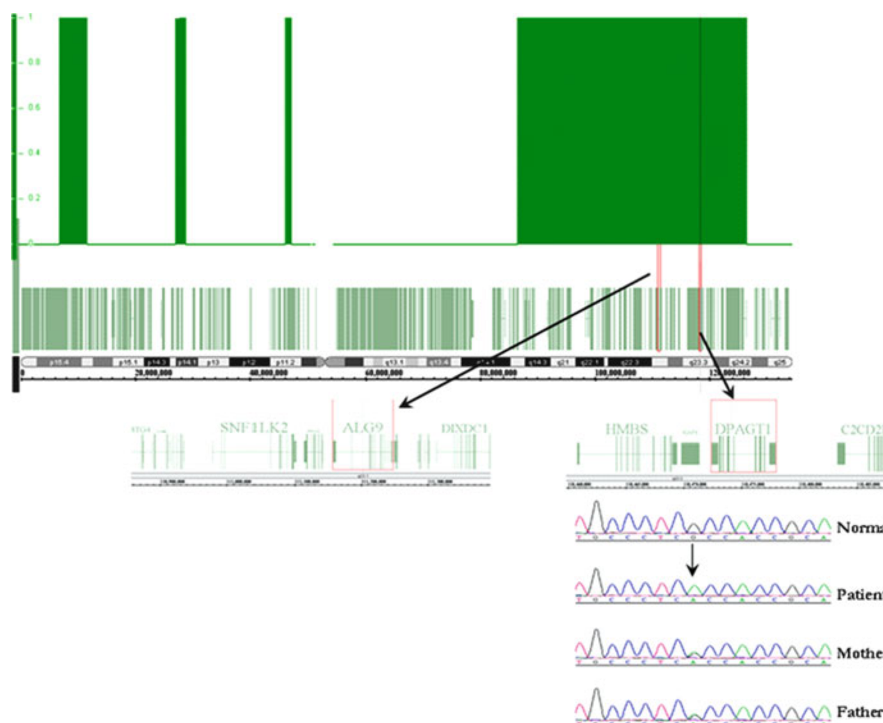

Fig. 2 Block of homozygosity on chromosome 11p15.5-q25 in the affected patient harboring both of the known CDG-I causing *ALG9* and *DPAGT1* genes. Mutation analysis showed the presence of a novel pathogenic c.902G>A (p.R301H) mutation in *DPAGT1*, segregating with the disease phenotype in this family

addition, both Polyphen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org) programs predicted the variant to be probably damaging lending further support to the pathogenicity of this novel mutation.

Discussion

Congenital defect of glycosylation type DPAGT1-CDG (CDG-Ij) was identified for the first time in a female patient who was presented at the age of 4 months with infantile spasms (Wu et al. 2003). The patient showed global developmental retardation, microcephaly, severe hypotonia, and intractable seizures. Dysmorphic features were also apparent with arched palate, micrognathia, esotropia, fifth finger clinodactyly, single flexion creases, and skin dimples on the upper thigh. Her brain MRI was normal. To our knowledge, no other cases of DPAGT1-CDG (CDG-Ij) have been reported since then. We present a detailed description of the phenotype of DPAGT1-CDG (CDG-Ij) in a large consanguineous Saudi family. The neurodevelopmental phenotype in our patients was similar to the original case described by Wu et al. (2003) with respect to the severe hypotonia, global developmental delay, and microcephaly. Dysmorphic features, however, were not observed in our patients. The brain MRI in our index case revealed delayed myelination, a nonspecific neuroradiological finding that was previously detected in ALG2-CDG (CDG-Ii) (Thiel et al. 2003). Wu et al. (2003) reported that their patient had minimal speech at 6 years of age but survival beyond that age was not reported. In our family, the disorder was fatal in all affected members (18 individuals) and mostly in early infancy.

The muscle biopsy in our patient revealed fiber type disproportion (FTD) which is typically diagnosed when type-1 fibers are consistently smaller than type-2 fibers. This condition, however, is genetically heterogeneous and has been observed in several forms of congenital myopathies, neuromuscular disorders, and muscular dystrophies (Clarke and North 2003). Mutations in *ACTA1* (Laing et al. 2004), *SEPN1* (Clarke et al. 2006), *TPM3* (Clarke et al. 2008), *RYR1* (Wilmshurst et al. 2010), and more recently *MYH7* (Ortolano et al. 2011) have also been identified in patients with FTD. To add to its nonspecific nature, this morphological finding has been reported in association with several inherited metabolic disorders including Pompe disease (Martin et al. 1976), multiple sulfatase deficiency (Tachi et al. 1984), congenital lactic acidosis (Iso et al. 1993), metachromatic leukodystrophy (Krendel et al. 1994), carnitine palmitoyltransferase deficiency (Shintani et al. 1995), and Krabbe disease (Marjanovic et al. 1996). To our knowledge, FTD has never been described in patients with CDG. Supported clinically and biochemically with the presence of hypotonia and elevated creatine kinase,

our case expands the list of genetic conditions that are known to be associated with FTD to include DPAGT1-CDG (CDG-Ij).

