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RESEARCH REPORT

Molecular Analysis of Turkish Maroteaux-Lamy Patients and Identification of One Novel Mutation in the Arylsulfatase B (ARSB) Gene

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Abstract Mucopolysaccharidosis type VI (MPS VI, Maroteaux-Lamy syndrome) is an autosomal recessive disorder caused by the deficit of the arylsulfatase B (ARSB) enzyme, which leads to dermatan sulfate pathological storage, resulting in a wide spectrum of clinical phenotypes. To date more than 130 different mutations were reported, most of them being restricted to individual families. We here report the first study on the ARSB gene mutations in MPS VI patients of Turkish ethnogeographic origin. On the whole we analyzed 13 unrelated families recruited from 3 different Turkish clinical centers, for a total of 52 subjects, including patients, parents, and siblings. The molecular characterization of ARSB gene in these subjects lead to the identification of eight different mutations (6 missense mutations and two single-nucleotide deletions) one of which novel: c.532C>G (p.H178D). We characterized seven different genotypes, all homozygous except one. The analysis highlighted c.962T>C (p.L321P) as the most frequently detected mutation in the group of patients examined and the c.1072G>A (p.V358M) as the most frequent polymorphism. All parents and 50% of the healthy siblings analyzed carried in a heterozygous condition the mutation identified in the affected relative. The high number of homozygotes reported in this study reflects the high degree of consanguinity of the Turkish population, being the parents of most of the patients here examined, first-degree cousins. As consanguineous marriages are an integral part of the Turkish society, carriers identification accompanied by genetic counseling in families at risk is the eligible approach to minimize the effects of consanguinity in this population.

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Introduction

Maroteaux-Lamy syndrome (mucopolysaccharidosis type VI, MPS VI; MIM#253200) is an autosomal recessive lysosomal storage disorder caused by the deficit of the N-acetylgalactosamine-4-sulfatase (4-sulfatase, arylsulfatase B, ARSB, E.C.3.1.6.12) activity. This leads to dermatan and chondroitin sulfate pathological storage resulting in a wide spectrum of clinical phenotypes, the most severe of which being characterized by growth retardation, dysostosis multiplex, coarse facial features, joints stiffness, cardiac complications, respiratory difficulties, hepatosplenomegaly, hernias, corneal clouding, hearing loss, and hydrocephalus. Unlike some other storage disorders,

mental development is usually normal. Death generally occurs before or during the second decade of life in patients with rapidly progressive forms, often due to cardiopulmonary complications (Valayannopoulos et al. 2010). The birth prevalence ranges between 1 in 43,261 births in the German Turkish immigrants (Baehner et al. 2005) and 1 in 1,505,160 births in Sweden (Malm et al. 2008).

The ARSB locus spans a region of 21 kb located at 5q11-q13 and contains 8 exons; the gene specifies an mRNA transcript of 6089 bp, encoding a protein of 533 amino acids with a signal peptide of 37 amino acids (Litjens et al. 1989; Modaressi et al. 1993). The 57 kDa mature form of the enzyme is formed by three disulfide-linked polypeptides of 43, 8, and 7 kDa (Kobayashi et al. 1992). To date, over 130 different mutations have been described in the ARSB gene (www.hgmd.org). About 75% of all known variations are missense or nonsense mutations, distributed all over the exons; the remaining 25% is represented by small deletions, insertions, splice-site mutations and gross deletions. Most mutations are private or restricted to a small number of subjects. Moreover, some alleles are particularly frequent in certain populations like p. R315Q in Portugal (Karageorgos et al. 2007b) and p. R152W in Russia (Voskoboeva et al. 2000). For some genetic variations a clear genotype-phenotype correlation was established (Karageorgos et al. 2007b), but in most cases this is not the rule.

Characterization of ARSB gene mutations has been reported for many populations from the American to the Australian. Although several studies on lysosomal storage disorders in the Turkish population are reported in literature, only few of them describe MPS cases, including one study reporting 3 MPS VI patients (Elcioglu et al. 2009; Emre et al. 2000; Terzioglu et al. 2002) and, recently, a paper on MPS VI that described, among others, four Maroteaux-Lamy subjects (among which two siblings) of Turkish ethnicity (Brands et al. 2013). However, no studies specifically targeted to the analysis of the MPS VI Turkish patients have been so far conducted. Therefore, this is the first report on ARSB gene mutations characterized in a group of Maroteaux-Lamy patients of Turkish ethnogeographic origin. The Turkish population is very interesting from the genetic point of view, given its social and cultural features that render consanguineous marriages very common. With the 17% of unions contracted between first-degree cousins (Koc 2008), the genetic disorders, in particular the hemoglobinopathies and phenylketonuria, represent a very important public health issue.

This study was carried out on 18 MPS VI patients deriving from 13 unrelated families recruited from 3 different Turkish clinical centers. The molecular analysis of ARSB gene in these subjects lead to the characterization of 8 mutations, one of which previously undescribed.

Materials and Methods

Patients

A total of 13 unrelated Maroteaux-Lamy Turkish families, including 52 subjects (18 patients, 18 parents, and 16 healthy siblings) were recruited from the Department of Pediatric Metabolism and Nutrition-Çukurova University of Adana, the Department of Pediatric Genetics-Marmara University Hospital of Istanbul, and the Diyarbakr Children's Hospital-Department of Pediatrics of Diyarbakr, and evaluated in this study. All families belong to the Turkish ethnic group. Patients are offspring of consanguineous marriages between first-degree cousins (11 families), second-degree cousins (1 family), or "distant cousins" (1 family). Clinical evaluations were performed in the Turkish clinical centers. Arylsulfatase B enzyme activity assays were performed in leukocytes by 3 different laboratories: Willink Biochemical Genetics Laboratory-Manchester (United Kingdom), Gazi University Medical Faculty Pediatric Metabolism Laboratory-Ankara (Turkey), and Sahlgrenska's University Laboratory-Molndal (Sweden). ARSB molecular analysis was carried out at the Laboratory of Diagnosis and Therapy of Lysosomal Disorders, University of Padova (Italy). Informed consent for genetic analysis was obtained for all patients and their relatives included in the study. For a better comprehension, patients phenotype was classified in the two principal forms of rapidly progressing, or severe, and slowly progressing, or mild, according to a previously published description (Valayannopoulos et al. 2010).

ARSB Mutation Analysis

Genomic DNA was extracted from peripheral blood leukocytes or from skin fibroblasts using the commercial QIAmp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany). ARSB exons and their flanking regions were PCR-amplified using appropriate intronic primers as previously described (Zanetti et al. 2009). Sequence variations were confirmed by sequencing duplicate PCR products in both directions. Obtained sequences were compared to the genomic reference sequence NC_000005.9.

Sequence Variations Nomenclature

All sequence variations were described according to the mutation nomenclature [(den Dunnen and Antonarakis 2001) HGVS, http://www.hgvs.org/mutnomen].

Analysis of the New Missense Sequence Variation

In order to establish if the new non-synonymous variation was a polymorphism or a mutation, an in silico prediction of the pathogenicity was obtained through the software PONP (Pathogenic-or-Not-Pipeline) which integrates five tools (PhD-SNP, SIFT, PolyPhen-2, SNAP, I-Mutant) to predict the probability that variations may affect protein function and may consequently be disease-related (Olatubosun et al. 2012). Interrogation of dbSNPs [http://www.ncbi.nlm.nih.gov/projects/SNP/] and 1000 genomes [http://www.1000genomes.org/] databases for alleles and genotype frequency was also performed.

Results

The molecular characterization of the ARSB gene mutations in the 18 Turkish Maroteaux-Lamy patients allowed the identification of eight point mutations: six missense mutations and two single-nucleotide deletions. One sequence variation had never been described before: c.532C>G (p.H178D). Five out of eight mutations are located in exon 5.

On the whole, we observed seven different genotypes of which only one is a composite heterozygote while the others are homozygotes. Nine individuals (including one couple of twins and two of brothers) carried the genotype c.[962T>C]+[962T>C]; 3 subjects (including 2 siblings) revealed the genotype c.[1036delG]+[1036delG].

Interestingly, patients P3, P6, P11, and P12 come from the same city and all of them except patient P3 carry the genotype c.[962T>C]+[962T>C]. Also patient P15 and the two brothers identified as P13 and P14 come from the same town and present with the same mutation c.1036delG.

Most of the patients analyzed in this study did not present a significant residual ARSB enzymatic activity; five out of the seven patients in whom such activity was found showed less than 1% of the lower level normally given as control value; in one patient we measured an activity of 2.81% and in another one 8.23% of the control levels.

Genotype-Phenotype Correlation

Patients' phenotypes ranged from mild to severe and are reported in Table 1.

The mutation c.962T>C (p.L321P) was first described in homozygosis in a patient with an intermediate phenotype, who was born from a consanguineous marriage (Isbrandt et al. 1994). Then it was reported, again in homozygosis, in one patient, with a non-specified phenotype (Karageorgos et al. 2007b).

In this study it was detected in a homozygous state in six families including nine patients, among which two couples of siblings and one of twins. The phenotypes of these nine cases range from severe to mild, while the arylsulfatase B activity was undetectable for all patients except for patient P9 showing a very low activity and a moderate phenotype.

The c.1036delG mutation leads to the production of a 176 amino acids shorter protein with respect to the wild-type ARSB. It was first reported in a homozygote German patient with a very high level of urinary GAG (699 mg/ml creatinine) who showed symptoms of a rapidly progressing disease (Karageorgos et al. 2007b).

In our analysis, the deletion was detected in the two siblings P13 and P14 and in patient P15. One of the two siblings presents with a severe pathology, while the other one, in ERT since 10 months of age, seems to carry an attenuated form of the disease. The third subject shows mild symptoms.

The genetic variation c.1079T>C (p.L360P) was detected for the first time in an attenuated phenotype (Voskoboeva et al. 2000).

In this study, this variation was diagnosed in two siblings (P16 and P17) both presenting with moderate symptoms.

The deletion c.1577delC was previously reported in a severe homozygote patient, son of consanguineous parents (Isbrandt et al. 1994).

Our patient (P18) carrying this mutation had a severe phenotype and died at 8 years of age for post-surgery complications.

The mutation c.903C>G (p.N301K) was recently described for the first time in homozygosis condition, in a Turkish patient with a rapidly progressing phenotype, child of consanguineous parents. Through the in vitro expression of the mutated protein they evidenced a slightly reduced amount of the 66 kDa ARSB precursor, while they did not detect the 46 kDa mature form of the enzyme (Brands et al. 2013).

In this study, the p.N301K mutation was diagnosed in homozygosis in patient P3 who presented with moderate symptoms.

The only compound heterozygote detected in our analysis is patient P1 carrying the genotype c.[160G>A]+[1057T>A] (p.[D54N]+[W353R]) and a severe form of the disease. Both mutations were previously described (Karageorgos et al. 2007b): the p.D54N was detected in a homozygote and in a compound heterozygote patients both of Portuguese origin; this mutation, directly affecting the ARSB catalytic site, might result in an early-onset and rapidly progressing pathology. The p.W353R mutation was found in a compound heterozygote with a non-specified phenotype.

Table 1 Genotypes and clinical phenotypes encountered in the 13 MPS VI families included in the study. Arylsulfatase B activity at diagnosis is reported; the assay was performed in three different laboratories as reported in the materials and methods section: (a) Turkey, (b) the United Kingdom, (c) Sweden. Enzyme activities are expressed as percentage of activity with respect to the lowest limit of the normal range (range of values detected in a healthy control population) or to the value of the normal control (contemporarily examined). *F* family, *pt* patient, *n.d.* not defined

F	Pt	c.DNA alteration	Amino acid substitution	Reference	Polymorphisms	Arylsulfatase B activity	Phenotype
1	P1	c.[160G>A]+ [1057T>A]	p. [D54N]+ [W353R]	Karageorgos et al. 2007b (HGMD: CM074027; HGMD: CM070023)	c.[1072G>A]+ [1072G>A]	0%[b]	Severe
2	P2	c.[532C>G]+ [532C>G]	p.[H178D]+ [H178D]	Previously undescribed	c. [1191G>A] + [1191G>A]	2.81%[a]	Mild
3	P3	c.[903C>G]+ [903C>G]	p.[N301K]+ [N301K]	Brands et al. 2013	c.[1151G>A]+ [1151G>A]	0%[b]	Moderate
4	P4	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	Isbrandt et al. 1994 (HGMD: CM940120)	c.[1072G>A]+ [1072G>A]	0%[b]	Severe
	P5	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	0.35%[b]	n.d.
5	P6	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	0%[b]	Severe
6	P7	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	0%[b]	Mild-moderate
	P8	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	0%[b]	Mild-moderate
7	P9	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	8.23%[c]	Moderate
	P10	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	n.a.	Moderate
8	P11	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	0.37%[b]	Moderate
9	P12	c.[962T>C]+ [962T>C]	p.[L321P]+[L321P]	As above	c.[1072G>A]+ [1072G>A]	0.07%[b]	Mild
10	P13	c.[1036delG]+ [1036delG]	—	Karageorgos et al. 2007 (HGMD: CD075326)	c.[1072G>A]+ [1072G>A]	0%[a]	Severe
	P14	c.[1036delG]+ [1036delG]	—	As above	c.[1072G>A]+ [1072G>A]	0%[a]	Mild
11	P15	c.[1036delG]+ [1036delG]	—	As above	c.[1072G>A]+ [1072G>A]	0.07%[b]	Mild
12	P16	c.[1079T>C]+ [1079T>C]	p.[L360P]+[L360P]	Voskoboeva et al. 2000 (HGMD: CM003998)	c.[1151G>A]+ [1151G>A]	0%[b]	Moderate
	P17	c.[1079T>C]+ [1079T>C]	p.[L360P]+[L360P]	As above	c.[1151G>A]+ [1151G>A]	0.75%[b]	Moderate
13	P18	c. [1577delC]+ [1577delC]	—	Isbrandt et al. 1994 (HGMD: CD941599)	—	0%[b]	Severe

Laboratory in which was performed the ARSB activity assay:

[a] Gazi University Medical Faculty Pediatric Metabolism Laboratory-Ankara (Turkey)

[b] Willink Biochemical Genetics Laboratory-Manchester (United Kingdom)

[c] Sahlgrenska's University Laboratory-Molndal (Sweden)

The novel variation diagnosed in this study is the c.532C>G (p.H178D), detected in a homozygous condition in patient P2, showing mild symptoms. This undescribed nucleotide substitution is not reported in both dbSNPs and 1,000 genomes databases. Moreover, in silico prediction of its pathogenicity through PONP integrated tools revealed that these variations belong to the "pathogenic" *Predicted class* with a *Probability of pathogenicity* of 0.72 (standard error 0.1 e^{-1}) confirming that the variation could not be considered a polymorphism but a disease-causing mutation.

The c.1072G>A (p.V358M) is the most frequent polymorphism encountered in the group of patients analyzed: 13 patients out of 18 were homozygotes for this variant. The polymorphism c.1151G>A (p.S384N) was detected in homozygosis in 3 patients, while only one patient was homozygote for the polymorphism c.1191G>A (p.P397P). Patient P18 did not carry anyone of the polymorphisms mentioned above. No patients carried the quite common polymorphism c.1126G>A (p.V376M).

Family Studies

Both parents of each patient were genetically characterized for 11 families out of 13; of the two remaining families, for one only the mother sample was available, for the other one no parents' samples were collected due to social problems of the family. All parents analyzed carried the child's mutation in heterozygous condition. We also performed carrier analysis in 16 healthy siblings: eight of them resulted carrier of the mutation detected in the affected relative.

Three families presented a family history reporting at least one previous child dead for worsening of MPS VI clinical conditions; two families had a history reporting at least one miscarriage; one family reported stillbirth siblings.

A pedigree was reconstructed for the families of patient P6 and of patients P13-P14 (Fig. 1a and b, respectively). Among the families evaluated in this study, these two presented with the highest number of affected children and/or miscarriages or termination of pregnancy events in their clinical history. The high degree of consanguinity is clearly shown in the two pedigrees reported, being the parents first-degree cousins.

Discussion

In this study, we report the results of the molecular analysis of the ARSB gene in a group of Turkish MPS VI patients. Turkish population is a very young heterogeneous population composed mainly by the Turkish ethnic group but also by other minor communities (Kurdish, Arabic, Greek, Circassian, Georgian, Armenian, and Jewish). Data from the 2003 Turkey Demographic and Health Survey indicates that Turkey is a country with a high level of consanguinity, as other Muslim populations of North Africa, Middle East, South and Central Asia. At present consanguineous marriages account for 22% of all unions, 76% of which being contracted between first-degree cousins. However, no later than 4 decades ago the percentage assessed around 27, the gradual reduction being due to the modernization of the Turkish society (Koc 2008). This social habit is motivated by cultural, religious but also economical reasons. The highest prevalence of consanguinity is found in the less developed regions of the country, in families with a low socioeconomic status in which women, but also their husbands, tend to have lower level of education.

Turkish population represents a challenge for genetic studies as its high rate of consanguineous marriages could be a contributing factor to the high incidence of some rare autosomal recessive diseases registered in the country; in particular, hereditary blood disorders (hemoglobinopathies and thalassemia) and phenylketonuria are the genetic pathologies presenting the highest frequencies (Tuncbilek and Ozguc 2007). On the other side, groups of the Turkish population might also represent a rare opportunity to evaluate specific pathological alleles in homozygous condition and in a quite conserved genomic background.