The homozygous mutation in the gene *DPAGT1* in our family was identified after homozygosity mapping, an approach that we have adopted in this case to overcome the tremendous locus heterogeneity of CDG. In the presence of an abnormal CDT assay, consanguinity, and multiple affected family members, homozygosity mapping is potentially a powerful, and likely cost-effective, approach to identify the specific defect in contrast to the laborious biochemical assays of the glycosylation pathways or to sequential sequencing of genes implicated in the various types of CDG.

In summary, we describe a detailed phenotype of DPAGT1-CDG in a large consanguineous family. This family includes CDG as a cause of FTD.

Acknowledgements We thank members of the Saudi Diagnostics Laboratory sequencing core facility for technical assistance. This work reports findings identified during the course of routine molecular diagnostic services undertaken by the Research Centre of King Faisal Special Hospital and Research Centre.

Synopsis

Better understanding of the phenotype of DPAGT1-CDG (CDG-Ij) defect. Utilization of homozygosity mapping to identify the specific gene defect in consanguineous families suspected to have an undefined type of CDG.

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RESEARCH REPORT

Galactosemia Screening with Low False-Positive Recall Rate: The Swedish Experience

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Abstract Newborn screening was implemented in the 1960s with screening for phenylketonuria (PKU). In the same decade, it became possible to screen for classical galactosemia, a rare autosomal recessive inherited disorder, which is potentially life threatening if not treated. While newborn screening for PKU has become almost universal, galactosemia is included only in a minority of European newborn screening programs. The major arguments why galactosemia is excluded from newborn screening programs are that the disease can be diagnosed clinically, there is a high rate of false positives and long-term complications are common despite early diagnosis.

Here, we report how we have decreased the number of false-positive galactosemia recalls to less than 0.01%, using a two-tier test strategy. All samples are tested with the Beutler blood spot test, a method that measures galactose-1-phosphate uridyltransferase activity. Samples with less than ≤15% activity are tested for galactose with a galactose dehydrogenase test (the rapid GAL-DH test), which catalyzes the oxidation of galactose and the reduction of NAD^+ to NADH that is estimated visually by fluorescence under UV-light. Both tests are semiquantitative.

With this strategy, screening for galactosemia is inexpensive, does not demand a heavy workload, and has a low false-positive re-call rate. The incidence of classical galactosemia in Sweden is 1/100,000, which is lower than the reported incidence in other European countries. Despite this, newborn screening for galactosemia has never been questioned.

Concise sentence: Screening for galactosemia using well-established methods to reduce the false-positive rate.

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Abbreviations

DG	Duarte galactosemia
G-1-P	Galactose-1-phosphate
Gal	Galactose
GAL-DH	Galactose dehydrogenase
GALE	Galactose epimerase
GALK	Galactokinase
GALT	Galactose-1-phosphate uridyltransferase
NBS	Newborn screening
PKU	Phenylketonuria

Introduction

Galactosemia is a rare autosomal recessive inherited disorder. Depending on which enzyme is affected the disorder can be divided into three subgroups; galactose-1-phosphate uridyltransferase deficiency (GALT; OMIM 230400), galactokinase deficiency (GALK; OMIM 230200), and galactose-4-epimerase deficiency (GALE; OMIM 230350). GALT-deficiency, also known as classical galactosemia, was first described in 1917 by Goppert (1917) and in 1956 galactose-1-phosphate uridyltransferase (EC 2.7.7.12) was identified as the enzyme that was deficient in this disease (Isselbacher et al. 1956). Galactosemia due to GALT-deficiency is the most common of the

galactosemia disorders, with a frequency of approximately 1/60,000 in Caucasians.

Galactosemia became a candidate disease for newborn screening (NBS) programs, which were under development in the 1960s. A bacterial inhibition assay was introduced in 1964 followed by the Beutler enzyme spot test in 1966 (Beutler and Baluda 1966). With the bacterial inhibition assay, all galactosemias can be detected while the Beutler method only detects patients with GALT-deficiency. Today, there are other screening methods for galactosemia as well, the most common being measurement of galactose (Gal) and galactose-1-phosphate (G-1-P) in blood spots. A major disadvantage with these screening methods for galactosemia has been the high frequency of false-positive results (Jeong et al. 2007; Freer et al. 2010).

GALT-deficiency is a potentially lethal disease with symptoms occurring within the first weeks of life. Untreated the neonate will show progressive symptoms from weight loss, vomiting and diarrhea, lethargy and hypotonia to jaundice, cataracts, hepatomegaly, prolonged bleeding time, and septicemia leading to neonatal death. With the initiation of a galactose and lactose restricted diet, the acute symptoms will regress, but despite early treatment long-term complications such as mild speech and language delay, premature ovarian failure (POF), and neurological defects are not uncommon (Waggoner et al. 1990).