Until now, analyses of ARSB gene mutations have been reported for many world populations including the North (Isbrandt et al. 1994; Jin et al. 1992; Karageorgos et al. 2007a; Simonaro and Schuchman 1995) and the South American (Karageorgos et al. 2007a; Petry et al. 2003, 2005), the Western European (Arlt et al. 1994; Isbrandt et al. 1994, 1996; Karageorgos et al. 2007a; Villani et al. 1998, 1999), the Eastern European (Isbrandt et al. 1994; Jurecka et al. 2012; Voskoboeva et al. 1994, 2000), the Asiatic (Dou et al. 2006; Wicker et al. 1991; Wu et al. 2000), and the Australian populations (Litjens et al. 1992, 1996; Wicker et al. 1991). Three studies have been published describing few cases of mucopolysaccharidoses in the Turkish population (Elcioglu et al. 2009; Emre et al. 2000; Terzioglu et al. 2002). Among these, Emre and colleagues characterized biochemically and genetically a group of MPS patients including three MPS VI cases (Emre et al. 2002). Recently, Brands described four MPS VI patients of Turkish ethnogeographic origin (Brands et al. 2013). Hence this is the first study reporting the molecular characterization of the ARSB mutations in a quite large group of Maroteaux-Lamy cases from Turkey. Eighteen patients coming from 13 unrelated families were genetically characterized. Since all MPS VI subjects analyzed have consanguineous parents (in most cases first-degree cousins), we evidenced, as expected, a lower degree of genetic heterogeneity with respect to what is generally described for other populations, confirming the effect of the social

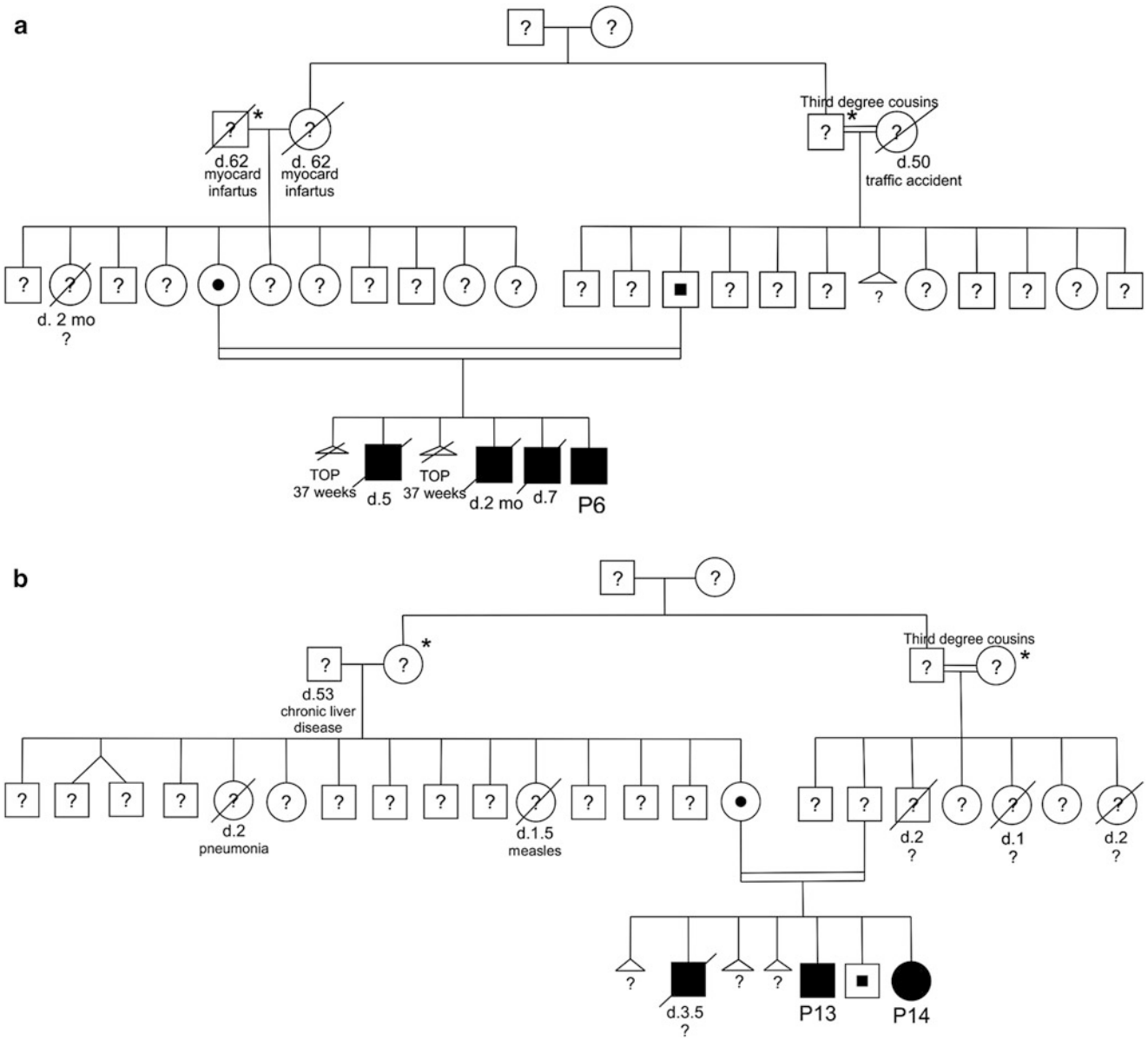

Fig. 1 Pedigrees of patient P6 (**a**) and patients P13-P14 families (**b**). Subjects identified with * and # are first-degree cousins. Molecular characterization of ARSB gene was performed only for the probands P6, P13, and P14, their parents and siblings. For all other relatives reported in the pedigrees, molecular status of ARSB gene was not investigated. *TOP* termination of pregnancy

habits mentioned above. In addition, founder effects might be postulated, given the small number of different mutations detected in the group analyzed, for a gene as ARSB, which shows in most populations a wide variety of private mutations. Also the high frequency of homozygous genotypes (12 out of 13 families) detected in this study reflects the above-mentioned aspects.

To date more than 130 different mutations have been described in the literature for the ARSB gene confirming the high genetic heterogeneity of MPS VI, a feature shared with all the other MPSs. This large number of different mutations, which are often restricted to single families or are novel, renders the analysis of genotype-phenotype correlation quite difficult in the MPS VI disease, although this might result easier in homozygous subjects carrying the same mutation. An attempt to establish a genotype-phenotype correlation was therefore made for the patients characterized in this study, considering the high rate of consanguinity and hence a likely similar genetic background which might help in establishing such a relationship. However the picture we revealed was not so unambiguous. For example, clinical evaluation of the 8 subjects homozygote for the p.L321P mutation showed a continuum of phenotypes from the mild-slowly to the

severe-rapidly progressing form of the disease. Also, for the c.1036delG mutation we observed both a mild phenotype and a severe phenotype. These findings allow to hypothesize the existence of other genetic or epigenetic factors which could be potentially important in the phenotype determination.

The most frequent polymorphisms detected in the Maroteaux-Lamy population analyzed are the p.V358M and p.S384N. In particular the p.V358M, when present, was found in homozygosis, suggesting a co-segregation with the disease-causing mutations. In fact, all patients carrying the mutations p.L321P or c.1036delG, presented also the polymorphism p.V358M. The allele frequency was 36.1%, slightly higher than that (32%) found in the population previously analyzed (Karageorgos et al. 2007b). Also the frequency of the allele p.S384N is higher with respect to the frequency detected by Karageorgos (16.6% versus 6.3%), although in that paper it was erroneously identified as pathological mutation. On the contrary, the polymorphism p.P397P was found in the 30.5% of the alleles here examined, less than half of the frequency (74.3%) calculated on the cases previously reported by the cited author (Karageorgos et al. 2007b).

Most of the patients analyzed in this study did not present a significant residual ARSB enzymatic activity; five out of the seven patients in whom such activity was found showed less than 1% of the lower level normally given as control value; in 1 patient we measured an activity of 2.81% and in another 8.23% of the control levels. All these seven patients showed a mild to moderate phenotype. None of the patients with a severe-defined phenotype reported residual enzymatic activity, however also for some of the mild/moderate patients we registered null ARSB activity. Therefore, from our data we might infer that the presence of a residual enzymatic activity usually correlates with a mild/moderate phenotype, but a total lack of activity is not always associated with a severe phenotype/prognosis.

In conclusion, analysis of the Turkish population has confirmed for MPS VI similar influence of the cultural habits on the incidence of inherited diseases, already denounced for hemoglobinopathies and phenylketonuria, as an important public health issue (Koc 2008). Similar issues are taken into consideration in other countries such as Israel, characterized by a high level of consanguineous marriages due to the existence of few separate ethnic groups (Bach et al. 2007) and in which counseling measures adopted in recent years have significantly reduced the prevalence of genetic disorders in the population.

In particular, measures addressed to identify in the population the most frequent genetic diseases and the set up of screening programs for them would help the individuation of carriers; more restricted screenings as well as prenatal diagnosis may also be conducted within at-risk families. All this should allow a possible reduction of the incidence of MPS VI as well as other recessive genetic diseases.

One-Sentence Take-Home Message

First ARSB molecular analysis extensive report of MPS VI Turkish patients.

Details of the Contributions of Individual Authors

AZ: conception and design of the study, performing of molecular analysis, analysis and interpretation of data, writing and critical revision of the manuscript

NO: conception and design of the study, clinical data collection, and critical revision of the manuscript

NE: clinical data collection and critical revision of the manuscript

MNO: clinical data collection and critical revision of the manuscript

DK: clinical data collection and critical revision of the manuscript

EL: cells culture and critical revision of the manuscript

MS: conception and design of the study and critical revision of the manuscript

RT: conception and design of the study, writing and critical revision of the manuscript, final approval of the manuscript

Name of One Author Who Serves as Guarantor

Rosella Tomanin

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Details of Ethics Approval

Ethics approval was not required to perform the described studies.

A Patient Consent Statement

Informed consent for genetic analyses in patients and relatives was obtained from all subjects involved in the study, including parents or tutors.

Conflict of Interest

Maurizio Scarpa has received research grants and honoraria and travel support for speaking engagements from Actelion, Shire HGT, Genzyme Corporation, and BioMarin.
Alessandra Zanetti, Neslihan Önenli-Mungan, Nursel Elcioglu, Mehmet Nuri Özbek, Deniz Kör, Elisabetta Lenzini, and Rosella Tomanin declare no conflicts of interest.

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CASE REPORT

Danon Disease Due to a Novel *LAMP2* Microduplication

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Abstract Danon disease is a rare X-linked disorder comprising hypertrophic cardiomyopathy, skeletal myopathy, intellectual disability, and retinopathy; mutations of the lysosome-associated membrane protein gene *LAMP2* are responsible. Most affected persons exhibit "private" point mutations; small locus rearrangements have recently been reported in four cases. Here, we describe the clinical, pathologic, and molecular features of a male proband and his affected mother with Danon disease and a small *LAMP2* microduplication. The proband presented at age 12 years with exercise intolerance, hypertrophic cardiomyopathy, and increased creatine kinase. Endomyocardial biopsy findings were nonspecific, showing myocyte hypertrophy and reactive mitochondrial changes. Quadriceps muscle biopsy demonstrated the characteristic autophagic vacuoles with sarcolemma-like features. LAMP2 tissue immunostaining was absent; however, *LAMP2* sequencing was normal. Deletion/duplication testing by multiplex ligation-dependent probe amplification (MLPA) assay revealed a 1.5kb microduplication containing *LAMP2* exons 4 and 5. RT-PCR studies were consistent with the inclusion of these two duplicated exons in the final spliced transcript, resulting in a frameshift. The proband's mother, who had died following cardiac transplantation due to suspected myocarditis at age 35, was reviewed and was shown to be affected upon immunostaining of banked myocardial tissue. This case constitutes the second report of a pathogenic microduplication in Danon disease, and illustrates a number of potential diagnostic pitfalls. Firstly, given the imperfect sensitivity of *LAMP2* sequencing, tissue immunostaining and/or MLPA should be considered as a diagnostic adjunct in the workup for this disorder. Secondly, the pathological findings in myocardium may be falsely indicative of relatively common conditions such as myocarditis.

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Introduction

Danon disease (OMIM #300257) is a rare X-linked disorder originally described as a Pompe disease phenocopy with normal acid maltase activity (Danon et al. 1981). The cardinal features of this condition are hypertrophic cardiomyopathy, preexcitation and tachyarrhythmias, and skeletal myopathy, with some affected persons manifesting intellectual disability and/or pigmentary retinopathy. Symptoms in affected males are highly penetrant, progressive, and severe, with mean onset in the preadolescent years; survival beyond 25 years is unlikely without cardiac transplantation (Boucek et al. 2011). Although heterozygous females have an attenuated phenotype, potentially fatal cardiac sequelae (preexcitation, arrhythmias, and cardiomyopathy) do occur in a large proportion, and median female

survival is approximately 45 years (Boucek et al. 2011; Miani et al. 2012). The disease process is characterized pathologically by progressive interstitial fibrosis, muscle fiber hypertrophy, periodic-acid-Schiff-positive sarcoplasmic vacuolation, and myofibrillar disarray (Murakami et al. 1995; Sugie et al 2005). Affected tissues accumulate abnormal autophagosomes containing bulk cytoplasmic debris and glycogen, bounded by a membrane expressing peculiar sarcolemma-like features (e.g., dystrophin and sarcoglycan staining) (Danon et al. 1981; Sugie et al. 2005; Tanaka et al. 2000; González-Polo et al 2005).

Danon disease results from loss-of-function mutations of the lysosome-associated membrane protein gene *LAMP2* (Nishino et al. 2000). *LAMP2*'s three protein products (LAMP2-A, LAMP2-B, and LAMP2-C) are integral membrane proteins, sharing a common N-terminal (luminal) domain, but with distinct transmembrane domains and cytoplasmic tails. These proteins permit the targeted import of cytoplasmic proteins (LAMP2-A, LAMP2-B) and RNA (LAMP2-C) into lysosomes for degradation (Cuervo and Dice 1996; Bandyopadhyay et al. 2008; Kaushik et al. 2011; Demirel et al. 2012; Fujiwara et al. 2013). The majority of reported Danon disease alleles are private, with nonsense and frameshift mutations predominating (Boucek et al 2011). Recently, nonrecurrent microdeletions and microduplications at the *LAMP2* locus have been demonstrated in four unrelated cases (Yang et al 2010; Majer et al 2013). Herein, we report the clinical, pathological, and molecular findings in a mother and son with Danon disease due to a small intragenic *LAMP2* microduplication, a novel category of mutation in this disorder.

Methods

Human Subjects—All studies were undertaken with the prior free and informed consent of the patient or legally responsible guardian, according to principles of the October 2008 revision of the Declaration of Helsinki. Written, informed consent was obtained prior to publication. In the case of the proband, clinical data were obtained in the course of providing standard clinical genetic care, rather than under a research protocol; clinical data for patient's mother were gathered by retrospective chart review after receiving the appropriate written permissions.

Molecular Analyses—Genomic DNA was isolated from blood leukocytes by salt precipitation on the Gentra Autopure LS workstation (Qiagen, Valencia, California). *LAMP2* sequencing and MLPA analysis were performed by a third-party clinical sequencing facility (Emory Genetics Laboratory, Decatur, GA) according to standard protocols. For RNA studies, total RNA was extracted from cultured skin fibroblasts using the PerfectPure RNA Cultured Cell Kit (5 Prime, Hamburg, Germany); first-strand cDNA synthesis was performed with the SuperScript III system (Invitrogen, Carlsbad, California) and oligo-(dT) priming. PCR analysis was performed according to standard protocols; products were then sequenced by standard fluorescent dideoxy terminator sequencing and separated on a 3730xl DNA analyzer (Applied Biosystems, Foster City, California). *LAMP2* primers used for genomic PCR (Fig. 3b) are ATGGAATTCTGATGGCCAA (forward) and GCTGCAGCTGAACATCACT (reverse). Primers used for *LAMP2* RT-PCR (Fig. 3c–d) are CTGGCTTTTC CTGGATTGCG (forward) and TAGAGCAGTGTGA-GAACGGC (reverse).

Results

Clinical Presentation–The proband, a male, was referred to our center at age 12 for evaluation of leg weakness and reduced exercise tolerance. Pregnancy, delivery, and early developmental milestones were normal. He presented initially to his primary care physician at age 6 for fatigability, poorly coordinated running gait, and post-exertional thigh pain; a persistently elevated creatine kinase (900–1,630 U/L) was documented. Weakness, more pronounced proximally in the lower extremities, followed an indolent course, such that he remained able to walk unassisted. The only other presenting complaint was poor academic performance, for which he received special schooling from age 9 onward. Family history was significant for his mother having undergone catheter ablation and defibrillator implantation at age 32 for persistent ventricular tachycardia; she subsequently developed severe heart failure, and died of perioperative complications following cardiac transplant at age 35. Pertinent physical examination findings in the proband included a partial Gower's sign, proximal lower extremity weakness (4+), intact reflexes, and an S3-S4 "gallop." Selected investigations were as follows: EMG was normal. ECG (Fig. 1) showed impressive left ventricular hypertrophy with a shortened PR interval, compatible with preexcitation and nonspecific intraventricular conduction delay. Plain chest film showed cardiomegaly with enlarged central suprahilar pulmonary vessels. Echocardiography showed an impressive degree of concentric left ventricular hypertrophy (Fig. 2; Supplementary Table 1), which, despite significant progression over 4 years, remained nonobstructive. Holter monitor demonstrated only rare supraventricular ectopy, and very rare ventricular ectopy, the predominant rhythm being sinus. Electrophysiology study revealed no accessory pathway, compatible with a diagnosis of "pseudopreexcitation"; however, he developed inducible ventricular tachycardia deteriorating to ventricular fibrillation. On formal psychometric testing (WISC-IV), his full-scale IQ was

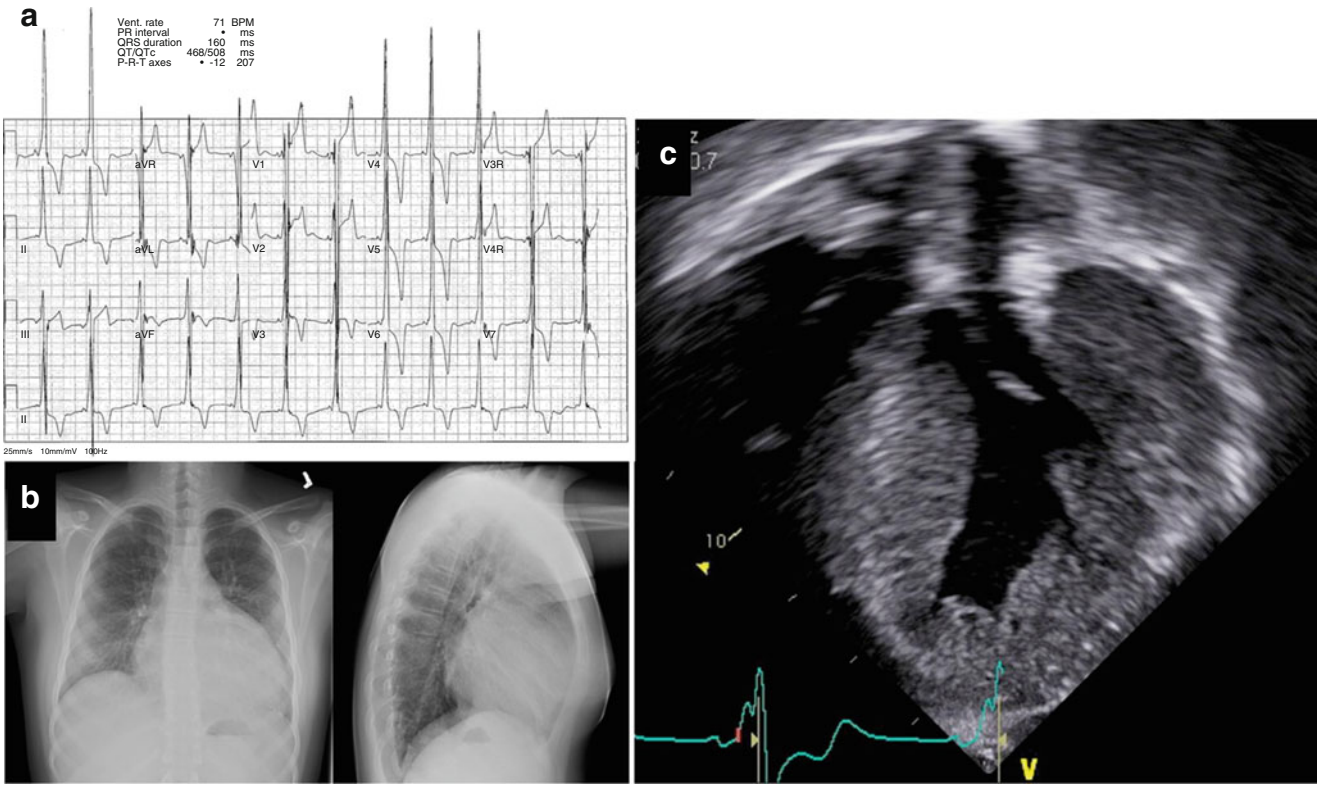

Fig. 1 Hypertrophic cardiomyopathy. (**a**) Standard 12-lead ECG showing shortened PR interval (~80 ms), left axis deviation with LV hypertrophy, nonspecific intraventricular conduction delay, and diffuse T-wave inversions. The patient was asymptomatic during the study. (**b**) Plain films showing massive cardiomegaly, prominent perihilar vessels, enlarged cardiothoracic ratio (~0.68), and absence of retrosternal clear space. (**c**) Echocardiogram shows massive LV hypertrophy (see also Supplementary Table 1)

scored as "borderline" (third centile). Cranial MRI appearance was nonspecific, showing a few tiny bilateral foci of increased T2 white matter signal intensity.

A diagnostic endomyocardial biopsy was performed at 13 years of age. This showed a degree of myocyte hypertrophy, but was otherwise unremarkable, with no glycogen accumulation or abnormal inclusions, and normal-appearing contractile filaments. Electron microscopy showed a nonspecific reactive increase in the number of mitochondria; mitochondria were also seen to demonstrate more than the usual number of cristae. The pathological examination of the native heart of the proband's mother had shown myocyte hypertrophy and extensive chronic inflammation, with muscle fiber necrosis, fibrofatty infiltration, and biventricular thinning (more pronounced in the right ventricle). The epicardium was described as having significant, virtually diffuse inflammation. These had been described in the original pathology summary as indicative of an active myocarditis, with changes consistent with possible arrhythmogenic right ventricular dysplasia (ARVD).

As a definitive diagnosis remained elusive, the proband further underwent a quadriceps muscle biopsy (Fig. 2). Routine stains showed no evidence of myofiber necrosis, regeneration, atrophy, or hypertrophy. Vacuolated fibers containing basophilic, granular, periodic acid-Schiff-positive material were identified. Electron microscopy showed patches of myofibrillar disarray, but no abnormal glycogen accumulation. Collections of subsarcolemmal mitochondria were seen, but again without any specific mitochondrial abnormality. Respiratory chain histochemical stains were normal; ATPase stain showed mild clustering of type I fibers. Plasma membranes exhibited normal staining for dystrophin, sarcoglycans alpha through delta, dysferlin, merosin, and caveolin. Importantly, these same stains also highlighted the rim of many of the abnormal vacuoles. LAMP-2 immunostaining was absent in skeletal muscle, in the proband's banked myocardial biopsy, and in banked myocardium from the proband's mother. Somewhat unexpectedly, *LAMP2* sequencing in the proband was normal; multiplex ligation-dependent probe amplification (MLPA) analysis identified a small intragenic microduplication of approximately 1.5kb, containing exons 4 and 5 (minimal hg19 coordinates chrX:119581626–119583100) (Fig. 3a). This interval is bounded by Alu repeats showing 83% overall sequence identity, suggesting nonhomologous recombination as a likely mechanism. Confirmatory genomic PCR and RT-PCR analyses (Fig. 3b-d) confirmed the presence of an additional copy of *LAMP2* exons 4 and 5 (344 coding bases)

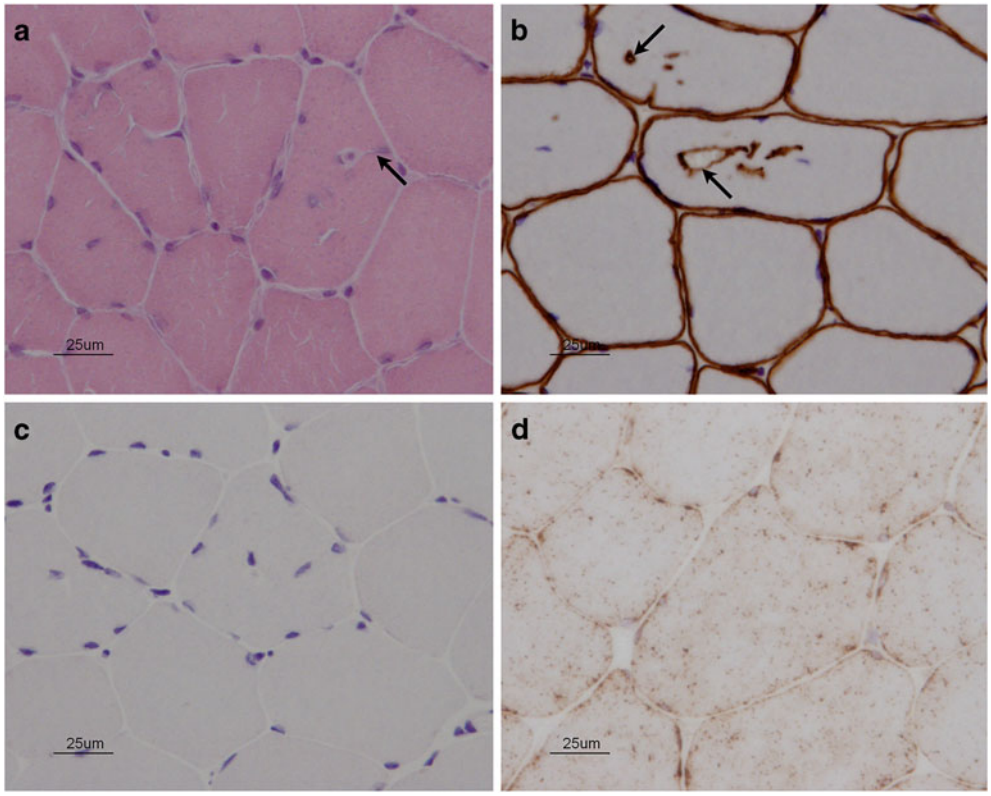

Fig. 2 Vacuolar myopathy with absent LAMP2 in skeletal muscle. (**a**) Hematoxylin and eosin-stained section shows cytoplasmic vacuolation. Arrow indicates a vacuole in continuity with plasma membrane. (**b**) Adhalin (alpha-sarcoglycan) stain. This marker, ordinarily present in the plasma membrane, also highlights vacuolar membranes. (**c**) Absent LAMP2 immunostaining in the proband. (**d**) Normal LAMP2 immunostaining in control muscle tissue

into the final spliced transcript, with an ensuing frameshift. X-inactivation studies could not be undertaken in the proband's mother due to limitations on the small amount of (paraffin-embedded) tissue remaining.

Discussion

Although consensus guidelines have yet to be established, follow-up for our patient consists of regular clinical assessment of Cardiology and Metabolics, Neurology (regarding his weakness), appropriate rehabilitative services (physical and occupational therapy), and the ongoing involvement of a genetic counselor. An implantable cardioverter-defibrillator has been offered but (so far) declined. He is currently being assessed for possible cardiac transplantation, a difficult decision given this family's prior experience.

This report constitutes the second, and smallest, reported *LAMP2* microduplication of which we are aware. Unlike microdeletions, which should be readily detectable in males due to their failure to amplify, microduplications are likely to be missed unless sought specifically using MLPA or a comparable technique. As this case illustrates, LAMP2 immunofluorescence is a useful diagnostic adjunct in males, with the caveats that "leaky" or nontruncating (e.g., missense) mutations may exhibit normal immunoreactivity (van der Kooi et al. 2008). In cases in which myocardial tissue is unavailable, or a less invasive testing option is preferred, alternative assays for LAMP2 protein expression have been described in peripheral blood leukocytes (by immunoblot) and in cultured skin fibroblasts (by immunofluorescence) (Fanin et al. 2006; Alroy et al. 2010). Because the presenting features of Danon disease (weakness, hypertrophic cardiomyopathy, arrhythmias, and intellectual disability) are individually rather common, a minimally invasive approach might be to combine leukocyte immunoblot with *LAMP2* sequencing in patients with compatible clinical features.

Also noteworthy in this case is that routine endomyocardial examination was initially nondiagnostic in both affected members of this pedigree. The reason for the discordant cardiac and skeletal muscle findings in our proband is unclear, but is unlikely to be due to differing tissue expression, as the duplication identified should completely abolish expression of all three LAMP2 isoforms. Although we are unaware of prior reports of

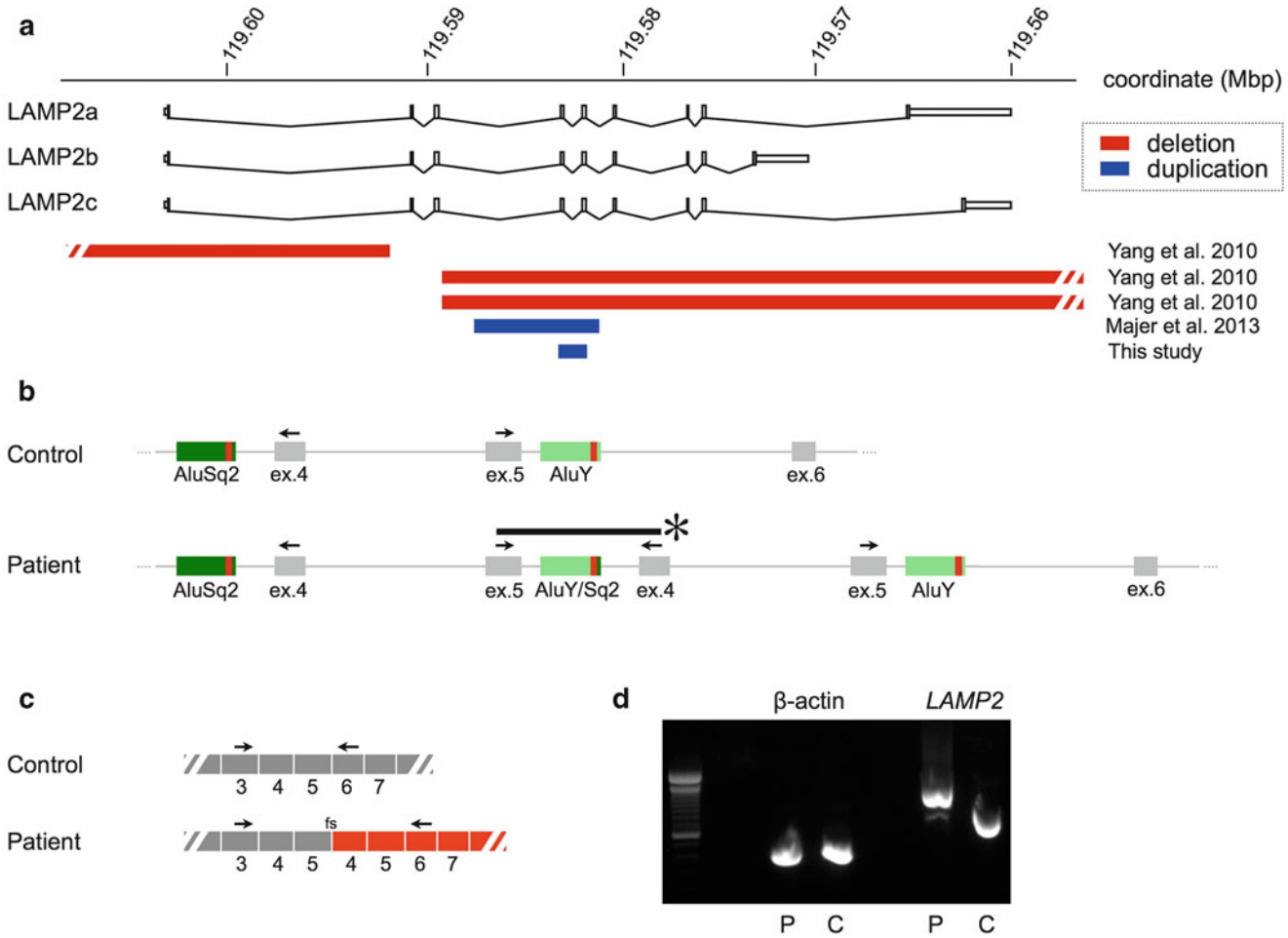

Fig. 3 Disruption of *LAMP2* by a small microduplication. (**a**) Overview of all reported *LAMP2* microdeletions (*red*) and microduplications (*blue*). Our patient's duplication encompasses *LAMP2* exons 4 and 5. B: Genomic PCR, which amplifies a novel junction fragment (*) in the patient (primer sites indicated with *arrows*); sequencing of this fragment situates the breakpoints within a 31bp region of high identity (*red bar*) between two flanking Alu repeats (data not shown). (**c**) The resulting spliced transcript contains an extra copy of exons 4 and 5 (344 coding bases), with an ensuing frameshift (fs). *Arrows* indicate primers used for RT-PCR shown in panel D. (**d**) RT-PCR (fibroblast RNA) yields a ~344bp larger product in the patient, consistent with the splicing scheme depicted in panel C

"false-negative" endomyocardial biopsy in patients with characteristic skeletal muscle findings, the reciprocal situation (vacuolar myopathy in cardiac but not skeletal muscle biopsy) has been described (Taylor et al. 2007). Findings in the proband's mother illustrate that the later stages of illness are sufficiently destructive that characteristic findings may not be readily apparent. In light of the surprisingly high sample prevalence of 6/75 (8%) and 3/50 (6%) of *LAMP2* mutations in two recent molecular screens of unselected hypertrophic cardiomyopathy patients (Arad et al. 2005; Cheng et al 2012), a high index of suspicion for this condition is warranted, and routine screening using a combination of methods appears appropriate.

Acknowledgments

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Synopsis

The diagnosis of Danon disease was established in a mother and son with a small intragenic *LAMP2* microduplication; myocardial pathologies and *LAMP2* sequencing having been initially misleading.

Compliance with Ethics Guidelines

Conflict of Interest: Matthew Lines, Stacy Hewson, William Halliday, Peter Sabatini, Tracy Stockley, Anne Dipchand, Sarah Bowdin, and Komudi Siriwardena declare that they have no conflict of interest.

Informed Consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of 1975, as revised in 2008. Informed consent was obtained from each patient (or legal delegate) for inclusion in the study. Additional informed consent was obtained from each patient identified in this report.

Author Contributions

Matthew Lines: Analysis/interpretation of data, drafting and revision of manuscript

Stacy Hewson: Analysis/interpretation of data, critical revision of manuscript

William Halliday: Analysis/interpretation of data (pathology), critical revision of manuscript

Peter Sabatini: Analysis/interpretation of data (molecular), critical revision of manuscript

Tracy Stockley: Analysis/interpretation of data (molecular), critical revision of manuscript

Anne Dipchand: Analysis/interpretation of data (echocardiography), critical revision of manuscript

Sarah Bowdin: Analysis/interpretation of data (clinical), critical revision of manuscript

Komudi Siriwardena: Conception and design, critical revision of manuscript, guarantor of manuscript

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CASE REPORT

Secondary Mitochondrial Respiratory Chain Defect Can Delay Accurate PFIC2 Diagnosis

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Abstract Multiple respiratory chain deficiencies represent a common cause of mitochondrial diseases and often result in hepatic failure. There is no gold-standard test for diagnosing mitochondrial disease, and the current diagnosis relies on establishing a consistent pattern of evidence from clinical data, neuroimaging, tissue biopsy, and biochemical investigations. In some patients, the mitochondrial respiratory chain defect (MRCD) diagnosis is confirmed by genetic investigations. In most cases, genetic investigations are not informative and a number of cases remain unexplained.

Here, we report on two children presenting with liver disease in whom first investigations suggested MRCD, due to decreased liver respiratory chain activities and decreased mitochondrial DNA copy number. However, sequencing of the genes known to be associated with mitochondrial DNA instability did not identify any pathogenic mutations. Further investigations including exome analysis, biliary bile salt analysis, and/or BSEP immunostaining detected a defect in the bile salt export pump (BSEP). Diagnosis of progressive familial intrahepatic cholestasis type 2 (PFIC2), a hereditary disorder in bile formation due to BSEP deficiency was confirmed by *ABCB11* gene sequencing. Deleterious mutations were identified in both patients: one harboring compound heterozygous mutations (p.Arg470*/c.1308 +2T>A) and the other homozygous nonsense mutation (p. Tyr354*). This report increases awareness of a possible secondary mitochondrial respiratory chain defect in the liver tissue associated with other underlying causes such as PFIC2.

List of Abbreviations

BSEP	Bile salt export pump
DGUOK	Deoxyguanosine kinase
GGT	Gamma-glutamyl transferase
MRCD	Mitochondrial respiratory chain defect
mt	Mitochondrial
PFIC2	Progressive familial intrahepatic cholestasis type 2
POLG	Polymerase gamma
UDCA	Ursodeoxycholic acid

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Introduction

Mitochondrial respiratory chain deficiencies may present as liver disease with cholestasis and/or liver failure within the first months of life (Molleston et al. 2013). There is no gold-standard test for mitochondrial respiratory chain defect (MRCD) diagnosis, which usually relies on establishing a pattern of evidence from clinical, biochemical, pathological, and radiological data (Cormier-Daire et al. 1997; Rustin et al. 1994). The primary origin of MRCD is likely when a specific genetic defect is identified, a situation that rarely occurs due to the huge number of candidate genes (Rustin et al. 1994; Gaignard et al. 2013; Molleston et al 2013). Recently, Molleston et al. (2013) established a tiered approach to guide the evaluation of suspected mitochondrial disease. Clinical presentation of MRCD is heterogeneous and several organs may be affected including liver, brain, muscle, and/or kidney (Cormier-Daire et al. 1997; Morris 1999). Isolated liver disease, observed in some cases, may represent an indication to liver transplantation, whereas multiorgan injury represents a contraindication (Dubern et al. 2001). We report here on two children in whom liver disease initially thought to be akin to MRCD was due to progressive familial intrahepatic cholestasis type 2 (PFIC2).

Case Reports

Patient 1 was born at term from healthy non-consanguineous parents and experienced physiological neonatal jaundice without discolored stools. She was referred to Bicêtre hospital at age 6 months for cholestatis, pruritus, hepatosplenomegaly, and growth delay since age 4 months. Serum liver tests showed increased bilirubin and bile salt levels, elevated transaminase activities, and slight increase of gamma-glutamyl transpeptidase (GGT) activity. Evidence for mild liver failure and increased alpha-fetoprotein level were observed. Normal lactate levels were found in blood and cerebrospinal fluid (Table 1). Liver ultrasonography showed signs of cirrhosis with portal hypertension and nodules and normal biliary tract. Liver histology revealed hepatocellular cholestasis, micronodular cirrhosis associated with giant hepatocytes. Cytoplasmic aspect of hepatocytes was eosinophilic (oncocytic) as seen in MRCD (Fig. 1), but no steatosis was observed. Neurological examination, brain MRI, and heart ultrasonography were normal. MRCD was suspected due to low respiratory chain complex levels and reduced copy number of mitochondrial DNA (mtDNA) in the liver (Table 2) and liver histology. Genetic investigations, including mtDNA analysis by long range PCR, *MPV17*, *TRMU*, deoxyguanosine kinase (*DGUOK*), and polymerase gamma (*POLG*) gene sequencing, failed to find any mutation. Liver function worsened, ursodeoxycholic acid (UDCA) therapy failed, and the child underwent a liver transplantation at 2 years for MRCD isolated to the liver. Bile was collected at this time and biliary lipid analysis revealed bile salt secretion deficiency (biliary bile salt 0.17 mmol/L, N>10), a sign suggestive of PFIC2. Immunostaining on explanted liver showed absence of BSEP (bile salt export pump) at the canalicular membrane, while canalicular MDR3 expression was normal (Fig. 1). All coding exons of *ABCB11* were amplified together with their respective exon/intron boundaries and sequenced. This analysis identified biallelic heterozygous mutations: a nonsense variation (c.1408 C>T; p.Arg470*) previously reported (Davit-Spraul et al. 2010) and a novel splice variation of exon 12 (c.1308+2T>A). In silico analysis of this latter variation predicted exon skipping due to disappearance of the donor splice site.

Patient 2 was born at term from healthy consanguineous parents and had intrauterine growth retardation. She presented with cholestatic jaundice from birth. She was referred to Bicêtre hospital at age 3 months for investigation of cholestasis. She had hepatosplenomegaly and growth delay. Liver tests showed hyperbilirubinemia, elevated serum transaminase activities, and slightly elevated serum GGT activity (Table 1). At 5 months of age, she had liver failure and development delay with axial and peripheral hypotonia. Blood tests showed severe lactic acidosis and increased alpha-fetoprotein level. She had severe hypoglycemia (Table 1), which remained unexplained despite appropriate investigations (data not shown). Heart ultrasound examination and brain MRI were normal. Ultrasonography showed an enlarged homogeneous liver. Liver histology showed giant hepatocytes, hepatocellular cholestasis, and panlobular fibrosis. No steatosis was observed. The neurological status led to the decision to contraindicate liver transplantation. UDCA therapy failed and the child died of liver failure at 7 months of age. Respiratory chain complexes showed low levels of complex I and III in liver biopsy and qPCR showed a reduced copy number of mtDNA (Table 2). MtDNA analysis by long range PCR excluded large deletion of mtDNA. At that time no other genetic investigation of MRCD was performed, but exome analysis was performed 10 years later in order to explain MRCD. This analysis revealed *ABCB11* defect, which was further confirmed using direct sequencing of *ABCB11* (homozygous mutation: c.1062T>A; p.Tyr354*).

In both children, other causes of cholestasis and/or liver failure known to present within the first year of live were excluded (Debray et al. 2001). Exon and exon-intron junctions of *POLG*, *DGUOK*, *MPV17*, *TRMU*, and *ABCB11 g*enes were sequenced as previously described as well as liver immunostaining analysis (Davit-Spraul et al. 2010; Gaignard et al. 2013).