While newborn screening for phenylketonuria (PKU; OMIM 261600) has become almost universal, galactosemia screening is included only in a minority of European NBS programs. In 2007, nine countries in Europe had a nationwide screening program for galactosemia while Finland was the only country that did not have a nationwide screening program for PKU (www.isns-neoscreening.org). There are three major arguments why galactosemia is excluded from the NBS programs (1) the disease can be diagnosed clinically, (2) high rate of false positives, and (3) long-term complications are common in spite of early treatment.

Most newborns with classical galactosemia will show symptoms of the disease at the time of recall but by screening most deaths in *Escherichia coli* sepsis will be avoided. In Sweden, only four patients with classical galactosemia born before the start of screening in 1967 are alive. After 1967, one patient has died due to galactosemia. Considering these results, neonatal deaths in galactosemia decrease significantly if screening for the disease is performed. It is possible to perform screening for classical galactosemia with a low false-positive rate using well-established methods and appropriate cut-off values, which is described in this article. Long-term complications cannot be avoided with the knowledge that is available today but this may change in the near future when new methods for treatment and follow up have been developed and in use (Coman et al. 2010). Screening for galactosemia also makes it possible to offer parents genetic counselling and prenatal diagnosis in subsequent pregnancies.

Screening in Sweden

The first method for galactosemia screening in Sweden was based on a bacterial inhibition assay (collaboration with Thalhammar, Vienna) in line with the Guthrie method for the screening of PKU (Guthrie and Susi 1963). The method was included in the NBS program in 1967 and used until 1985 when the Beutler spot test was employed (Fig. 1). During the period 1967–1985, 22 cases of GALT-deficiency and 3 cases of GALK-deficiency were identified but no cases of GALE-deficiency. In 1983, the Beutler spot test was introduced as a second tier test and in 1985, we decided to change to the Beutler spot test as the first step (Beutler and Baluda 1966). From then on we have only been screening for GALT-deficiency. Throughout the years a quantitative determination of Gal and G-1-P has been performed on all positive cases, but different cut-off values have been employed.

The Beutler Spot Test

The Beutler spot test is a semiquantitative, fluorometric method, which measures GALT-activity. With the Beutler spot test, patients with GALT-deficiency are identified. In addition, severe deficiencies of glucose-6-phosphate

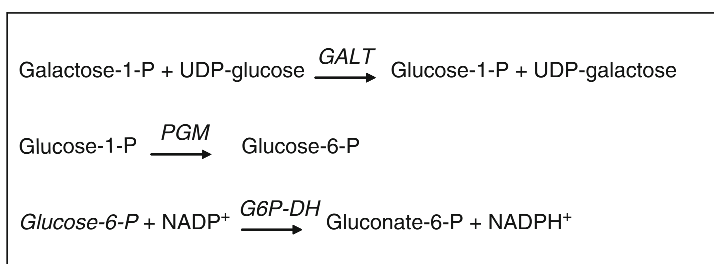

Fig. 1 Determination of GALT-activity according to Beutler. *GALT* galactose-1-phosphate uridyltransferase, *PGM* phosphoglucomutase, *G6P-DH* glucose-6-phosphate dehydrogenase

dehydrogenase (EC 1.1.1.49) and phosphoglucomutase (EC 5.4.2.2) can be picked up, since these enzymes are required for the test to function (Fig. 1).

A disadvantage with the Beutler spot test is that it can show a false-positive result (absent enzyme activity) if the sample contains EDTA. This is common in samples taken from older children, i.e. adoptive and immigrants up to the age of 18 years, which are offered the screening test on arrival in Sweden. It is not uncommon that samples containing EDTA that are positive in the Beutler screening also show false-positive results in the screening for congenital adrenal hyperplasia (OMIM 201910) when using the Auto DELFIA method, a fact that is assisting in the evaluation of the Beutler results. Samples that have been exposed to heat are another source of false-positive results.

Two-Tier Testing

When a case of galactosemia is suspected due to a positive Beutler spot test it is of importance to verify the result rapidly. Quantitative analysis of Gal and Gal-1-P in our laboratory takes 4 h, which we consider to be too long. We therefore introduced a rapid and simple method to determine total Gal semiquantitatively in samples with low activity in the Beutler test – "the rapid galactose dehydrogenase (EC 1.1.1.120) test" (rapid GAL-DH test). This method has been in use for the past 20 years. The Beutler spot test is ready in the same day as the screening sample arrives in the laboratory. If a sample shows an activity of 15% or less in the Beutler spot test, the rapid GAL-DH test is performed. The rapid GAL-DH test is a semiquantitative, fluorometric spot test where total galactose is approximated visually within an hour. With this two-tier approach, the diagnosis of galactosemia can be confirmed on the same day the sample arrived in the laboratory. In addition, a quantitative determination of Gal and G-1-P is always performed the following day.

Method

Gal in filter paper spots is determined enzymatically with the enzyme GAL-DH, which catalyzes the oxidation of galactose. At the same time nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH, which is estimated visually by fluorescence under UV-light (Fig. 2).