Table 1 Biochemical investigations in the two patients with PFIC2

Patient age at investigation	Patient 1 6 months	Patient 2 3 months	Patient 2 5 months
Blood tests*			
Total/conjugated bilirubin (<17 μmol/L)	417/276	223/143	359/247
Bile salts (<15 μmol/L)	310	nd	nd
GGT (<45 IU/L)	69	49	59
Alanine aminotransferase (<40 IU/L)	634	2064	1041
Creatine phosphokinase (<260 IU/L)	70	nd	46
Alpha-fetoprotein[a] (<7 IU/mL)	117000	nd	13400
Prothrombin time[b] (>70 %)	55	100	49
Factor V (>80 %)	61	100	50
Albumin (>35 g/L)	39	nd	28
Fasting glucose (3.2–5.5 mmol/L)	3.8	nd	0.9
Lactate (0.5–1.7 mmol/L)	1.6	nd	8.0
Pyruvate (<0.15 mmol/L)	0.111	nd	0.300
Lactate/pyruvate ratio (8–18)	14	nd	27
Ammonium (<50 μmol/L)	58	nd	69
Cerebrospinal fluid analysis*			
Lactate (0.6–1.9 mmol/L)	1.3	nd	3.8
Pyruvate (<0.15 mmol/L)	nd	nd	0.18
Lactate/pyruvate ratio (8–18)	nd	nd	21

nd not determined

*Normal values are given in parenthesis

[a] Values after intravenous injection of vitamin K. GGT, gamma-glutamyl transferase; normal GGT range for age: 1 month (10 to 270 IU/L), 2 months (10 to 160 IU/L), 4 months (7 to 100 IU/L), >5 months (5 to 45 IU/L)

[b] Normal alpha-fetoprotein range for age: 1 month (<5,600 IU/mL), 3 months (< 180 IU/mL), 5 months (<70 IU/mL), 6 months (<20 IU/mL), > 6 months (<7 IU/mL)

Discussion

In most circumstances, the diagnosis of MRCD affecting the liver is based on the analysis of mitochondrial respiratory chain complex activities measured on liver tissue (Cormier-Daire et al. 1997; Rustin et al. 1994). Experimentally it has been shown that cholestasis and cirrhosis may affect the function of mitochondrial respiratory chain complexes (Krähenbühl et al. 1994, 1995). Therefore, in case of cholestasis and/or liver failure, there is a risk to overdiagnose liver MRCD, the decrease in mitochondrial respiratory chain complex activities and/or in mtDNA copy number being secondary to liver injury. The data reported here in two children show that secondary MRCD may be due to liver disease such as PFIC2.

Since cases of MRCD affecting the liver have been previously described, it was difficult to not consider MRCD in the two patients reported here, all the more that both had additional signs that could evoke MRCD (Cormier-Daire et al. 1997; Dubern et al. 2001). These findings described below, together with the decrease of liver mitochondrial respiratory chain complex activity and of mtDNA copy number, have led to a wrong diagnosis of MRCD. Patient 1 had an oncocytic aspect of hepatocytes, a finding not reported so far in PFIC2 and described in MRCD (Morris 1999; Davit-Spraul et al. 2010). Analysis of biliary bile acids performed at the time of liver transplantation has suggested PFIC2, biliary bile acid concentration being <1 mmol/L (Davit-Spraul et al. 2010). This diagnosis was confirmed by BSEP immunostaining and *ABCB11* sequencing. Patient 2 had intrauterine growth retardation and development delay with hypotonia, hypoglycemia, and elevated blood and cerebrospinal fluid lactate/pyruvate ratios – findings not reported so far in PFIC2 and described in MRCD (Cormier-Daire et al. 1997; Dubern et al. 2001; Davit-Spraul et al. 2010). Exome analysis performed to elucidate the genetic basis of MRCD established PFIC2 diagnosis. It is very likely that in both children, signs evocative of MRCD were due to a secondary dysfunction of mitochondrial respiratory chain induced by primary liver injury of PFIC2. Indeed, both patients presented with a moderate reduction of mtDNA copy number (20–30%), which retrospectively is likely due to a secondary MRCD rather than a primary genetic defect. The presence of mtDNA depletion syndrome with liver mtDNA copy number below 10% is highly suggestive of a primary genetic defect (Molleston et al. 2013, Sarzi et al. 2007). Nevertheless, in patients exhibiting mtDNA depletion syndrome with a less pronounced decrease in liver mtDNA copy number (10–40 %), a primary defect cannot be

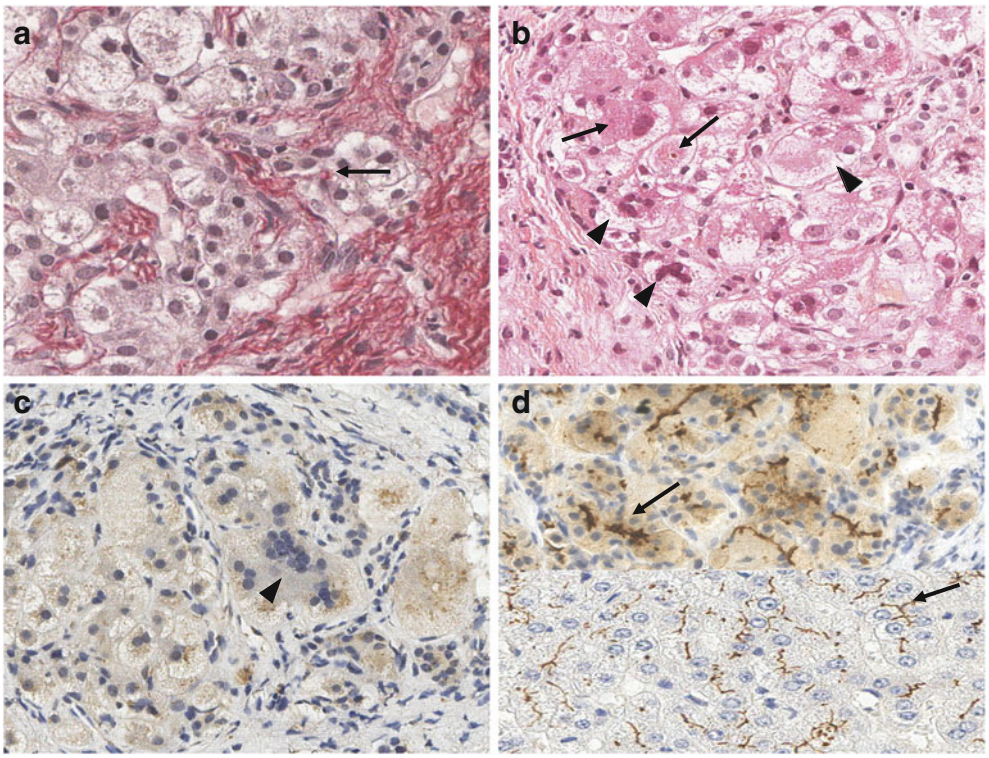

Fig. 1 Liver histology and liver immunostaining studies in patient 1. (**a**) Patient: Picro Sirius staining, x40. Presence of hepatocellular cholestasis with a canalicular bile plug (*arrow*) and of lobular fibrosis. (**b**) Patient: Hematoxylin and eosin (HES), x40. Note the oncocytic aspect of hepatocytes (*arrow*). Presence of multinucleated and giant hepatocytes (*arrowheads*, also in panel C). (**c**) Patient: BSEP immunostaining, x40. Absence of BSEP canalicular expression. (**d**) *Upper panel*: Patient, MDR3 immunostaining, x40. MDR3 is expressed at the canalicular membrane (*arrow*). *Lower panel*: Normal human liver, BSEP immunostaining, x40. BSEP is expressed at the canalicular membrane (*arrow*)

Table 2 Enzyme activities of mitochondrial respiratory chain complexes and molecular analysis of mitochondrial DNA in the liver of the two patients with PFIC2

	Respiratory chain activities in liver biopsy[a]		
	P1	P2	Control values
Complex activities(nmol/min/mg proteins)			
Complex I	5	5	19–26
Complex II	83	75	168–277
Complex III	73	114	143–192
Complex I + III	29	11	41–84
Complex II + III	31	40	66–103
Complex IV	102	216	202–319
Citrate synthase	65	58	63–131
Complex IV/CS	1.6	3.7	2.5–3.3
Complex IV/II	1.2	2.9	0.9–2.1
Complex IV/III	1.4	1.9	1.0–1.8
Complex IV/I	20.4	43.2	6.0–10.0
Molecular analysis			
Mt DNA long range PCR	Normal	Normal	Normal
Mt DNA copy number	27%	30%	100%

[a] Surgical liver biopsies were performed in patients 1 and 2, at 6 and 5 months of age, respectively, when patients had liver failure (Table 1). *Mt DNA* mitochondrial DNA, *CS* citrate synthase. Mitochondrial enzymatic activities of respiratory chain complexes (I, II, III, and IV) and mitochondrial enzyme marker (citrate synthase) were measured in liver biopsies (2). Mt DNA was analyzed by long range PCR and mt DNA copy number was measured in liver biopsies by quantitative PCR based on the ratio of mt DNA to nuclear DNA (*MTND2*/*ATP5B*), according to Chabi et al. 2003

excluded. In such situation, a secondary defect can also be observed (Helbling et al. 2013). Previous studies reported bile acid toxicity on oxidative mitochondrial metabolism affecting complex II, III, and IV activities (Krähenbühl et al. 1994, 1995). Thus, cholestasis could be responsible for and explained the reduced mitochondrial respiratory chain complex activities observed in our patients. Also, the reduced mtDNA copy number seen in our patients might be due, at least in part, to liver failure as it has also been shown in other patients undergoing liver transplantation for acute liver disease (Helbling et al. 2013).

The data presented here show that in presence of signs of MRCD in a child with cholestasis and liver failure, a PFIC2 should be considered. This, all the more that routine blood biological markers (increased alanine aminotransferase > fivefold normal, increased alpha-fetoprotein for age, normal or slightly elevated GGT, increased bile acids), pruritus, liver histology (giant hepatocytes, hepatocellular cholestasis), may suggest PFIC2 and that the proof for MRCD remains uncertain (Davit-Spraul et al. 2010). Otherwise, there is a risk to overdiagnose MRCD and to wrongly contraindicate liver transplantation in case of extrahepatic involvement, such as a neurological injury, potentially reversible and not due to primary MRCD. Another risk is to overdiagnose PFIC2 in case of MRCD isolated to the liver. This risk has minimal consequences since a good outcome is expected after liver transplantation, while the appearance of a delayed neurological involvement cannot be formally excluded (Dubern et al. 2001). Currently, in such situation, BSEP liver immunostaining and *ABCB11* sequencing should be performed first and may help to differentiate between both entities.

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One-Sentence Take-Home Message

Hepatic mitochondrial respiratory chain defect may be secondary to severe cholestasis and liver failure.

Contribution of Individual Authors

Anne SPRAUL: identification of patient defect (genetic analysis), manuscript writing

Marine BEINAT: identification of patient defect (exome analysis)

Dominique DEBRAY: diagnosis and follow-up of patient 2, revising manuscript

Agnes ROTIG: identification of patient defect (exome analysis), revising manuscript

Abdelhamid SLAMA: biochemical and genetic analysis of MRCD, revising manuscript

Emmanuel JACQUEMIN: diagnosis and follow-up of patient 1, manuscript writing and supervision

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RESEARCH REPORT

Newborn Screening for Hunter Disease: A Small-Scale Feasibility Study

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Abstract Hunter disease (Mucopolysaccharidosis type II, MPS II) is an X-linked lysosomal storage disorder caused by deficiency of iduronate-2-sulfatase (IDS). Two main therapies have been reported for MPS II patients: enzyme-replacement therapy (ERT) and hematopoietic stem-cell transplantation (HSCT). Both treatment modalities have been shown to improve some symptoms, but the results with regard to cognitive functioning have been poor. Early initiation of therapy, i.e., before neurological symptoms have manifested, may alter cognitive outcome. The need for early identification makes Hunter disease a candidate for newborn screening (NBS). Our objective was to explore the use of a fluorometric assay that could be applicable for high-throughput analysis of IDS activity in dried blood spots (DBS). The median IDS activity in DBS samples from 1,426 newborns was 377 pmol/punch/17 h (range 78–1111). The IDS activity in one sample was repeatedly under the cutoff value (set at 20% of the median value), which would imply a recall rate of 0.07%. A sample from a clinically diagnosed MPS II individual, included in each 96-well test plate, had IDS activities well below the 20% cutoff value. Coefficients of variation in quality control samples with low, medium, and high IDS activities (190, 304, and 430 pmol/punch/17 h, respectively) were 12% to 16%. This small-scale pilot study shows that newborn screening for Hunter disease using a fluorometric assay in DBS is technically feasible with a fairly low recall rate. NBS may allow for identification of infants with Hunter disease before clinical symptoms become evident enabling early intervention.

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Abbreviations

4MU	4-methylumbelliferrone
BMT	Bone marrow transplantation
DBS	Dried blood spot
ERT	Enzyme-replacement therapy
HSCT	Hematopoietic stem-cell transplantation
IDS	Iduronate-2-sulfatase
MPS	Mucopolysaccharidosis
NBS	Newborn screening

Introduction

Hunter disease (Mucopolysaccharidosis type II, MPS II, OMIM 309900) is an X-linked lysosomal storage disorder caused by deficiency of iduronate-2-sulfatase (IDS) (Wraith et al. 2008). IDS deficiency results in accumulation of

dermatan sulfate and heparan sulfate in various tissues and elevated levels in urine. Like many other lysosomal storage disorders, MPS II is clinically heterogeneous with a spectrum of disease severity ranging from childhood to adult presentation. Typical clinical features include coarse facies, dysostosis multiplex, joint stiffness/contractures, obstructive and restrictive airway disease, recurrent infections, hepatosplenomegaly, umbilical/inguinal hernias, and cardiac valve disease (Burton and Giugliani 2012). Children with severe MPS II also suffer from mental retardation, while patients with a more attenuated form usually have normal intelligence (Holt et al. 2011).

Two modes of therapeutic interventions are applied in MPS II: enzyme-replacement therapy (ERT) and bone marrow or hematopoietic stem-cell transplantation (BMT/HSCT). ERT has been shown to improve a number of symptoms (Muenzer et al. 2012; Wraith et al. 2008; Scarpa et al. 2011), but does not prevent mental retardation since the intravenously administered ERT does not cross the blood–brain barrier. HSCT has the potential advantage that transplanted cells are able to cross the blood–brain barrier and to differentiate into microglia providing an IDS enzyme source in the CNS. Unfortunately, the results of BMT/HSCT in MPS II have been variable (Vellodi et al. 1999; Krivit 2004; Guffon et al. 2009; Scarpa et al. 2011; Tanaka et al. 2012). As reported for ERT, positive effects included resolution of hepatosplenomegaly and stabilization of cardiac abnormalities, but neurological function in patients with the severe disease phenotype did not improve (Guffon et al. 2009). However, these data are limited to BMT performed in patients that already had cognitive decline. The possibility remains that early intervention by HSCT (e.g., before the age of 1 year) may change disease progression with respect to neurological symptoms. Early HSCT of patients with Hunter syndrome has not been reported to date. However, for other lysosomal storage disorders the benefits of early treatment have been shown, including Hurler disease (Boelens et al. 2013). Early intervention requires identification of patients at a very early age, before clinical features have become evident, i.e., by newborn screening (NBS) (Marsden and Levy 2010; Zhou et al. 2011). NBS has been reported for a number of lysosomal storage disorders including Pompe disease (Chien et al. 2008; Dajnoki et al. 2008), Fabry disease (Dajnoki et al. 2010), and Krabbe disease (Orsini et al. 2009), but not for MPS II. Enzymatic assays for measuring the IDS activity in dried blood spots have been described in fluorometric (Oemardien et al. 2011; Tolun et al. 2012) and in tandem mass spectrometric (Wolfe et al. 2011) platforms. In these studies small numbers of samples (<100) were used to show that the assays could be performed successfully, but to date analysis of IDS activity in large number of DBS samples has not yet been reported. Our objective was to investigate the suitability of the 4MU-based fluorometric assay for a routine diagnostic laboratory setting using microplate technology with a relatively large number of 1,426 DBS samples.

Materials and Methods

DBS Samples

Anonymous DBS samples, residues from routine newborn screening, were obtained from the National Institute for Public Health and the Environment (RIVM). To obtain a representative selection of the Dutch population, random samples were taken from all Dutch provinces in numbers proportional to the number of newborns per province. DBS were thoroughly air dried and stored at 4°C in sealed plastic bags. The samples were 2–3 years old at the time the enzyme assays were performed. Disease-positive DBS were obtained from a clinically diagnosed MPS II individual. Quality control DBS, manufactured to contain low (QC low), medium (QC medium), and high (QC high) lysosomal enzyme activities, were provided by Dr. Hui Zhou from the Centers for Disease Control and Prevention, Atlanta, Georgia (De Jesus et al. 2009).

This study was approved by the Erasmus University Medical Center Institutional Review Board.

Enzyme Assay

Iduronate-2-sulfatase (IDS) activities were determined essentially as described previously (Voznyi et al. 2001; Civallero et al. 2006; Oemardien et al. 2011), but using 96-well microplates instead of reaction vials. Briefly, a 3.2 mm DBS punch was placed in a microplate well and incubated for 17 h with 40 µL reaction mixture (1.25 mmol/L 4-methylumbelliferryl-α-L-iduronate 2-sulfate (Moscerdam, Oegstgeest, The Netherlands), 10 mmol/L lead acetate, 100 mmol/L sodium acetate pH 5), and 20 µL of a solution containing 1 µg/mL recombinant human α-L-iduronidase (Tolun et al. 2012) (R&D Systems, commercial preparation diluted in water containing 0.2% BSA). Following 17-h incubation at 37°C, the plates were placed on ice and protein precipitation was performed by addition of 30 µL 16% trichloroacetic acid. After 10 min, the plates were centrifuged for 5 min at 1,500*g* and 4°C. Subsequently, 60 µL supernatant from each well was transferred to the corresponding well of a 96-well Optiplate (Perkin Elmer) using a Microlab Star liquid handling workstation (Hamilton), and 200 µL sodium carbonate buffer (pH 10.7) containing 0.25% Triton X-100 was added to enhance the fluorescence of the reaction product 4-methylumbelliferone (4MU).

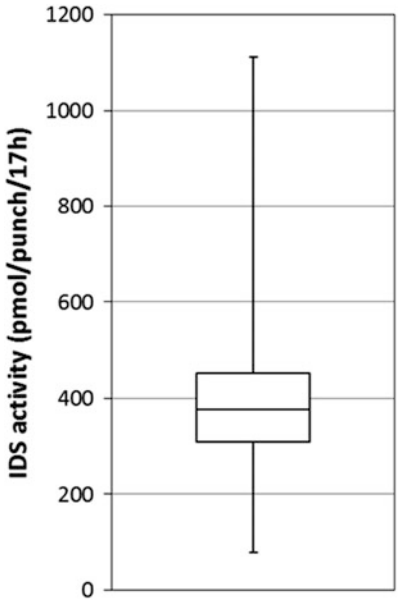

Fig. 1 Iduronate-2-sulfatase activities in anonymous newborn screening filter cards ($n = 1426$). The *box* represents those results within the 25th to 75th percentiles, the *error bars* show the range of enzyme activities and the horizontal line the median

Following mixing, fluorescence intensity was measured with a fluorometer (Varioskan, Thermo Electron Corporation) at an excitation wavelength of 365 nm and an emission wavelength of 448 nm. Each 96-wel plate included 90 unknown samples, two blanks (blank filter paper punch), one MPS II patient sample, and one of the three different QC samples. The last two wells were used for a calibrator (60 μL of 12.5 μmol/L 4-methylumbelliferone), which was added after the 17-h incubation and included in the subsequent sample processing steps. IDS activities were calculated by subtracting the blank value and converting fluorescence readings to pmol 4MU per 3.2 mm punch per 17 hours (pmol/punch/17 h).

Results and Discussion

IDS activity was determined in 1,426 DBS from newborns. The median activity was 377 pmol/punch/17 h (Fig. 1) (range 78–1111, average 388, SD 118). For each 96-well plate, specimens with an activity less than 20% of the plate median value (in total 7 of the 1,426 samples; 0.5%) were retested using a second punch from the original blood spot card. Six of the seven originally positive samples had normal IDS activity in the second testing. The false-positive outcomes must have been caused by erroneous sample or liquid handling. Inclusion of a second enzyme assay in the first testing could have allowed discriminating between these two possibilities, but would have required blood-spot extraction and sample splitting before substrate addition. The IDS activity in one of the seven originally positive samples remained below the cutoff value upon retesting. Since the NBS cards used in our study were de-identified and not traceable to a person, we could not confirm the low IDS activity in a fresh blood sample from this newborn. A disease-positive control sample (i.e., from an MPS II individual) was blindly loaded at different positions in each of the 17 microplates that were used to analyze the 1,426 samples. All these MPS II samples had IDS activity well below the 20% cutoff value (Fig. 2; mean activity −21 pmol/punch/17 h). The negative value is due to the method we used to calculate the activity, which includes subtraction of the blank.

Intra-assay variation was 11% (mean value of two different samples). QC samples provided by the CDC had the following mean IDS activities in pmol/punch/17 h: QC low 190 (SD 23; $n = 5$), QC medium 304 (SD 41; $n = 6$), and QC high 430 (SD 69; $n = 5$). Inter-assay variation coefficients calculated from these values were 12% to 16%. The QC low and QC high specimen clearly had distinct values, but a relatively high activity was recorded for the QC low specimen. In contrast, other lysosomal enzymes, such as α-glucosidase, α-galactosidase, and α-L-iduronidase, had very low activities in the QC low sample compared to QC medium and QC high in our laboratory (results to be published elsewhere), as well as other laboratories (De Jesus et al. 2009). We do not have an obvious explanation for high IDS activity in the QC low sample, but this may be accounted for by the DBS preparation procedure, which includes mixing of several blood fractions, including serum, and the notion that

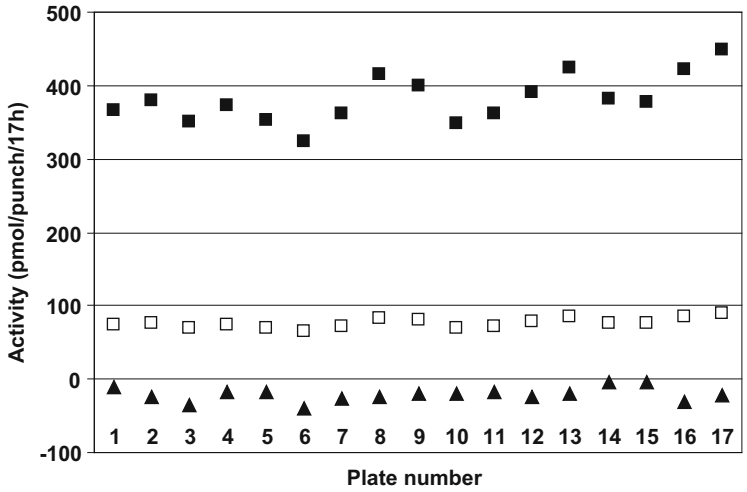

Fig. 2 Iduronate-2-sulfatase activities in anonymous newborn screening filter cards ($n = 1426$) and DBS obtained from an established MPS II patient. Seventeen microplates were required to test all samples. On the horizontal axis, the microplate number is indicated. On the vertical axis, the following IDS activities are depicted: the median IDS activity calculated for 90 newborn screening DBS samples included in each plate (■), the value corresponding to 20% of the plate median activity (□, used as the cutoff value to identify samples that were analyzed a second time), and the value obtained for an MPS II sample included in each plate (▲)

considerable IDS activity is present in plasma. IDS activity was stable for at least 2 months in DBS stored at 4°C. After 2 months of storage, IDS activities in the three different QC samples were 86% to 103% of the original value confirming previous results (Tolun et al. 2012).

Importantly, in our study only one infant would be recalled for confirmation in a second sample. Based on this result, the recall rate would be 0.07%. Although this number remains to be confirmed by larger studies, it is comparable to the results obtained in studies for other lysosomal storage disorders: Pompe disease (0.039%) (Dajnoki et al. 2008) and Krabbe disease (0.06%) (Orsini et al. 2009). A higher recall rate (0.82%) was reported by Chien et al. for Pompe NBS in Taiwan, but this likely results from a common α-glucosidase variant with low enzyme activity present in the Asian population (Chien et al. 2008). After several more years' experience with implementation of NBS for Pompe in Taiwan, the screening algorithm has been recalculated to achieve a recall rate of 0.009% (41 of 473,738 infants, calculated by us on the basis of the study by Chiang et al. (2012)). Our study did not allow estimation of a false-negative rate.

The fluorometry-based method is easily implemented in a routine diagnostic laboratory, but has as disadvantage that the 4MU-based enzyme assay used to determine IDS activity is rather expensive due to the high substrate cost (5 US$ per sample). Downscaling the assay volume, for instance, by employing digital microfluidics (DMF) as recently described (Sista et al. 2011), might reduce the costs, although the cost structure using disposable cartridges is not yet clear.

Conclusion

This small-scale pilot study shows that newborn screening for Hunter disease using a fluorometric assay in DBS is feasible and has a fairly low recall rate. The assay is ideally performed overnight, requires 1–2-h hands-on time after punching, and gives a result within 24 h of sample receipt. NBS may allow for identification of infants with Hunter disease before clinical symptoms become evident enabling early intervention by, e.g., ERT or HSCT.

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Conflict of Interest

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AMB, JvdB, ATvdP, LHE, and AJReuser declare that they have no conflict of interest.

Informed Consent

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

This study was approved by the Erasmus University Medical Center Institutional Review Board.

Details of the Contributions of Individual Authors

G. Ruijter, A. van der Ploeg, S. Weinreich, and A. Reuser contributed to the planning, conduct, and reporting of the work described in the article.

D. Goudriaan, A. Boer, J van den Bosch, and L. Elvers contributed to the conduct and reporting of the work described in the article.

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CASE REPORT

Abnormalities in Glycogen Metabolism in a Patient with Alpers' Syndrome Presenting with Hypoglycemia

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Abstract Intermittent hypoglycemia has been described in association with Alpers' syndrome, a disorder caused by mutations in the mitochondrial DNA polymerase gamma gene. In some patients hypoglycemia may define the initial disease presentation well before the onset of the classical Alpers' triad of psychomotor retardation, intractable seizures, and liver failure. Correlating with the genotype, *POLG* pathogenicity is a result of increased mitochondrial DNA mutability, and mitochondrial DNA depletion resulting in energy deficient states. Hypoglycemia therefore could be secondary to any metabolic pathway affected by ATP deficiency. Although it has been speculated that hypoglycemia is due to secondary fatty acid oxidation defects or abnormal gluconeogenesis, the exact underlying etiology is still unclear. Here we present detailed studies on carbohydrate metabolism in an Alpers' patient who presented initially exclusively with intermittent episodes of hypoglycemia and ketosis. Our results do not support a defect in gluconeogenesis or fatty acid oxidation as the cause of hypoglycemia. In contrast, studies performed on liver biopsy suggested abnormal glycogenolysis. This is shown via decreased activities of glycogen brancher and debrancher enzymes with normal glycogen structure and increased glycogen on histology of the liver specimen. To our knowledge, this is the first report documenting abnormalities in glycogen metabolism in a patient with Alpers' syndrome.

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Introduction

The integrity of physiological regulation of blood glucose levels is of utmost importance to brain metabolism, with prolonged states of hypoglycemia ultimately leading to encephalopathy and death. Therefore, blood glucose levels are tightly controlled to remain within a range of 2.5 and 7.5 mmol/L (45–135 mg/dL) (Lonlay et al. 2004). Several metabolic pathways work in unison to avoid the hypoglycemic state. Mutations in genes involved in glycolysis and gluconeogenesis, glycogen synthesis and degradation, as well as fatty acid oxidation pathways and their hormonal regulators may all lead to primary hypoglycemia syndromes (Haymond 1989). To identify the defective pathway and ultimately the specific enzymatic block requires methodical analysis of various metabolic markers and tissues, which can be costly and time consuming (Mochel et al. 2005). However when hypoglycemia is a secondary finding of a known metabolic disorder, these detailed investigations are not usually performed. Mitochondrial disorders are prime candidates for secondary hypoglycemic syndromes, since their underlying energy debt may deem them unable to

supply pathways of glucose metabolism with sufficient ATP. The Stanford Mitophenome Database is a tool generated to assist clinicians in phenotype/genotype correlations for mitochondrial disease patients (http://www.mitophenome.org/). Close to one third of the 174 genes in the database list hypoglycemia as a phenotypic feature (Scharfe et al. 2009). Over a decade after the initial discovery of the disease correlation between progressive external ophthalmoplegia (PEO) and mutations in the mitochondrial DNA polymerase gamma, the *POLG* gene has emerged as one of the most common causes of mitochondrial disease (van Goethem et al. 2002; Nguyen et al. 2005; Wong et al. 2008). With close to 200 pathogenic mutations described to date, the gene is associated with a wide spectrum of phenotypes and may follow autosomal dominant as well as autosomal recessive patterns of inheritance (Tang et al. 2011; Stumpf et al. 2013). As the sole polymerase responsible for mtDNA replication and repair, abnormalities in *POLG* function lead to increased mtDNA mutability and mtDNA depletion (Del Bo et al. 2003; Naviaux and Nguyen 2004). Severity of mutations correlates with the actual disease presentation, and Alpers' disease is representative of the severe end of the phenotypic spectrum (Stumpf et al. 2013). Unlike most infants with Alpers' disease, which initially present with intractable seizures and eventually progress to liver failure, our patient was ascertained due to isolated intermittent hypoglycemia. Aggressive early investigations into the underlying etiology allowed insights into the disease progression of this *POLG* patient and led us to the finding of abnormal glycogen storage in the liver, with respective absent and severely decreased activities of glycogen debrancher (GDE) and brancher enzymes (GBE) prior to the development of frank liver disease. To our knowledge, absent GDE activity has not been previously described in Alpers' patients.

Case Report

The patient was delivered by cesarean section due to breech presentation; postnatal course was only significant for transient neonatal jaundice. At 4 months of age, she had two episodes of difficult arousal in the morning following uneventful nights, resulting in emergency room visits. Review of outside medical records showed that isolated glucosuria was present, without documented abnormal blood glucose values. This raised the suspicion of a renal tubular defect, but repeated urine glucose and biochemical studies did not confirm this possibility. Neurological consultation, including EEG, was unrevealing. At 10 months of age, fasting morning blood glucose was 2.4 mmol/L while the patient was asymptomatic. This prompted admission to our hospital for further studies. Physical exam, including developmental milestones, was normal and there was no clinical or laboratory evidence of muscle or liver involvement. A fasting study was performed at night, after the patient had been feeding regularly during the day. After 8 h of fast, a finger stick blood glucose level was 2.7 mmol/L and venous blood samples for critical labs were obtained (Table 1).

Results were consistent with ketotic hypoglycemia. The normal lactic acid and low alanine suggested intact gluconeogenesis. The response of growth hormone and insulin at the time of hypoglycemia were appropriate. Although serum cortisol level was within the normal reference range, it was considered inappropriately low for a child with hypoglycemia. The initial diagnosis of hypocortisolism was made, and the patient was discharged on treatment with hydrocortisone and frequent feeds. MRI of the brain and pituitary gland was normal. Two days before her first birthday, the patient had early morning lethargy, with a blood sugar of 1.7 mmol/L. She had missed her 2 am feed which resulted in an 11-h fast. The patient fully recovered after formula and orange juice were given. On the day of her birthday, she was asymptomatic and medications and meals were given as scheduled. However in the middle of the night, the patient had a seizure. Blood sugar was normal at 96 mg/dL and she was taken to the emergency room where repeat blood sugar was 3.7 mmol/L. She was admitted to intensive care unit due to lethargy, seizures, and apnea requiring intubation and mechanical ventilation. Sepsis work up and ammonia were normal, cortisol (after stress dose in ER) was high at 5,904 nmol/L (214 μg/dL), and comprehensive metabolic panel was normal, except for elevated AST and ALT of 173 and 141 U/L, respectively.

Due to hypoglycemia while on steroids and mildly elevated LFTs, repeat metabolic testing was obtained. Organic acids once more showed ketosis, whereas lactic acid, acylcarnitines, and CK were normal and alanine was again low at 163 umol/L. MRI of brain (2nd) showed bilateral symmetric diffusion abnormalities in both occipital lobes (Fig. 1 #2) and subtle T2 prolongation in the same areas, which, in view of the normal MRI two weeks prior, was interpreted to be secondary to the recent hypoglycemic episode. A new fasting study was done while on hydrocortisone, after the patient was on frequent feedings for a few days (Table 2). The patient developed again hypoglycemia and increased ketone body production; however, postprandial samples revealed high blood sugar and lactic acid. Glycogen storage disease (GSD) type 0 was suspected, but no mutation was found after full sequencing of *GYS2* (Prevention Genetics, Marshfield, WI). The patient did not have further hypoglycemia or seizures while on scheduled feeds and steroid therapy. Repeat MRI of brain (3rd, 18 days following 2nd MRI) showed resolution of previous diffusion abnormalities, but persistent abnormal T2 prolongation in the medial aspects of both occipital lobes

Table 1 Fasting study #1

	Glucose *(3.6–6.1 mmol/L)*	CO2 *(15–28 mmol/L)*	ALT *(10–32 U/L)*	Cortisol *(77.3–634.6 nmol/L)*	GH*(2–10 ng/mL)*	Insulin *(0–90.3 pmol/L)*	Lactic acid *(0.5–2.2 mmol/L)*	β-OH-Butyrate *(0.05–0.19 mmol/L)*	Alanine *(246–486 μmol/L)*	CK *(24–206 U/L)*
Results	**1.5 mmol/L**	21 mmol/L	**151 U/L**	331.08 nmol/L	**29 ng/mL**	<6.95 pmol/L	1.6 mmol/L	**1.32 mmol/L**	**175μmol/L**	36U/L

All reference laboratory ranges are in parentheses. All out of range values are in bold. Reference laboratory ranges were developed for the normal population with normal blood glucose values.

Abbreviations: *ALT* Alanine aminotransferase, *GH* Growth hormone, *CK* Creatine kinase

Age 10 months: post 8-h fast. Organic acid: significant ketosis, mild increase in dicarboxylic acids. Acylcarnitine: slight elevations of C2 and several other acylcarnitines from C12:1 to C18:1 with no diagnostic pattern

was interpreted as evolution of prior insult. By the time of discharge (20 days stay), the patient had returned to baseline except for cortical visual impairment with expected resolution given improved MRI findings and normal visual evoked potentials. Two months later, the patient had new seizures and developed respiratory failure despite being compliant with every 4-h feeding regimen and hydrocortisone treatment. Blood sugar was normal at home as well as in the emergency room. MRI of the brain (4th) showed further improvement of T2 prolongation in occipital lobes without new lesions. The patient recovered fully within a few days and was playful and interactive. No hypoglycemia was documented during this admission, but lactic acid and ALT were persistently elevated and were as high as 17.7 mmol/L (0.5–2.2 mmol/L) and 103U/L (10–32 U/L), respectively. A muscle biopsy was ordered, and skin and liver biopsies were obtained at 15 months of age. Muscle biopsy was studied on cryostat sections by H&E, modified trichrome stain, ATPase, NADH-TR, PAS, PAS diastase, Oil red 0, phosphorylase A, B kinase, cytochrome oxidase, and acid phosphatase (Bonilla et al. 1992). Results showed <1 % COX negative fibers, but were otherwise normal (no ragged red fibers). Muscle electron microscopy also showed normal results (Long Beach Memorial Medical Center). Electron transport chain studies performed on muscle were at the low end of the control range for NADH cytochrome C reductase (complex I/III) and cytochrome c oxidase (complex IV) (CIDEM, Cleveland, OH). An in vitro fatty acid oxidation probe assay performed in skin fibroblasts showed normal results (Mayo Clinic, Rochester MN) (Blau et al. 2008).

Liver pathology showed relatively uniform nodules divided by fibrous bands with ductular proliferation. The majority of the hepatocytes exhibited cytoplasmic fine vacuolization containing abundant glycogen (confirmed by PAS with and without diastase). The overall histology was reported as consistent with a glycogen storage disease. Liver enzyme activities and glycogen content were measured on frozen liver biopsy tissue using standard spectrophotometric methods that have already been published (Wapnir 1985; Brown and Brown 1989). The residual enzyme activity was measured indirectly by measuring the amount of glucose or phosphate released using glucose or phosphate reagent. Liver glycogen content was borderline elevated at 7.1 % (normal 3.3 ± 1.7); glucose-6-phosphatase, glycogen phosphorylase, and glycogen phosphorylase kinase activities were all repeatedly normal with a G-1P/Glu ratio of 50 % indicating normal glycogen structure. Branching enzyme activity was low at 20 um/min/g of tissue (normal 85 ± 31), and glycogen debrancher enzyme (GDE) activity assessed in liver tissue was undetectable (performed twice at Duke University Medical Center).

Sequence of the debrancher (*AGL*) gene was normal (Prevention Genetics, Marshfield, WI).

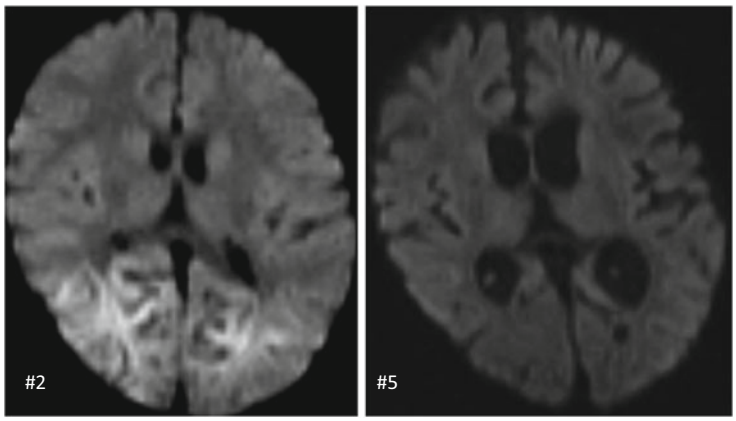

Fig. 1 Diffusion-weighted images (MRI #2 and #5). MRI #2 shows bilateral symmetric diffusion abnormalities in both occipital lobes. MRI #5 shows brain atrophy with periventricular and subcortical white matter signal abnormality and full resolution of occipital lesions

Table 2 Fasting study #2

Fasting time	4 h	5 h	6 h	7 h	8 h	8.5 h	9 h	9.5 h	1 h*	2 h*	3 h*
Glucose (3.6–6.1 mmol/L)	5.2	5.8	4.2	**3.3**	**3.4**	4.9	3.9	**3.1**	**8.9**	6.8	6.7
Alanine (246–486 μmol/L)								281			
Lactate (0.5–2.2 mmol/L)								2	**3.3**	**4.3**	**4.3**
β-OH-Butyrate(0.05–0.19 mmol/L)								**0.64**	**0.26**	0.18	**0.21**
Cortisol (77.3–634.6 nmol/L)			129.7**								
FFA (0.5–0.9 mmol/L)								**1.2**			

All reference laboratory ranges are in parentheses. All out of range values are in bold. Reference laboratory ranges were developed for the normal population with normal blood glucose values.

Abbreviations: *FFA* Free fatty acids, β-*OH-Butyrate* Beta-hydroxybutyrate

**Although cortisol value is within normal range it is considered low for a child with hypoglycemia

*Postprandial studies; all specimens are from central line

Age 24 months: during hydrocortisone treatment + after frequent feedings for +2 days

Two weeks after discharge from her third hospitalization, the patient presented with refractory myoclonic seizures. MRI of brain (5th, 1 month apart from 4th) revealed, for the first time, mild brain atrophy with periventricular and subcortical white matter signal abnormality and full resolution of the occipital lesions (Fig. 1 #5). The patient progressed rapidly to encephalopathy, lactic acidosis, and hepatic failure. *POLG* sequencing revealed compound heterozygous mutations c.1399G>A, p.[A467T]+c.2542G>A, p.[G848S], as underlying cause of the disease.