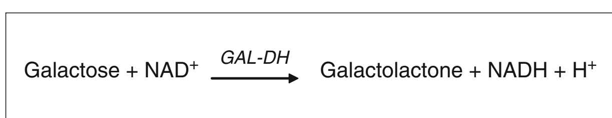

Fig. 2 Principle for rapid GAL-DH. *GAL-DH* galactose dehydrogenase

The analysis has five controls consisting of one blank filter paper spot, blood filter paper samples containing none, 2, 4, and 8 mM Gal, respectively. Blood spots from a healthy child are used as a negative control. Two dried blood spots (diameter 3.0 mm) from each control and the samples to be analyzed are incubated at 37 °C in glass tubes for 30 min in a reaction mixture of 100 µl containing 50 µl Tris-buffer (170 mM, pH 8.0) and 50 µl G-1-P-mix containing: 19.61 µl Tris-buffer (50 mM pH 8.6); 24.51 µl NAD (0.013 M); 0.98 µl Gal-DH (2.5 mg/ml) and 4.9 µl alkaline phosphatase (300U/ml). An aliquot of 10 µl from each incubation mixture is spotted on a filter paper and dried in room temperature. The fluorescence is visually interpreted under long-wave ultraviolet light. The intensity of the fluorescence corresponds to the level of total Gal. Samples showing fluorescence greater than 2 mM total Gal are considered positive (Fig. 3).

Results and Discussion

During 1985–2009 when the Beutler spot test has been performed, patients have been diagnosed at an average age of 6.5 days (range 4–14 days). In 2007, as a preparation for expanded screening with tandem mass technique, the recommended time of sampling was changed from as soon as possible after 72 h to after 48 h of age. The samples are taken at an average of 2.9 days after birth and reach the laboratory at an average time of 5.5 days after birth. This implies that a child with galactosemia born in Sweden will have a diagnosis and be on treatment within the first week of life.

Two cut-off levels are in use for the Beutler spot test, one for samples with an activity of 10% or less and one for

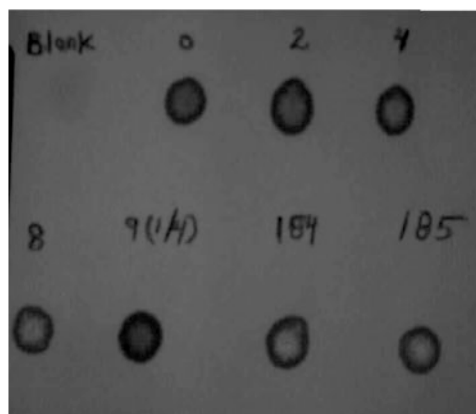

Fig. 3 Rapid GAL-DH test. *First line*: samples containing 0, 2, 4 mM galactose. *Second line*: sample containing 8 mM galactose, number 9 healthy child, number 184 and 185 twins with classical galactosemia. Number 184 had 7.3 mmol/l galactose and number 185 had 5.3 mmol/l galactose when determined quantitatively

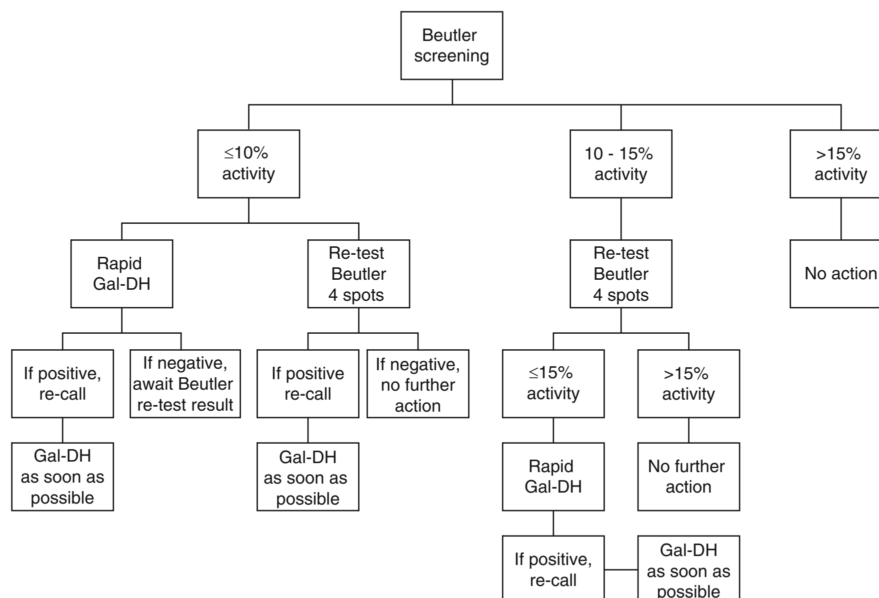

Fig. 4 Algorithm for newborn screening for galactosemia using the two-tier approach with Beutler screening and the rapid GAL-DH on the initial screening sample. Positive Beutler test ≤15% activity. Positive rapid Gal-DH test approximately ≥2 mM galactose

samples with activity over 10% and up to 15%, see algorithm (Fig. 4). Infants with ≤10% activity are re-called promptly.