Discussion

Since the initial discovery of the causal relationship between *POLG* mutations and Alpers' syndrome, an enormous body of literature elucidating this disease mechanism has accumulated. Large studies exploring phenotype/genotype correlations are mostly focused on the primary clinical features involving liver pathology, encephalopathy, neuropathy, and dysphagia (Horvath et al. 2006; Wong et al. 2008; Tang et al. 2011). Although multiple reports have associated hypoglycemia with Alpers' disease, the underlying etiologies have not been delineated, or remain speculative (Naviaux et al. 1999; Ferrari et al. 2005; Nguyen et al. 2005; Bortot et al. 2009). In a recent article by Scalais et al., an Alpers' patient with the same p.[A467T]+p.[G848S] genotype presented with hypoglycemia and hyperlactatemia as the prominent initial findings. In contrast to our patient who initially presented with isolated ketotic hypoglycemia, normal lactic acid level, and normal physical exam at 10 months of age, this infant had already developed severe liver dysfunction at 3.5 months of age with jaundice, ascites, and hepatomegaly. A liver biopsy at ~9 months of age showed micronodular cirrhosis and bile ductular proliferation (Scalais et al. 2012). At 18 months a second liver biopsy showed Kupffer cells with PAS positive large granules and glycogen rosettes, a finding usually considered secondary to glycogen storage

(Roels et al. 2009). Enzymatic studies for glucose 6-phosphatase activities were normal in both biopsy samples and fructose 1 phosphate aldolase/fructose 1,6 bisphosphate activities were normal in the first biopsy. Unfortunately, there is no mention whether liver glycogen content or glycogen brancher and debrancher enzyme activities were measured. Interestingly the authors describe that their patient did not respond to glucagon, a response that could be consistent with GDE deficiency depending on the fasting time. Due to the many similarities between our patient and the one previously published (Roels et al. 2009; Scalais et al. 2012), one may speculate whether our findings are genotype specific. A search of the Human DNA Polymerase Gamma Mutation Database (http://tools.niehs.nih.gov/*polg*) for p.[A467T]+p.[G848S] revealed 18 additional published cases. Of these, only two publications mention hypoglycemia and neither of them address glycogen metabolism (Ferrari et al. 2005; Nguyen et al. 2005). The relatively high numbers of this particular genotype and the lack of prior pursuit of glycogen studies may encourage retrospective chart review and enzymatic analyses on stored liver samples for this patient population.

To further broaden our search for derangement in glycogen metabolism in mitochondrial disease, we took a close look at other mtDNA depletion syndromes which can be associated with hypoglycemia. Insights derived from studies on *MPV17*, a gene of unknown function leading to progressive liver failure in infancy, prove particularly interesting (Karadimas et al. 2006). Patients with mutations in *MPV17* have shown an exceptionally high rate of secondary hypoglycemia. In a recent publication on *MPV17* deficiency, Uusimaa reports on 12 different families with *MPV17*-related mtDNA depletion syndrome and 50 % of them had hypoglycemia (Uusimaa et al. 2013). Furthermore detailed metabolic studies on 2 *MPV17* patients revealed early fasting intolerance (3–4 h postprandially) with physiological parameters resembling glycogen synthesis and glycogenolysis defects. Unfortunately, enzymatic tests in liver elucidating the precise etiology of disrupted glycogen metabolism were not done in these patients (Parini et al. 2009). Interestingly a study exploring the potential function of *MPV17* via the yeast homologue Sym1 showed that Sym1 deficiency induces disturbed glycogen metabolism. The authors speculate that this finding may likely be secondary to abnormal levels of mitochondria-derived TCA cycle intermediates (Dallabona et al. 2010). Patients with mutations in another mtDNA depletion syndrome associated with hypoglycemia, deoxyguanosine kinase (*DGUOK*), also showed increased glycogen in liver tissues in multiple cases (Dimmock et al. 2008). A lack of enzymatic glycogen metabolism studies in this publication again did not allow further insights into the exact mechanisms of hypoglycemia secondary to liver failure.

The complete absence of GDE activity in our patient's liver sample was confirmed by repeat assay. Additionally control enzymes that run in parallel were normal, ruling out the possibility of technical problems. Since the *AGL* gene had been sequenced previously with normal result, it is possible that the absence of GDE activity was secondary to decreased mRNA and protein levels or due to decreased enzyme activity secondary to a damaged, misfolded protein. We were not able to address this question directly due to the lack of remaining liver sample. Changes in gene expression secondary to *POLG* mutations have been observed previously in fibroblast cell lines derived from *POLG* patients (Voets et al. 2012) as well as in the *POLG* mutator mouse skeletal muscle (Hiona et al. 2010). We accessed the mRNA data deposited in the gene expression omnibus repository (GEO http://www.ncbi.nlm.nih.gov/geo/) (GSE21189) and did not find evidence of altered mRNA levels for the glycogen debrancher enzyme in mouse skeletal muscle (Edgar 2002; Hiona et al. 2010; Barrett et al. 2012). Since the POLG mutator mouse is a model for premature aging rather than for Alpers' disease, we also reviewed another recently submitted GEO dataset by Falk et al.: "Transcriptome profiling in primary human mitochondrial respiratory disease" (GSE 42986). Here we compared mRNA data collected on human skeletal muscle from three mtDNA depletion syndrome patients (*POLG, MPV17, RRM2B*) to three control patients with abnormalities in the pyruvate dehydrogenase complex and normal mitochondrial electron transport. The comparison suggested robust and unaltered expression levels for the *AGL* gene, in human skeletal muscle (data not shown) which may not be representative of expression levels in the liver.

AGL is a large protein comprised of 1,532 amino acids (NP_000019.2). It is unusual insofar that it is a monomer with two active sites, catalyzing two different enzymatic reactions (4-alpha-glucotransferase and amylo-1,6-glucosidase) (Nakayama et al. 2001). Its large size and structure may deem the protein more vulnerable to the effects of free radical damage and low levels of ATP both of which have been observed in POLG-related phenotypes (Vries et al. 2007; Voets et al. 2012). Potential increased production of free radicals in our patient may have been further compounded by conditions of low glucose and hypoxia which may add to the unfolded protein response and free radical damage (Elanchezhian et al. 2012). Abnormalities in glycogen metabolism secondary to POLG mutations may also translate to other organs involved in glycogen metabolism. Particularly a closer look at astrocytes may prove to be interesting since it has recently been shown that polyglucosan polar bodies confined to this tissue are able to cause a mitochondrial disease phenocopy with leukodystrophy and diaphragmatic failure (Dainese et al. 2013) and that cultured astrocytes derived from mice deficient in glutathione activity and increased free radicals have altered glycogen metabolism (Lavoie et al. 2011).

Ultimately hypoglycemia associated with mitochondrial DNA depletion syndromes may have different etiologies depending on the function of the gene involved, the patient's specific genotype, mutations in modifier genes as well as disease stage, progression, and degree of liver involvement.

To our knowledge, this is the first report on a child with Alpers' syndrome in which the underlying cause for intermittent hypoglycemia has been linked to abnormal glycogen metabolism. This may not only carry significance for Alpers' patients or patients with other *POLG*-related syndromes but may hold interesting information for mitochondrial disease in general.

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Competing Interest

The authors have no competing interests.

Synopsis

Hypoglycemia associated with mitochondrial disease mimicking glycogen storage disease may be underrecognized or underreported and may manifest prior to the onset of typical mitochondrial disease symptoms like seizures or liver failure.

Compliance with Ethics Guidelines

Conflict of Interest

Mariella Simon declares that she has been partially supported by Hailey's wish foundation, a charitable foundation interested in supporting causes concerning mitochondrial disease.

Richard Chang declares that he has no conflict of interest.

Deeksha Bali declares that she has no conflict of interest.

Lee-Jun Wong declares that she has no conflict of interest.

Ying Peng declares that she has no conflict of interest.

Jose Abdenur declares that he has no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). The study does not qualify as human subject research since the patient is deceased and there are no subject identifiers.

Animal Rights

This article does not contain any studies with animal subjects performed by the any of the authors.

Contributions of Individual Authors

Mariella Simon wrote the abstract, introduction, and discussion.

Richard Chang wrote the case report and cared for the patient.

Deeksha Bali was in charge of enzyme studies for glycogen storage disease.

Lee-Jun Wong was in charge of POLG testing.

Ying Peng oversaw neurological testing and cared for the patient.

Jose E. Abdenur designed clinical and laboratory testing strategy and planned and edited the manuscript.

All authors have reviewed the final version of the manuscript.

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CASE REPORT

Assessment of Basal Metabolic Rate and Nutritional Status in Patients with Gaucher Disease Type III

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Abstract Gaucher disease type III (GD III) is a rare form of GD characterized by neurological involvement and severe systemic disease. The objective of this study was to assess the nutritional status and energy metabolism of patients with GD III. **Methods:** The basal metabolic rate (BMR, measured by indirect calorimetry) and anthropometric parameters (height, weight, body mass index (BMI), and arm circumference) of three patients with GD III (p.L444P/L444P genotype) were assessed at different time points. The clinical severity of GD was assessed by means of physical examination, laboratory tests, imaging findings, and the severity scores proposed by Zimran (SSI) and Davies (SSNI). **Results:** The measured BMR of patients 1 (age 14 years, not on enzyme replacement therapy (ERT), SSI score 33, SSNI score 14.5), 2 (age 17 years, on ERT, SSI score 33, SSNI score 16), and 3 (age 20 years, on ERT, SSI score 33, SSNI score 7.5) was, respectively, 47%, 72%, and 15% higher than that estimated by the Harris–Benedict equation. Patients with a more severe phenotype had more marked hypermetabolism. Patients 1 and 2 had BMI-for-age z scores of −1.09 and −1.39, respectively, and height-for-age z scores of −4.27 and −3.02, respectively; patient 3 had a BMI of 24.7 kg/m^2. **Conclusion:** All three patients showed hypermetabolism; however, the two patients with the highest BMR had more severe GD and were malnourished. Additional studies are warranted to assess whether hypermetabolism may be a biomarker of disease severity in GD.

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Abbreviations

BMR	Basal metabolic rate
ERT	Enzyme replacement therapy
GD	Gaucher disease

Introduction

Gaucher disease (GD, OMIM 230800) is the most prevalent lysosomal storage disorder. It is the result of mutations in the

GBA gene, which codes for the enzyme glucocerebrosidase (GBA, EC 3.2.1.45), located on chromosome 1q21.31. GBA deficiency leads to a progressive buildup of its substrate, glucocerebroside, which in turn accounts for the clinical manifestations of Gaucher disease (Cox-Brinkman et al. 2008). Three forms of GD have been described: a) type I, the most prevalent form, characterized by visceral, hematological, and bone disease, with absence of neurological involvement; b) type II, or acute neuronopathic GD, with very early onset of clinical manifestations and major neurological involvement; and c) type III (OMIM: 231000), subacute or chronic neuronopathic GD, which is similar to type II but less severe (Schiffmann and Vellodi 2007).

Type III GD can be further divided into subtypes according to the nature and extent of neurological involvement: some patients have more severe neurological manifestations with progressive myoclonus epilepsy and mild visceral involvement (type IIIA), whereas others have severe visceral disease and oculomotor apraxia as the sole sign of neurological involvement (type IIIB) (Patterson et al. 1993). A third variant (type IIIC), characterized by oculomotor apraxia, moderately severe visceral involvement, and progressive cardiovascular calcifications, has also been described (Abrahamov et al. 1995). As GD III is even more rare than GD I, few patients have been studied, particularly with respect to nutritional status (Bembi et al. 1994; Erikson et al. 1995; Tylki-Szymanska and Czartoryska 1999; Altarescu et al. 2001). Assessments of the basal metabolic rate (BMR) of patients with Gaucher disease type III were not found in the literature.

The objective of this study was to assess the nutritional status and energy metabolism of patients with GD III treated at a reference center for Gaucher disease management in Rio Grande do Sul, Brazil.

Methods

This study included all three patients (two male, one female) with GD III followed at the Rio Grande do Sul State Referral Center for Diagnosis, Monitoring and Treatment of Gaucher Disease (CRDG), Brazil.

The diagnosis of GD had previously been established by the usual clinical, enzymatic, and molecular parameters. Energy metabolism was assessed by indirect calorimetry, performed between 7 and 9 a.m., after patients had been instructed to fast for 12 hours. The protocol recommended to all patients was the following: 8 hours of sleep, a restriction of physical activity and caffeine base products for 24 hours; 30 minutes of rest before the assessment; transportation by car; and assessment of gases for 30 minutes, discarding the first 10 minutes. All three patients underwent assessment in the same season. BMR was measured with a MedGraphics CPX-D breath-by-breath cardiopulmonary exercise testing system, using manufacturer-recommended calibration and gas collection protocols, as well as the equation proposed by Weir (1949). Briefly, as the indirect calorimetry evaluates the respiratory quotient, there are 15–30 measurements per minute. Each patient was evaluated during 30 minutes, but the first 10 minutes were not taken into account, and the more stable period was chosen for analysis. Means were obtained after the exclusion of outliers. Results were converted into kcal/day and compared with BMR as estimated by the Harris–Benedict equation (Harris and Benedict 1919). Differences up to 10% between BMR and the values of Harris–Benedict equation were not considered clinically relevant because studies have reported that the reassessment of indirect calorimetry in healthy individuals can result in alterations by 3–5% when conducted after 24 hours or up to 10% when conducted after weeks or months (Compher et al. 2006).

Nutritional assessment was based on height-for-age and body mass index (BMI)-for-age z scores for the adolescent patients (1 and 2) and on BMI for the adult patient (3); arm circumference was measured in all three patients. The nutritional status was diagnosed in accordance with the World Health Organization guidelines (WHO 1995, 2006 and 2007) and arm circumference was measured as per Frisancho (1990). GD severity was assessed on the basis of the systemic involvement score proposed by Zimran et al. (1992) and the neurological severity score proposed by Davies et al. (2007). This study was approved by the Hospital de Clínicas de Porto Alegre Research Ethics Committee (project n. 08/204), and all patients (or their legal guardians) provided written informed consents for participation.

Results

All three patients present the p.L444P/L444P genotype, subtype 3b. A summary of the clinical profile of all three patients is shown in Table 1. Clinical, laboratory, and imaging findings were indicative of a more severe disease phenotype in patients 1 and 2 as compared to patient 3; this was corroborated by neurological examination. At the time of inclusion, patient 1 was untreated due to the development of allergic reactions to imiglucerase (Genzyme Corp., Allston, MA) after 10 years of enzyme replacement therapy (ERT) and taliglucerase alfa (Protalix, Carmiel, Israel) as well (Vairo et al. 2013). Patient 2 was on imiglucerase ERT (60 IU/kg/infusion), as was patient 3 (60 IU/kg/infusion until age 16 and 30 IU/kg/infusion thereafter). Patients 2 and 3 are siblings and had undergone splenectomy – at age 2.9 and 5.7, respectively – before starting ERT.

All patients had severe bone changes that hindered height measurement: Patient 1 had major thoracic kyphosis, thoracolumbar scoliosis, and coxa valga; Patient 2 had

Table 1 Summarized profile of patients with Gaucher disease type III included in this study

	Patient 1			
	Assessment 1	Assessment 2	Patient 2[a]	Patient 3[a]
Gender	Male		Male	Female
Genotype	p.L444P/L444P		p.L444P/L444P	p.L444P/ L444P
Age (years)	11.8	14.2	17	20
Age at start of treatment (years)	1.8[b]		2.9	5.7
Treatment duration (years)	—[c1]	—[c2]	14	14
Imiglucerase dose (IU/kg/infusion)	—	—	60	30[d]
Weight (kg)	26.1	29.7	42.7	56
Height (cm)	130.0	132.0	153.0	150.5
Body mass index (kg/m^2)	15.4	17.0	18.2	24.7
Splenectomy (Y/N)	No	No	Yes	Yes
Splenomegaly (Y/N)	Yes[e]	Yes[e]	—	—
Hepatomegaly (Y/N)	No	Yes[e]	No	No
Osteoporosis (Y/N)[f]	—	—	No	No
Respiratory involvement[g]	—	RLD + ILD	RLD + ILD	None
Respiratory rate (mean)	—	30	20	—
Hemoglobin (g/dL)	9.8	8.0	13.5	11.4
Platelets ($\times$ 10^3/mm^3)	165	56	454	437
Chitotriosidase (nmol/mL/h).VR(a)	9939	19878	10409	10938
Albumin (g/dL)	—	2.9	4.3	4.6
SSI – Severity score[h]	25	33	33	33
SSNI – Neurological severity score[i]	—	14.5	16	7.5
Basal metabolic rate (kcal/kg/day)	43	51	52	28

— not measured/unavailable

[a] Patients 2 and 3 are siblings

[b] Patient discontinued enzyme replacement therapy after 10 years of treatment due to a severe allergic reaction (Vairo et al. 2013)

[c] At the time of assessment, the patient had been off ERT for (1) 8 months; (2) 38 months

[d] 60 IU/kg/infusion until age 16 and 30 IU/kg/infusion thereafter

[e] Spleen and liver assessed by ultrasound (volume):

Spleen: 1 evaluation = 306.5 cm^3; 2 evaluation = 2484.5 cm^3

Liver: 1 evaluation = 2,169 cm^3; 2 evaluation = 5,370 cm^3

[f] Assessed by bone density scanning. Not performed in patient 1

[g] Characterized by restrictive lung disease (RLD, assessed by spirometry) and interstitial lung disease (ILD, assessed by computed tomography). Lung function tests (spirometry) of patient 1 showed mild, proportionate reduction of FVC and FEV1 and intact air flow at assessment 1, which had progressed to severe restrictive lung disease unresponsive to bronchodilator therapy by assessment 2

[h] Zimran et al. 1992

[i] Davies et al. 2007

kyphoscoliosis and lumbosacral hyperlordosis; and Patient 3 had thoracolumbar hyperkyphosis and wedge-shaped vertebrae due to a history of pathological fractures.

Basal Metabolic Rate

Patient 1 underwent two assessments: at month 8 and month 38 after treatment discontinuation. In these assessments, BMR was, respectively, 14% and 47% higher than that estimated by the Harris–Benedict equation, corresponding to 43 and 51 kcal/kg/day. Patients 2 and 3 had a BMR 72% and 15% higher than that estimated by the Harris–Benedict equation, corresponding to 52 and 28 kcal/kg/day, respectively.

Nutritional Status

Before ERT, the weight-for-age z scores of patients 1, 2, and 3 (at age 1.8, 2.9, and 5.7 years, respectively) were −2.28, −1.20, and −4.45. Patient 1 had a height-for-age z score of −4.11. At initial assessment, patient 1 had

BMI-for-age and height-for-age z scores of −1.16 and −2.54, respectively, versus −1.09 and −4.27, respectively, at the second assessment. Patient 2 had BMI-for-age and height-for-age z scores of −1.39 and −3.02, respectively, and patient 3 had a BMI of 24.7 kg/m^2 (height-for-age z score at age 19 years: −1.93). Arm circumference was below the 5th percentile in patients 1 and 2 and between the 50th and 75th percentiles in patient 3.

Discussion

The findings of this study showed that hypermetabolism is very frequent in GD type III, regardless of the current status of ERT. This was expected since many chronic diseases are associated to hypermetabolism (Schols 2003; Kao et al. 2011). Indeed, one patient had a BMR 70% higher than the estimated rate despite treatment, which may explain the malnutrition diagnosis. Patient 1, who was not on ERT, underwent repeated metabolic assessments, which showed a marked increase in BMR concomitantly with a deterioration in the clinical status: worsening blood cell counts, development of hepatomegaly, progression of splenomegaly from mild to severe, and deterioration of airflow from intact to severe restrictive respiratory disease (Table 1). The organomegaly identified on the physical examination and imaging tests was also reflected in an increase in BMI during the assessment period despite frank malnutrition.

The pulmonary manifestations present in these three patients are consistent with data available in the literature, which reports an increased risk of these manifestations in patients homozygous for the p.L444P mutation (Santamaria et al. 1998; Kraoua et al. 2011). One hypothesis for this hypermetabolic state involves the pulmonary manifestations these patients experience. Lung compliance is affected by kyphoscoliosis, as shown by the evidence of restrictive lung disease on spirometry. The only patient with normal spirometry in this sample had relatively mild hypermetabolism. In previous studies, malnourished patients with chronic obstructive pulmonary disease were found to have BMRs 18% higher than those of healthy controls (Baarends et al. 1997; Schols 2003; Kao et al. 2011). This increase is explained chiefly by the increased respiratory rate that occurs secondary to alveolar involvement, medication use, and a chronic inflammatory state. However, in our series, patients experienced BMR increases of up to 70%, which suggests that pulmonary involvement may be a contributing factor to these abnormalities in energy metabolism rather than their sole cause. Studies have shown that patients with GD type I also exhibit a hypermetabolic state, the causes of which remain unclear (Barton et al. 1989; Corssmit et al. 1995; Doneda et al. 2011, 2013). The two patients in our series with pulmonary involvement and a more severe phenotype also had comparatively marked hypermetabolism. Within this context, hypermetabolism appears to be a biomarker of disease severity.

The application of indirect calorimetry, nonetheless, shows some limitations. It requires specific conditions for reproducibility of results, such as the following: a) adhesion to a protocol establishing fasting period, intake restriction of some substances, such as caffeine, ethanol and nicotine, as well as restriction to physical activities previously to the assessment; b) a physically comfortable place under temperatures between 20º and 25 °C; and c) patients must rest for 10 to 20 minutes before assessment, and then they must lay still in supine position for the collection of respiratory gases, whose quotient must be located in the interval >0,7 and <1 (Compher et al. 2006). This strict protocol for the assessment of BMR and the difficulties showed by some GD III patients in remaining in supine position may compromise its application as a biomarker of the disease. In relation to the patients assessed in this study, the protocol for assessing BMR through indirect calorimetry was strictly followed, and they showed no difficulties remaining calm.

Nutritional assessment of patients with GD III can be quite complex, due to the presence of skeletal deformities. Height could not be measured adequately in any of the three patients, thus making the BMI an unreliable indicator. Likewise, assessment of waist circumference is hindered by the presence of organomegaly, and subscapular skinfold measurement, by bone deformity. We were unable to find any published literature on the nutritional assessment of GD III patients; therefore, we are unaware of whether such patients treated at other centers present similar skeletal abnormalities or are similarly predisposed to malnutrition, as are two of the patients managed at our service. The patients reported herein have very severe disease, and, accordingly, were quite severely malnourished at the start of treatment. As treatment progressed, their weight z scores improved, although only patient 3 achieved an adequate weight. After a period of catch-up growth early in the course of treatment, the weight z scores of patients 1 and 2 appear to have plateaued at an unsatisfactory level.

On reviewing the literature, we found four studies that assessed the growth of children and adolescents with GD III, among other parameters. In the first study, three patients with GD III experienced substantial catch-up growth and two experienced catch-up weight gain after 12 months of ERT (Bembi et al. 1994). In the second study, the authors reported that patient growth returned to normal after ERT (Erikson et al. 1995). The third study reported the outcome of eight patients (five of whom children) after 28 months of ERT with alglucerase. Three non-splenectomized pediatric patients exhibited improved growth dynamics as compared

to the other two children; improvement in weight was less marked, probably due to the presence of visceromegaly and ascites at baseline (Tylki-Szymanska, Czartoryska 1999). The fourth study reported the weight and height outcomes of patients with GD III. According to the authors, ERT-responsive patients exhibited normal or accelerated growth during the study period (Altarescu et al. 2001).

These studies detected improvements in organomegaly, cell counts, and growth – similar to those seen in patients with type I disease – over the course of the observation period. However, we were unable to find any studies that addressed whether the improvements in weight and height seen in the early stages of therapy remain over time and whether such patients achieve adequate BMI and height by the end of the growth period. In this study, we observed that nutritional status indicators initially improved, but later plateaued at inadequate levels in two of the three patients.

Conclusion

Assessment of BMR showed that all three patients were in a hypermetabolic state, which was particularly marked in the patients with more severe disease. Whether hypermetabolism contributes to disease severity or severe disease leads to hypermetabolism is still unclear. Nutritional status assessment showed that the two GD III patients who were malnourished had more severe hypermetabolism, which may have had an adverse impact on their clinical condition. We believe evaluation of metabolism in this patient population may contribute to the development of nutritional guidance that is better suited to patients' real needs, and that an understanding of the causes of hypermetabolism may aid management of GD. Further studies are required to elucidate the causes of hypermetabolism and malnutrition in patients with GD III.

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Summary

Assessment of nutritional status and energy metabolism by indirect calorimetry in patients with Gaucher disease type III.

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CASE REPORT

Mutations in the Complex III Assembly Factor Tetratricopeptide 19 Gene TTC19 Are a Rare Cause of Leigh Syndrome

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Abstract We report a patient with Leigh syndrome shown to have two previously undescribed truncating mutations in the *TTC19* gene. Our patient is a 4-year-old boy with global developmental delay, language regression at 13 months, and brain MRI showing T2 high-signal lesions involving the putamen, caudate body, and the brainstem, which appear to be progressing. Molecular testing showed our patient is heterozygous for two previously undescribed mutations in the *TTC19* gene, c.577G>A (p.Trp186Stop) and c.964_967delGGCT (p.Gly322MetfsX8), both of which are predicted to cause loss of protein function due to either protein truncation or nonsense-mediated mRNA decay. *TTC19* encodes tetratricopeptide 19 (TTC19) and is thought to be a complex III (CIII) assembly factor that is embedded on the inner mitochondrial membrane as part of two high-molecular-weight complexes, one of which coincides with CIII. The initial presentations of previously described patients with *TTC19* mutations are heterogeneous and can be from childhood to adulthood. In summary, *TTC19* mutations have been shown to affect CIII complex function, which results in a heterogeneous clinical phenotype including Leigh syndrome.

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Introduction

TTC19 encodes tetratricopeptide 19 (TTC19) and is thought to be a CIII assembly factor that is embedded on the inner mitochondrial membrane as part of two high-molecular-weight complexes, one of which coincides with CIII. This complex transfers electrons from coenzyme Q to cytochrome c, which is critical in generating the mitochondrial electrochemical potential. *TTC19* mutations were first shown to be detrimental to mitochondrial complex III (CIII) function in 2011 (Ghezzi et al. 2011).

Since that time, there have been at least five cases documented in the medical literature as having truncating mutations in the *TTC19* gene (See Table 1) (Ghezzi et al. 2011; Balasubramaniam et al. 2012). The age of presentation is broad, with our patient presenting at age four with global developmental delay, language regression at 13 months of age, and brain MRI abnormalities consistent with Leigh syndrome, and another patient presenting at age 42 with subacute rapid neurological failure with death occurring 2 years later (Ghezzi et al. 2011). All mutations in the six patients currently identified are homozygous nonsense-truncating mutations. Additional biochemical testing performed in some patients has identified isolated CIII deficiency (Ghezzi et al. 2011).

Our patient is a 4-year-old Hispanic boy born to a 33-year-old gravida 7 para 3 mother with global developmental delay, language regression at 13 months, and brain MRI showing T2 high-signal lesions involving the putamen, caudate body, and the brainstem, which appear to be progressing (Fig. 1). He was considered a well infant until his parents became concerned at 7–8 months of age for developmental delay in his gross motor skills due to him not yet sitting independently. Between the age of one and two, he suffered language regression with loss of a 19-word vocabulary at

Table 1 Clinical presentation patients with *TTC19* mutations

	Age of presentation (years)	Ethnic origin	Symptoms	Muscle biopsy	Functional status	Reference
Patient 1	5	Italian	ID, ataxia	Isolated CIII defect	Wheelchair bound (37 years)	(Ghezzi et al. 2011)
Patient 2	10	Italian	ID, ataxia	Isolated CIII defect	Bedridden (26 years)	(Ghezzi et al. 2011)
Patient 3	5	Italian	Psychomotor regression, ataxia	Isolated CIII defect	Bedridden (19 years)	(Ghezzi et al. 2011)
Patient 4	42	Italian	Subacute rapid neurological failure	Isolated CIII defect	Deceased (45 years)	(Ghezzi et al. 2011)
Patient 5	8	Iraqi	Encephalomyopathy	Isolated CIII defect	Unavailable	(Balasubramaniam et al. 2012)
Patient 6	1	Hispanic	DD, language regression	Reduction in complex I + III (79 %), II (52 %), II + III (36 %) and IV (46 %) activities	DD, ataxia	N/A

ID intellectual disability; *DD* developmental delay

13 months of age, with subsequent MRI suggestive of mitochondrial disease, particularly Leigh syndrome. A Leigh syndrome panel was sent, which showed two novel mutations in the *TTC19* gene. Additionally, biochemical analysis of the electron transport chain (ETC) in muscle showed decreased activity in complex I–IV.

Methods Used: Direct sequencing of a Leigh syndrome nuclear gene panel was performed along with a review of the electronic chart and medical literature. A commercial laboratory was used to perform the molecular analysis.

Summary of Results: Molecular testing showed our patient is heterozygous for two previously undescribed mutations in the *TTC19* gene, c.577G>A (p.Trp186Stop) and c.964_967delGGCT (p.Gly322MetfsX8), both of which are predicted to cause loss of protein function due to either protein truncation or nonsense-mediated mRNA decay. The c.964_967delGGCT mutation is a four-base-pair deletion that causes a frameshift at glycine codon 322, changing it to a methionine residue and thus creating a premature stop codon at position 8 of the new reading frame. Neither of these two mutations in the *TTC19* gene have previously been described in the medical literature. ETC activity on muscle demonstrated an increase in citrate synthase (1.45X mean), suggesting mitochondrial proliferation and decreased complex I + III (79 %), II (52 %), II + III (36 %) and IV (46 %) activities. We note, however, these values were not sufficiently low to fulfill minor criterion of the modified Walker criteria. We note all other patients with *TTC19* mutations to date had isolated CIII deficiency identified in muscle.

Conclusions

Leigh syndrome is a subacute, necrotizing encephalomyopathy with an estimated prevalence of 1:40,000 live births. It is characterized by an initial period of normal development during the first few months of life, after which children present with progressive delay or regression in psychomotor development, nystagmus, opthalmoparesis, optic atrophy, ataxia, dysphagia, hypotonia, and dystonia. It is clear that *TTC19* mutations affect mitochondrial function and therefore they should be included in nuclear gene panels for evaluation of mitochondrial dysfunction, including the Leigh syndrome phenotype. The clinical presentation of patients with *TTC19* mutations is broad, with initial presentation from childhood to adulthood. Additionally, the background of patients with *TTC19* mutations is varied, with one patient from consanguineous Iraqi parents, two of nonconsanguineous Italian descent and one of Hispanic descent. In summary, *TTC19* mutations have been shown to affect CIII complex function, which results in a heterogeneous clinical phenotype including Leigh syndrome.

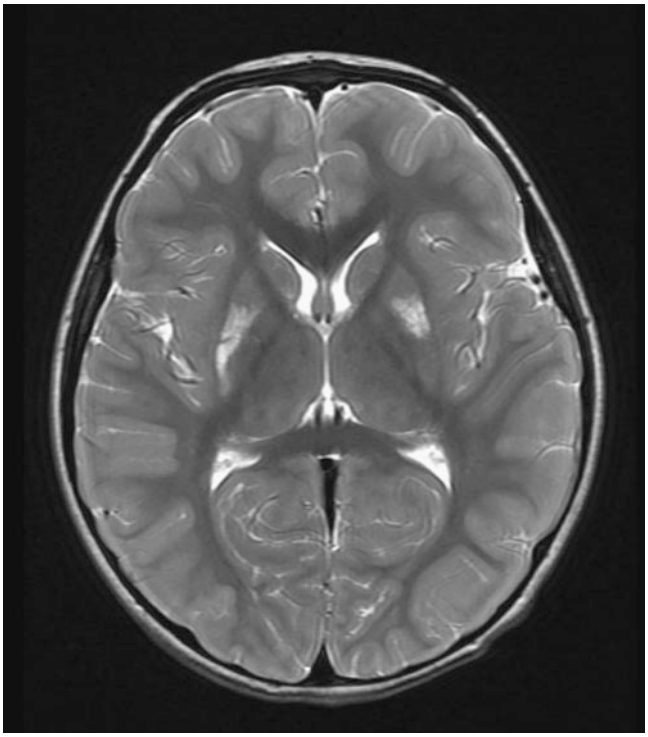

Fig. 1 MRI of brain showing increased T_2-weighted signal intensities in the caudate bodies and putamen characteristic of Leigh syndrome

Compliance with Ethics Guidelines

Paldeep Atwal declares that he has no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

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RESEARCH REPORT

Quality of Life in Adult Patients with Glycogen Storage Disease Type I: Results of a Multicenter Italian Study

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Abstract Background: Glycogen storage disease type I (GSD I) is a chronic metabolic disease that requires a lifelong strict dietetic treatment to avoid hypoglycemia and can lead to severe complications during adult age. Impaired quality of life (QoL) has been reported in affected children, but this aspect has not been previously investigated in adults.

Objective: To assess QoL in adult patients with GSD I.

Patients and Methods: Italian patients with GSD type Ia and Ib, who were 16 years or older, were asked to complete the SF-36 questionnaire, assessing their QoL. Data on demographic characteristics and clinical history were collected from clinical records and interviews.

Results: Thirty-eight patients (22 females, 16 males; 27 with GSD Ia, 11 with GSD Ib, median age 26.5 years) completed the SF-36 questionnaire. Overall, when compared to normal values, patients with GSD I had lower median scores in *general health perception* and *social functioning,* but better median scores for *bodily pain* and *mental health*. Patients with GSD Ib had a lower Z-score than GSD Ia patients for *emotional health problems*. Male patients showed better Z-scores in *physical functioning*, *general health perception,* and *social functioning* when compared to females. *Emotional health problems* Z-score was lower in nephropathic patients.

Conclusion: QoL can be impaired in adult patients with GSD I. The results of this study show that patients with GSD type Ib, women, and those with renal complications are more likely to experience a poorer QoL.

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Introduction

Glycogen storage disease type I (GSD I) is a rare inherited metabolic disease affecting carbohydrate metabolism. Two variants of the disease have been clinically and genetically distinguished: GSD Ia, due to defects of the glucose 6-phosphatase (G6Pase) hydrolytic enzyme (coded by the G6Pase gene, band q21 chromosome 7); and GSD Ib, due to defects of the glucose G6P transporter (coded by the G6P translocase gene, band q23 chromosome 11) (Chen 2001). Both forms are rare conditions, considering that the estimated prevalence of GSD (types I–IV) is about 0.00004 %, but GSD Ib is less frequent, representing about 20 % of GSD type I (Wolfsdorf and Weinstein 2003).

GSD Ia is characterized by hypoglycemia (induced by a short fasting period), hyperuricemia, hyperlipidemia, hepatomegaly, truncal obesity, and bleeding tendency (Rake

et al. 2002a). GSD Ib shares its main features with GSD Ia, but it is also characterized by neutropenia and neutrophil dysfunction, associated with frequent infections and possible chronic inflammatory bowel disease (Visser et al. 2000). Furthermore, long-term complications such as chronic anemia, kidney disease, arterial hypertension, hepatic adenomas with a small risk of malignant transformation, osteopenia, and, rarely, pulmonary hypertension may occur in both types (Talente et al. 1994).

The disease generally presents during early infancy (Rake et al. 2002a) and the main treatment is lifelong diet therapy, consisting of frequent meals, rich in complex carbohydrates in order to avoid hypoglycemic episodes and secondary metabolic derangements. Fasting tolerance is variable among patients, and it may be different according to age. However, affected subjects are usually forced to eat every 3–4 h during daytime, and to consume uncooked cornstarch (UCCS) before going to bed, or to use continuous nocturnal gastric drip feeding (CNGDF) to prolong sleep time (Rake et al. 2002b; Visser et al. 2002).

In modern medicine, it has become more accepted that the aims of medical care should not only be to alleviate the physical symptoms of the disease but also to improve the overall quality of life (QoL). In GSD I, QoL can be impacted by the disease and its complications, by the strict diet regimen and the eventual medications, and by the medical procedures needed in a comprehensive follow-up, such as blood samples, ultrasounds, magnetic resonances, and bone densitometry. A reduced QoL has been previously demonstrated in children affected by GSD Ia and Ib (Storch et al. 2008).

Life expectancy in GSD I is still undefined, but it has dramatically improved, compared to 30 years ago, with more and more patients surviving to adulthood (Martens et al. 2008). As a consequence, patients have to deal with new issues connected with self autonomy, integration into society, traveling, competitive working, and having a family of their own. In this context, investigating QoL in adult GSD I patients is of particular interest, but, to our knowledge, no data are available so far on this topic. Therefore, a cross-sectional study was conducted in order to depict a profile of the QoL of adult patients affected by GSD I and to identify possible groups at higher risk of a low QoL.

Methods

Patients affected by GSD type Ia or Ib were enrolled between 2009 and 2012 in seven different Italian metabolic centers. Diagnosis had to be confirmed by enzyme assay on hepatic biopsy or by molecular analysis. Patients with a liver or kidney transplant or on a waiting list for transplantation were excluded. A written informed consent was obtained from all participants.

Information on sociodemographic characteristics and clinical history were gathered both from clinical records and face-to-face interviews. Clinical records were reviewed to retrieve information on anemia (Hb $<$ 12 g/dl for males and Hb $<$ 11 g/dl for females), hepatic adenomas (identified through ultrasound scan or MRI), hypertension (defined as blood pressure repeatedly $>$140/90 mmHg or use of antihypertensive drugs for hypertension treatment), microalbuminuria (defined as albumin excretion 30–300 mg/24 h), proteinuria (defined as albumin $>$300 mg/24 h or protein $>$ 150 mg/24 h and diagnosed from a 24-h urine collection), chronic renal failure (CRF, defined as a glomerular filtration rate (GFR) $<$ 60 ml/min), and chronic inflammatory bowel disease (IBD, defined as the presence of perianal infection and protracted diarrhea).

Health-related QoL was assessed through the Italian version of the Short Form Health Survey (SF-36) questionnaire. This toll has been validated in several experimental and observational studies regarding different chronic diseases and has been widely used in clinical studies involving Italian subjects since 1990 (Apolone et al. 1997). The SF-36 questionnaire consists of 36 items combined into eight scaled scores: *physical functioning*, *role physical* (limitations caused by physical health problems), *bodily pain*, *general health* perception, *vitality*, *social functioning*, *role emotional* (limitations caused by emotional health problems), and *mental health*.

The raw score of each scale was transformed into a 0–100 scale and then normalized to a Z-score using normative Italian data. The physical and mental component summary measures (PCS and MCS, respectively) are also computed and transformed to norm-based scores (mean = 50, standard deviation = 10) using the mean, standard deviation, and scoring coefficients from the US general population (Ware and Sherbourne 1992). Both for combined scales and summary measures, higher scores indicate more favorable physical functioning or psychological well-being.

Statistical Analysis

The standard SF-36 protocol was applied to perform the analyses of SF-36 questionnaires (Apolone et al. 1997; Ware and Sherbourne 1992). Briefly, after checking for out-of-range answers, missing values were replaced by scale means where valid responses were available for at least half of the scale items. Scores were then recoded, recalibrated, and converted into 0–100 scores. Finally, summary measures were computed using the US population normative values (Ware and Sherbourne 1992) since these provide reliable estimates even when applied to non-US populations (Apolone et al. 1997).

All categorical variables are described as frequencies and percentages, while median and interquartile ranges are reported for continuous variables. The rank sum test was used to compare the QoL scores in different groups.