The false-positive rate has dropped to less than one newborn a year after lowering the cut-off value for the Beutler spot test to ≤15%, and increasing the cut-off for G-1-P to ≥1.5 mmol/l (Table 1). After including the rapid GAL-DH test in 1991 as a second tier test all patients with classical galactosemia have had a verified diagnosis at the time of recall.

The two most important changes to reduce the false-positive rate in the galactosemia screening were change of method from bacterial inhibition assay to the Beutler spot test in 1985 and lowering the cut-off value for the Beutler spot test in 1992. When the Beutler spot test was introduced as the test method for galactosemia screening in 1985, the cut-off value was set at an activity of 30% or less. With this cut-off value, newborns with Duarte galactosemia (DG) (newborns carrying one Duarte allele and one classic galactosemia allele) who have approximately 25% rest activity and also some patients heterozygous for one severe mutation were recalled giving a high false-positive incidence (1/8,500 newborns). When patients with DG were caught in the NBS for galactosemia, our program chose to recommend treatment and follow up of the newborns with half of the breast milk exchanged for galactose free formula for 4–6 months. This was routine in 1983–1989. Already at this time it was uncertain if newborns with DG needed treatment with a galactose restricted diet (Gitzelmann and Bosshard 1995). Our experience was that at the end of the follow-up at 6 months of age the patients had normal galactose metabolites. A decision was made in 1992 that the goal of the galactosemia screening was to detect patients with classical galactosemia excluding newborns with DG. Evaluation of the true positive cases showed that all cases with classical galactosemia have an enzyme activity of 8% or less in the Beutler test. Hence, the criteria for recall were changed to an activity of ≤15% and a positive rapid Gal-DH test. With this approach, almost all newborns with DG are unrecognized. There is still an uncertainty of whether DG patients need treatment or not but several publications indicate that infants with DG have a favorable outcome

Table 1 Newborn screening for galactosemia in Sweden 1967–2010

Years	Newborns	True positives	Incidence	False positive	Incidence	Method	Cut off%	Quantitative determination	
								Gal mmol/l	G-1-P mmol/l
1967–1985	1,780,600	22	1/81,000	336	1/5,300	Bacterial inhibition assay		>0.6	>0.6
1986–1991	677,900	3	1/226,000	80	1/8,500	Beutler	30	>0.6	>0.6
1992–2010	1,973,400	18	1/108,000	10	1/194,000	Beutler	15	>0.5	>1.5
Total	4,401,900	43	1/103,000	424	1/10,000				

on galactose unrestricted diet (Ficicioglu et al. 2008, 2010; Powell et al. 2009; Fernhoff 2010).

Some laboratories combine metabolite screening and enzymatic testing, either performing both or as a two-tier test. If the Beutler spot test is the second test in a two-tier test combination where Gal and Gal-1-P are measured first, galactosemia due to GALK-deficiency and GALE-deficiency can also be identified. The latter conditions are, however, extremely rare. When metabolite screening is the approach, ingestion of galactose/lactose containing formula is necessary. Another disadvantage when using the galactose metabolites Gal and G-1-P to detect galactosemia is a high rate of false-positive cases, due to an increase of Gal and G-1-P seen in newborns with DG and in some newborns who are heterozygous for one classical galactosemia allele. There are also newborns who have a transient increase of galactose metabolites. With our two-tier approach the false-positive rate has decreased to almost null and furthermore no missed cases of galactosemia have been reported to the laboratory.

A drawback with the present lower age at sampling is an increased risk of the child not having been fed lactose when the screening sample is taken. In this case the rapid GAL-DH test will be negative. This has been verified in two cases of samples taken from younger siblings with galactosemia having not been given any Gal containing food. In these cases, we have found only a slight elevation of Gal-1-P in the screening sample, 0.53 and 0.90 mmol/l respectively (reference value <0.1 mmol/l) but no increase in Gal when measured quantitatively. To assure that we do not miss any cases, all samples with less than 10% activity and negative on the rapid GAL-DH tests are evaluated considering the age of the child at sampling and an assurance that the child has been fed galactose.

Screening for galactosemia using the Beutler method is inexpensive. The total cost per child for working time, reagents, and machines is less than 0.25 Euro.

Conclusion

We find it surprising that galactosemia is not included in more screening programs, whilst organic acidurias and urea cycle disorders are. Several of the disorders included in the expanded screening by tandem mass result in severe sequelae in spite of early treatment. Neonatal screening cannot be governed by methodology when cheap and well-performing methods are available.

The incidence of classical galactosemia in Sweden is 1/100 000, which is lower than the reported incidence in other European countries. Despite this, newborn screening for galactosemia has never been questioned. One reason could be that we have had a well-working strategy for the screening procedure with a low false-positive rate especially after 1992. The screening methods we use are well established, inexpensive, and do not demand a heavy workload.

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RESEARCH REPORT

Efficacy of Vigabatrin Intervention in a Mild Phenotypic Expression of Succinic Semialdehyde Dehydrogenase Deficiency

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Abstract We report a patient with succinic semialdehyde dehydrogenase deficiency who presented a mild phenotype including developmental language delay, in association with the typical elevations of 4-hydroxybutyric acid (GHB) in biological fluids and MRI alterations. Two pathogenic mutations were identified one transversion (c.278 G>T) in exon 1 and another (c.1557 T>G) in exon 10. Both parents are carriers of one of the mutations, confirming compound-heterozygosity in their affected child. To reduce the GHB levels in body fluids, a treatment with vigabatrin at low dose (25 mg/kg per day) was started, monitoring its efficacy by clinical and neurochemical follow-up. After 9 months of therapy with vigabatrin, a significant reduction of GHB concentrations in urine and CSF was observed; after 36 months, a significant improvement of communicative skills, not previously reported, was referred. These results support the hypothesis that the clinical improvement is correlated to the reduction in the GHB levels and the importance of considering the SSADH deficiency in the differential diagnosis of patients with mental retardation and language delay.

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Introduction

Succinic semialdehyde dehydrogenase (SSADH) deficiency (OMIM 271980) is a rare autosomal recessive disorder of the 4-aminobutyric acid (GABA) catabolic pathway. In the absence of SSADH activity, succinic semialdehyde (SSA) is converted into 4-hydroxybutyric acid (GHB) that accumulates in plasma, urine, and cerebrospinal fluid (CSF) and represents the biochemical hallmark of this disorder. Patients are identified by detection of elevated GHB in body fluids and SSADH deficiency confirmed by enzyme assay in lymphocytes (Gibson et al. 1991, 1994, 1998).

The SSADH gene (*ALDH5A1*) has been mapped on chromosome 6p22 (Trettel et al. 1997; Blasi et al. 2002) and multiple different mutations have been identified (Gibson et al. 1997; Gibson and Jakobs 2001; Akaboshi et al. 2003) in approximately 450 patients thus far diagnosed (Jakobs et al. 1981; Kim et al. 2010). The clinical picture of SSADH deficiency is highly heterogeneous, characterized by neurological symptoms including such as varying degrees of mental retardation, seizures, hypotonia, ataxia, and developmental language delay. Other clinical features, including movement disorders, oculomotor apraxia, and nystagmus have been reported (Gibson et al. 1997; Gordon 2004).

The most common abnormalities on MRI consist of increased T2-weighted signal involving the cerebellar dentate nuclei, globus pallidus, and subthalamic nuclei

symmetrically as well as the subcortical white matter and brainstem (Ziyeh et al. 2002; Pearl et al. 2003). Proton spectroscopy has variably revealed elevated levels of GABA and GHB in the white and gray matter (Ethofer et al. 2004; Kim et al. 2010).

Currently, therapeutic intervention in patients with SSADH deficiency has been limited to antiepileptic drugs, mainly vigabatrin (GVG), which irreversibly inhibits GABA transaminase activity penultimate to the SSADH enzyme. The metabolic outcome of this inhibition should yield increased free and total GABA concentration in brain with concomitant reduction of SSA and GHB levels in biological fluids. Therapy with vigabatrin has been tried in a moderate number of patients with good results in some and little efficacy in others (Gibson et al. 1989, 1995; Jaeken et al. 1989; Matern et al. 1996; Gropman 2003; Ergezinger et al. 2003; Leuzzi et al. 2007).

We report an SSADH-deficient patient with a mild phenotype and the follow-up during treatment with vigabatrin, which revealed encouraging results.

Case Report

A 8.2 years old girl, second child of unrelated healthy parents, was admitted in our department at 4.5 years to evaluate idiopathic mental retardation with a severe speech delay. She was born at term after an uneventful pregnancy; APGAR score after 1 and 5 min was 9–10; she weighed 3,040 g and was 50 cm long; OFC was 35.5 cm. There was no family history of epilepsy, motor or psychiatric disorders. Her brother, 16 years old, presented an isolated speech delay but he graduated from high school. The child started to walk alone at 14 months demonstrating mild hypotonia with joint hyperlaxity and insufficient coordination. Her language development showed impressive delays, especially in expressive language with sufficient speech comprehension.

At first examination, the child presented clumsiness and difficulties in execution of oral and ideomotor praxis, severe language deficit with verbal dyspraxia, mild mental retardation and hyperactive behavior. Spontaneous language production was limited to few incomplete words. Verbal expression was integrated with a production of iconic, deictic and some referential gestures.

Karyotype (550 band level) and basal neurometabolic workup (aminoacids, creatine, organic acids) were performed. The organic acids analysis revealed a marked increased excretion of GHB (311 mmol/mol creatine; controls < 5), while the other investigations were normal. EEG showed rare paroxysmal abnormalities; motor-sensitive nerve conduction velocity of the lower limbs was normal.

MRI with proton spectroscopy (^{1}H-MRS), carried out based upon a suspicion of an SSADH deficiency, showed the typical neuroradiological pattern above described. There were abnormalities involving globus pallidus bilaterally and symmetrically, as well as abnormalities of the subcortical white matter and cerebellar dentate nucleus. No significant alterations were detected by ^{1}H-MRS.

SSADH activity in the patient's lymphocytes was strongly decreased (48 pmol/min per mg protein; controls 1,907–3,901 pmol/min per mg protein) confirming the suspected diagnosis.

DNA investigation of the SSADH gene (*ALDH5A1*) performed in the patient and her family identified two pathogenic mutations: a G>T transversion (c.278 G>T) in exon 1, which results in the substitution of cysteine by phenylalanine at position 93 (p.Cys93>Phe); the other a T>G transversion (c.1557 T>G) in exon 10 which results in the replacement of tyrosine by a termination codon at position 519 (p. Tyr519X). Both healthy parents carried one of the mutations, confirming compound-heterozygosity in their affected child; the brother is a carrier of the maternally inherited allele.

The assessment of cognitive abilities was performed using the Leiter International Performance Scales (Leiter 1997, Italian translation 2002). Language evaluation was performed using: the Italian version of the Infant's and Toddler's MacArthur Bates Communication Development Inventories (CDI) (Caselli and Casadio 1995) for productive vocabulary, the Peabody Picture Vocabulary Test (PPVT) for receptive vocabulary (Dunn and Dunn 1997, Italian standardization, Stella et al. 2000) and the Rustioni test (Rustioni 1994) for sentence comprehension. Voluntary oral praxis movements were evaluated on verbal request and on imitation too.

To reduce the GHB levels in body fluids, a treatment with vigabatrin at low dose (25 mg/kg per day) was started, monitoring its efficacy by clinical and neurochemical follow-up. The GHB and aminoacids (AA) levels in CSF and blood were monitored before starting therapy (T0), at T2 (6 months), T4 (12 months), T6 (30 months), and T7 (36 months).; the MRI-MRS was performed at the same times except for the T7 measurement. A more frequent monitoring of urinary concentrations of GHB and AA were performed at T1 (3 months), T2 (6 months), T3 (9 months), T4 (12 months), T5 (18 months), T6 (30 months), and T7 (36 months). To evaluate the possible side effect of irreversible constriction of the visual field under vigabatrin therapy (Gordon 2004), the child underwent periodic ophthalmologic evaluation (fundus oculi and visual field examinations).

Table 1 Laboratory findings during vigabatrin treatment

	T0	T1	T2	T3	T4	T5	T6	T7	Controls
Vigabatrin (mg/kg per day)	None	25	25	25	25	25	25	25	–
GHB urine (mmol/mol creatine)	311	298	95	71	71	51	52	60	< 5
GHB blood (μmol/L)	204	–	170	–	–	–	107	121	< 2
GHB CSF (μmol/L)	357	–	102	–	–	–	60	–	< 2

Results

At T0, GHB levels were markedly increased in urine (311 mmol/mol creatine), blood (204 μmol/L) and CSF (357 μmol/L) (Table 1). After 3 months (T1) of therapy with vigabatrin a mild reduction of GHB concentrations in urine was observed, with a further dramatic fall at T2 and T3; the same decrease was observed in CSF, whereas the levels in blood remained high even after 30 months of treatment.

Aminoacids levels in plasma, urine, and CSF were consistently normal, except for a mild increase in ornithine in the blood at T7.

The MRI abnormalities remained unchanged after more than two years of treatment; the EEG performed during follow-up disclosed sharp waves and spikes upon the central parietal regions, especially on the right hemisphere. Intermittent photic stimulation did not provoke photoparoxysmal response and no epileptic seizures were observed. At T7 the fundus oculi was still normal but a visual field constriction developed in the right eye.

The assessment for language skills performed at T0, T1, T5, and T7 showed a mild improvement in communicative abilities (Table 2). Moreover a more pronounced recovery in the behavior with reduction of hyperactivity and attention deficit was observed, while the cognitive abilities, evaluated at T4 and T7, remained stable.

Table 2 Raw scores obtained with neuropsychological test

	T0	T1	T5	T7
Rustioni test	32.7	47.7	56.4	95
PPVT	38	38	51	72
Oral praxis (on request/on imitation)	8 + 1	8 + 1	8 + 5	9 + 6
MacArthur questionnaire	12[a]	–	38[a]	46[a]

[a] Any level of referential expressions, including syllabes and simplified words

Discussion

SSADH deficiency is an autosomal recessive rare disorder caused by mutations in *ALDH5A1* gene, which maps to chromosome 6p22. Since the first report in 1981 (Jakobs et al. 1981), several other patients have been identified (Ziyeh et al. 2002; Pearl et al. 2003; Gropman 2003; Leuzzi et al. 2007; Di Rosa et al. 2009), showing marked differences between them regarding GHB concentrations in body fluids, residual enzyme activity, clinical symptoms, and efficacy of therapy. Neurological findings are predominant in SSADH deficiency but the clinical picture shows a wide spectrum from mild to severe developmental delay, especially involving the language. Other typical signs and symptoms include axial hypotonia, ataxia, movement disorder such as dystonia or choreoatetosis and epilepsy (Knerr et al. 2007). Psychiatric symptoms may be the most disabling and are manifest by hyperkineticism, inattention and sometimes aggression in early childhood, anxiety, and obsessive–compulsive disorder in adolescence and adulthood (Kim et al. 2010).

The clinical phenotype above reported, in which the mental retardation with prevalent involvement of communicative skills represents the core of the disease, is less severe than the majority of other patients described in the literature. So far the patient has never experienced movement disorders or psychiatric symptoms, despite the presence of the prototypical alterations in the basal ganglia.

In addition, we have performed the neuropsychological profile before and during vigabatrin treatment to better characterize the potential effectiveness of GVG therapy.

The first speech evaluation showed that the child was able to use 12 referential expressions including onomatopoeia and simplified words and to perform only few verbal and nonverbal oral movements. Imitation of oral movements did not bring any quantitative and qualitative improvement. During the treatment the verbal production increased and the child learned new verbal expressions even though referentiality was unstable. After 36 months of GVG therapy, lexical and phrasal comprehension had significantly improved. The vocabulary includes 46 words; some of them are juxtapositions of syllables but are still not fused; however, referentiality is stabilized. Imitation of oral praxis movements has also improved. Table 2 summarizes the raw scores obtained with the communication and cognition testing we employed.

Antiepileptics, mainly vigabatrin, are the most frequently used drugs in the treatment of SSADH deficiency aimed at reducing seizures and behavioral symptoms. The response to treatment varies greatly, as well as the severity of clinical phenotype and the residual SSADH activity in relation to the GHB levels. Moreover, the effects of vigabatrin on GABA transaminase are not entirely predictable, depending on the differences between brain and peripheral forms of the enzyme (Ergezinger et al. 2003). The partial effect on peripheral GABA transaminase would lead to an increase of GABA in the CNS, with the resultant clinical ineffectiveness. This could be one of the mechanisms to explain the lack of clinical response in some patients under vigabatrin therapy. Ergezinger et al. (2003) reported a significant improvement in a patient employing vigabatrin at a low dose (25 mg/kg per day) in parallel with a drop of the levels of GHB in body fluids and suggested that others monitor vigabatrin treatment not only by clinical assessment but also by repeated controls of GHB in body fluids. According to Ergezinger, the GHB concentrations in CSF of our patient decreased by 7 times during treatment with vigabatrin up to 30 months, and less markedly in urine and plasma (Table 1). These results appear to support the hypothesis that the clinical improvement is correlated with the reduction in the GHB levels, although the values remain always above the normal range. Unfortunately, not all patients reported in the literature have been subjected to plasma and/or CSF monitoring of GHB, so such a correlation remains unproven at this time. The mild increase of GHB levels in blood and urine of our patient after 36 months of therapy could be related to a slight reduction of the dose per kilogram per day due to a body weight gain. The clinical improvement of the child, after a static course period and speech therapy, and the absence of the onset of other neurological symptoms of the disease can be ascribed to the vigabatrin effect. The 3-year period may be still too short to clarify whether our patient truly benefits from vigabatrin the long run, although there is no doubt that the reduced levels of GHB depend on the therapy, which may have influenced the course of the disease.

After 36 months of treatment, biochemical analyses revealed higher levels of blood ornithine (102 μmol/L; 37–96 μmol/L n.v.) than at T0 (57 μmol/L) or in all previous examinations. The visual field constriction was observed for the first time at the final examination, coincident with the ornithine increase, seems to suggest a correlation between these observations. Recently, it has been suggested that increase of ornithine levels might be involved in vigabatrin-associated visual field defects (Roubertie et al. 1998; Sorri et al. 2010) as a result of ornithine-δ-aminotransferase (OAT) impairment. In some patients with gyrate atrophy, vitamin B6 substitution has been successfully employed to prevent the loss of vision (Wang et al. 2000; Ohkubo et al. 2005; Sorri et al. 2010). In line with this observation, and in order to prevent a worsening of the visual defect, we implemented a pyridoxine supplementation (300 mg/day) in conjunction with the same dosage of vigabatrin.

To the best of our knowledge, no other patients with an identical genotype as our have been described in the literature. The p.Cys93Phe mutation has been identified in several patients with SSADH deficiency; overexpression of the p.Cys93Phe allele in mammalian cells results in 3% SSADH activity as compared to overexpression of wild-type SSADH (Akaboshi et al. 2003). Therefore, the mutation should be considered pathogenic. The p.Tyr519X mutation results in a premature stop and may be subjected to nonsense mediated decay; it has not been reported before, but should be considered pathogenic due to the nature of the mutation. Both mutations were not detected in 210 control chromosomes.

In conclusion, we suggest that SSADH deficiency should be considered in the differential diagnosis of patients with mental retardation and language delay, and we confirm the clinical efficacy of vigabatrin treatment. However, careful monitoring of the visual field over time, together with measurement of ornithine plasma concentrations, should be considered to prevent the vigabatrin ocular toxicity and to evaluate the effectiveness of vitamin B6 supplementation.

Take-Home Message

SSADH deficiency should be considered in the differential diagnosis of patients with mental retardation and language delay; we confirm the clinical efficacy of vigabatrin treatment.

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