The statistical software Stata 11.0 (Stata Statistical Software: Release 11.0, 2009. StataCorp LP, College Station, TX, USA) was used to perform data analysis.

Results

Thirty-eight GSD I patients were enrolled, and their general characteristics are reported in Table 1. All were Caucasians. Two patients were sisters, while the others belonged to different families. Their median age was 26.5 years, the youngest being 16 and the oldest being 41. Their median age at diagnosis was 1 year, the earliest diagnosis being made at about 2 months of age and the latest at 24 years.

Most of the patients ($n = 27$) were affected by type Ia GSD, while 11 had type Ib. Their clinical relevant characteristics are described in Table 2. As regards comorbidities, one female patient had suffered from epilepsy since childhood, two males had a history of nephrolithiasis, one female had pulmonary hypertension, and two males with GSD Ib had chronic inflammatory bowel disease. One female with GSD Ib had autoimmune hypothyroidism. A disturbance of alimentary behavior was signaled in one female patient, but no other psychiatric problems were reported.

At the study time, all patients were following a diet consisting of frequent meals rich in complex carbohydrates, with variable restrictions in lactose or fructose intake, and were taking UCCS at least once a day. Detailed data on UCCS consumption were available for 32 patients. The median UCCS dosage was 120 g/day (interquartile range [IQR] 100–160; range min–max 40–325), considering patient's weight the median dosage was 1.9 g/kg/day (IQR 1.5–2.5; range min–max 1–5.1), the majority of patients assuming it twice a day (range min–max 1–6). Nine out of 32 patients (28.1 %) were forced to wake up during nighttime, interrupting sleeping, to take a dose of UCCS.

Three patients out of 38 were also using continuous nocturnal gastric drip feeding (CNGDF), while another 12 used CNGDF during childhood, but stopped it in adulthood. Thirty-five patients were taking medications; the detailed drug consumption is shown in Table 3.

Thirty-four respondents completed all SF-36 items, while four participants, all females, had at least one missing SF-36 item. In two cases, it was possible to input all missing values (1 in *physical functioning,* 1 in *mental health*, 1 in *general health*), in one the imputation could be made only for one *bodily pain* item but not for *role physical* and *role emotional* (all items were missing), and in another one *physical functioning* scale could not be calculated since all the items were left blank.

Table 1 General characteristics of the patients ($n = 38$)

	n	%	Median	Interquartile range
Gender				
Male	16	42.1		
Female	22	57.9		
Age (years)			26.5	20–32
Age at diagnosis (years)			1	0.4–3
Age at diet therapy start (years)			3	1–9
Education[a]				
Junior high school	12	34.3		
Senior high school	20	57.1		
University	3	8.6		
Occupation[a]				
Employed	18	51.4		
Unemployed	4	11.4		
Student	11	31.4		
Housewife	2	5.7		
Marital status[b]				
Unmarried[c]	33	86.8		
Married	5	13.2		
With one child	3			
With two children	2			

[a] Three patients did not report their educational level nor their occupation.

[b] There were no separated, divorced, or widowed people.

[c] No unmarried subjects had children.

The transformed scores for each scale are reported in Table 4, while Table 5 shows the SF-36 Z-scores.

Overall, *physical functioning*, *role physical*, *vitality*, and *role emotional* scores were consistent with the Italian reference values, while GSD I patients scored better in *bodily pain* and *mental health* scales and worse in *general health* and *social functioning* (Table 4).

Summary measures for both physical and mental component were consistent with normal US values (Table 4).

When a stratified analysis was performed by gender, males showed better scores in *physical functioning* (95 vs. 85; Z-scores 0.45 vs. 0.02; $p = 0.002$), *general health* (72 vs. 52; Z-scores 0.31 vs. −0.60; $p = 0.01$), and *social functioning* (94 vs. 69; Z-scores 0.70 vs. −0.37; $p = 0.03$). Furthermore, when differences were investigated according to GSD I type, *role emotional* showed to be much better

Table 2 Clinical characteristics of the patients ($n = 38$)

	n	%
Type		
Ia	27	71.1
Ib	11	29.0
Anemia	20	52.6
Adenomas	16[a]	42.1
Microalbuminuria	11	29.0
Proteinuria	9	23.7
Chronic renal failure	1	2.63
Hypertension	5	13.2
UCCS	38	100
CNGDF, ongoing	3	7.9
CNGDF, ever	15	39.5
Drug consumption	35	92.1
Number of drugs[b] (only patients assuming drugs, $n = 35$)	1	2–4

UCCS uncooked cornstarch, *CNGDF* continuous nocturnal gastric drip feeding

[a] Among these, two patients underwent to surgical resection (because of adenoma's diameter >5 cm)

[b] Median (interquartile range)

Table 3 Drugs consumed by the patients ($n = 38$)

Drug	Number of patients
Allopurinol	34
ACE inhibitor or Sartan	16
Calcium	9
RHGCSF	7
Iron	7
Multivitamins	6
Omega 3	4
Mesalazine	2
Antidepressant	0
Anxiolytic	0
Other[a]	5

RHGCSF recombinant human granulocyte colony-stimulating factor

[a] Anticonvulsants (1), furosemide (1), levotiroxine (1), fibrate (1), anti-inflammatory drugs (1)

in patients affected by GSD type Ia when compared to patients affected by GSD Ib (100 vs. 33; Z-scores 0.64 vs. −1.15; $p = 0.01$) (Table 5). Moreover, patients with renal complications (at least one of either microalbuminuria, proteinuria, or chronic renal failure) showed a worse *role emotional* than patients with unaffected kidneys (83.3 vs. 100; $p = 0.04$), while no differences were found according to anemia, hepatic adenomas, age (≤20 vs. >20 years), age at diagnosis (<3 vs. ≥3 years), age at diet therapy start (<3 vs. ≥3 years), UCCS dosage or timing, and CNGDF use (data not shown).

Discussion

GSD I is a rare metabolic disease that may result in different dysfunctions and long-term complications, and requires the adoption of specific dietary habits to avoid metabolic drawbacks. As a consequence of both the disease itself and of its treatment, affected patients may experience an impairment of their QoL, this being a multidimensional concept reflecting the patient's subjective evaluation of his/her functioning and emotional well-being. Investigating QoL is important to discover possible shortfalls in the management of patients, especially those with a chronic disease that is likely to have a long-term impact on all aspects of everyday life. Nevertheless, to our knowledge, this is the first study investigating QoL among adult patients with GSD I since this aspect was investigated only in children so far (Storch et al. 2008).

Social outcome (including interpersonal relationships, education, and professional carrier) is strictly linked with QoL, and it has been previously demonstrated that in inborn errors of metabolism impaired QoL can be associated with an altered social structure in comparison to healthy peers (Simon et al. 2008). Although QoL was not one of the aims of the study, the European Study on GSD I (ESGSD I), conducted on more than 200 GSD I patients reported that 11 % needed a special education or work, while 6 % were unable to have a profession because of mental disability (Rake et al. 2002a). In contrast, in our cohort, neither mental disability nor need for special education or work was reported. In fact, 20 patients had a senior high school diploma and 3 held a university degree. Moreover, 18 patients had a normal employment and 11 were students in normal schools or universities. However, it must be kept in mind that the ESGSD study was conducted in 2002, and many adult patients from this cohort had treatment pre-dating the use of UCCS during their childhood, hence several had probably poorer metabolic control at the time of their growth. The current standards of care prevent patients from suffering severe hypoglycemia during childhood, thus avoiding brain damage (Wolfsdorf and Crigler 1999) and allowing patients to have a regular education and job.

The present study showed that overall GSD I adult patients had lower scores in *general health* perception and *social functioning*.

The personal evaluation of *general health* given by GSD I patients was similar to that reported by patients affected by type 2 diabetes, another chronic disease requiring a lifelong diet (Lloyd and Orchard 1999). This suggests that the implications of the disease and its treatment on everyday life

Table 4 SF-36 scores

	Score	Reference
Scaled scores (0–100)	**Median (IQR) (range min–max)**	**Median (range min–max)**[a]
Physical functioning ($n = 37$)	95 (85–95) (0–100)	95 (0–100)
Role-physical ($n = 37$)	100 (75–100) (0–100)	100 (0–100)
Bodily pain ($n = 38$)	100 (62–100) (20–100)	84 (0–100)
General health ($n = 38$)	57 (47–72) (5–100)	70 (0–100)
Vitality ($n = 38$)	60 (45–75) (25–90)	65 (0–100)
Social functioning ($n = 38$)	75 (63–100) (37.5–100)	87.5 (0–100)
Role-emotional ($n = 37$)	100 (67–100) (0–100)	100 (0–100)
Mental health ($n = 38$)	80 (56–92) (28–96)	68 (0–100)
Summary measures	**Median (IQR)**	**Median (IQR)**[b]
Physical component ($n = 36$)	50.8 (45.8–55.7) (31.1–59.8)	53.3 (45.2–57.1)
Mental component ($n = 36$)	49.1 (39.3–57.6) (30.9–60.9)	49.3 (40.6–54.3)

[a] Reference: Italian population

[b] Reference: US population

have a stronger impact on the general well-being perception than the disease itself. Thus, even a very rare and a common disease may share similar scores in *general health*.

Social functioning represents the extent to which physical health or emotional problems interfere with normal social activities. Low scores found in GSD I patients can be explained with the need for frequent meals at scheduled times that may prevent affected adults from being flexible and adapting to the fast rhythm of the contemporary society. Furthermore, these patients need to restrict simple sugars, fat and alcohol intake, resulting in a diet that is different from their peers'. This can limit or even hamper many social activities, such as going out for dinner or participate in a party (Bhattacharya 2011).

Interestingly only 13.2 % patients among our cohort were married and had children, while the expected percentage would be 25.3 % (95 % CI 15.4–37.3), when the age- and gender-matched Italian population is used as a reference (Istat, Italian National Institute of Statistics www.istat.it). These data might reflect the lower scores in social functioning, with probably more difficulty in forming adult relationships in which to have children than healthy peers. Furthermore, difficulty in becoming independent from parents and starting their own family has been observed in people with inherited metabolic diseases in general. Considering the high level of parental involvement in disease management during childhood, this is not unexpected (Lee 2002).

Interestingly, adult patients with GSD I showed better scores than the general population in *bodily pain* and *mental health* scales. This can be explained by the coping phenomenon, thanks to which people with a chronic illness are able to emphasize the positive aspect of their lives, to reduce the general stress (Carver and Connor-Smith 2010), although we cannot exclude that participants were hesitant to report difficulties in these areas.

Compared to the study published by Storch and colleagues, describing a lower QoL in several areas and summary measures in youth GSD I patients (Storch et al. 2008), we found generally better results for adult patients, with the summary measures for physical and mental components being within the normal range. This difference between adults and children can have different interpretations. First of all, during adulthood the exogenous glucose amount required to maintain normoglycemia is lower than that during growth; as a consequence, fasting time can usually be prolonged, allowing patients both to have less frequent meals during the day and longer sleep periods (Moses 2002). In fact, in our cohort, many patients used CNGDF during childhood but were able to stop it during adulthood when the consumption of cornstarch was sufficient to achieve a good glycemic control during the night.

Secondly, having a chronic disease since early childhood can promote the development of an adaptive behavior in which adult patients, through years of experience with their disease, are able to use strategies (i.e., always carrying some food in their bags or consuming extra cornstarch before physical activity) that allow them to participate in normal activities (such as working, playing sports, or traveling), as well as their healthy peers.

Interestingly, in the study by Storch and colleagues, a high level of parental stress was found, and parents generally felt that their children were more impaired than reported by the children themselves. Most likely, this parental attitude negatively affects the QoL during childhood, while parental stress has probably a lower influence in adulthood, with a positive effect on QoL.

Table 5 SF-36 Z-scores

Scales	All *Median (IQR)*	Males *Median (IQR)*	Females *Median (IQR)*	p Value	Type Ia *Median (IQR)*	Type Ib *Median (IQR)*	p Value
Physical functioning	0.45 (0.02;0.45) $n = 37$	0.45 (0.45;0.67) $n = 16$	0.02 (−0.62;0.45) $n = 21$	0.002	0.35 (0.02;0.45) $n = 26$	0.45 (0.02;0.45) $n = 11$	0.92
Role-physical	0.61 (−0.09;0.61) $n = 37$	0.61 (0.61;0.61) $n = 16$	0.61 (−0.09;0.61) $n = 21$	0.22	0.61 (−0.09;0.61) $n = 26$	0.61 (−0.09;0.61) $n = 11$	0.42
Bodily pain	0.95 (−0.42;0.95) $n = 38$	0.95 (−0.39;0.95) $n = 16$	0.95 (−0.46;0.95) $n = 22$	0.91	0.95 (−0.46;0.95) $n = 27$	0.95 (0.37;0.95) $n = 11$	0.42
General health	−0.37 (−0.82;0.31) $n = 38$	0.31 (−0.15;0.60) $n = 16$	−0.60 (−1.27;−0.15) $n = 22$	0.01	−0.37 (−0.82;0.53) $n = 27$	−0.46 (−1.72;0.31) $n = 11$	0.29
Vitality	−0.09 (−0.82;0.63) $n = 38$	0.03 (−0.57;1.00) $n = 16$	−0.21 (−1.01;0.39) $n = 22$	0.29	0.15 (−0.82;0.63) $n = 27$	−0.57 (−1.30;1.12) $n = 11$	0.63
Social functioning	−0.10 (−1.71;0.97) $n = 38$	0.70 (−0.10;0.97) $n = 16$	−0.37 (−1.18;0.97) $n = 22$	0.03	−0.10 (−0.64;0.97) $n = 27$	−0.10 (−0.64;0.97) $n = 11$	0.75
Role-emotional	0.64 (−0.25;0.64) $n = 37$	0.64 (−0.25;0.64) $n = 16$	0.64 (−0.25;0.64) $n = 21$	0.35	0.64 (0.64;0.64) $n = 26$	−1.15 (−1.15;−0.64) $n = 11$	0.01
Mental health	0.64 (−0.51;1.22) $n = 38$	0.64 (−0.32;1.22) $n = 16$	0.35 (−1.08;1.02) $n = 22$	0.22	0.64 (−0.51;1.22) $n = 27$	0.07 (−0.70;1.22) $n = 11$	0.90

To explain the good summary measures found, we also need to consider that the present study was conducted in Italy, a country where the GSD nutritional requirements can easily match the common diet, rich in complex carbohydrates. Thus, we cannot exclude that a similar evaluation performed in other countries, where food habits are very different and not consistent with a GSD diet, could give different results.

Moreover, we also cannot exclude that there was a difference in disease severity between the two study populations since 10/38 patients in our cohort were diagnosed after 2 years of age, probably due to a mild disease. Unfortunately, no data about disease history were present in the Storch study. Finally, we must point out that the two studies are not fully comparable since different tests were used to assess QoL due to the different age of participants (Pediatric Quality of Life Inventory version 4.0 in the study by Storch and colleagues vs. SF-36 in the present study) (Storch et al. 2008). In spite of low numbers, we also tried to perform several stratified analyses and to compare different subgroups of patients. As a result, we found that patients with GSD type Ib reported lower values than type Ia. This can be explained clinically since GSD Ib subjects often suffer from frequent infections and bowel disease, which can affect everyday living (Froissart et al. 2011). However, interestingly, in our cohort, we found a lower rate of IBD than that in the ESGSD study (18 % vs. 77 %) (Visser et al. 2002). The reason could be that in our cohort 7/11 GSD Ib patients were receiving recombinant human granulocyte colony-stimulating factor, and this treatment could have reduced the proportion of patients affected by IBD.

Lower QoL was also found in patients with renal complications (microalbuminuria, proteinuria, or chronic renal failure). Only one patient suffered from chronic renal failure and this condition can be easily associated to a poorer QoL; on the other hand, those suffering from microalbuminuria or proteinuria usually take additional drugs, and this treatment probably strengthens the patient's consciousness of having a kidney disease that can progress further, thus affecting the QoL.

Moreover, female patients reported lower scores than males in several scales, irrespective of the differences in GSD type, number of complications, or number of drugs taken. This could be related to a gender difference in experienced stress. In particular, woman can be more concerned about their physical appearance, which can be altered by a prominent abdomen, a characteristic face, and, sometimes, by a short stature (especially the old generation). Furthermore, women can be more anxious about their health, and this can affect the perceived well-being.

As liver transplantation is thought to ameliorate GSD I patients' QoL, overcoming the problem of recurrent hypoglycemia and the necessity of a strict diet (Kido et al. 2013), it would be of interest to assess the difference between GSD I patients medically treated and those who underwent hepatic transplantation through a validated instrument of QoL assessment. Anyway, in Italy very few GSD I patients

underwent liver transplantation so far to allow a sufficient sample size to perform a comparison. Since transplantation completely changes the disease burden, transplanted patients were excluded from this study by design.

Conclusions

QoL can be impaired in adult patients with GSD I; therefore, the evaluation of QoL using validated scales should be included in their periodic follow-up. Particular attention should be paid to patients with GSD type Ib, women, and those suffering from renal complications since they are more likely to experience a poorer QoL.

QoL improvement should be one of the aims of the disease management. The development of new treatments (i.e., long-lasting starches, gene therapy) might ameliorate QoL of GSD I patients in the future.

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Compliance with Ethics Guidelines

All authors declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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RESEARCH REPORT

The Molecular Bases of Phenylketonuria (PKU) in New South Wales, Australia: Mutation Profile and Correlation with Tetrahydrobiopterin (BH_4) Responsiveness

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Abstract Phenylketonuria (PKU) is an autosomal recessive inborn error of phenylalanine metabolism predominantly caused by mutations in the phenylalanine hydroxylase (*PAH*) gene. Mutation screening was carried out in a large cohort of PKU patients from New South Wales, Australia. Pathogenic mutations were identified in 99% of the alleles screened, with the two most common mutations (p.R408W and IVS12+1G>A) accounting for 30.7% of alleles. Most individuals were compound heterozygotes for previously reported mutations, but four novel mutations (c.163+1G>T, c.164-2A>G, c.461A>T [p.Y154F], and c.510-1G>A) and a novel polymorphism (c.60+62C>T) were also identified. A number of patients have been previously tested for their response to dietary supplementation of tetrahydrobiopterin (BH_4), the cofactor of PAH. Correlation between genotype and the responses revealed that although genotype is a major determinant of BH_4 responsiveness, patients with the same genotype may also show disparate responses to this treatment. A clinical and biochemical evaluation should be undertaken to determine the effectiveness of PKU treatment by supplementation of BH_4.

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Introduction

Phenylketonuria (PKU, OMIM 261600) is an inborn error of metabolism of phenylalanine (Phe), with an autosomal recessive mode of inheritance. The severity of the disorder varies between patients and is classified as 'classical', 'moderate', 'mild' or hyperphenylalaninaemia (HPA), depending on the blood Phe level at the time of diagnosis or dietary Phe tolerance (Blau et al. 2010). The causative gene in the majority of PKU patients is phenylalanine hydroxylase (*PAH*), located on chromosome 12 (Woo et al. 1983). To date, more than 800 variants have been reported in *PAH* (http://www.biopku.org, last accessed 21 November 2013) and the majority of patients are compound heterozygotes. Mutations in *PAH* lead to impaired function of the hepatic enzyme, PAH (EC 1.14.16.1), which catalyses the conversion of the essential amino acid L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr), a precursor of the neurotransmitters dopamine, noradrenaline and adrenaline.

A study in Victoria, Australia (Boneh et al. 2005), reported the incidence of PKU to be 1 in 11,226, while in the state of New South Wales (NSW), Australia, the incidence is 1 in 8,900, mirroring the figures found in

other Caucasian populations (Scriver and Kaufman 2001). However, the pathogenic mutations in New South Wales have not been previously studied. Phenotype–genotype correlations in PKU have shown clear associations between some mutations and the severity of disease (Kayaalp et al. 1997; Bénit et al. 1999; Güttler et al. 1999; Zschocke 2003; Bercovich et al. 2008). Due to the large number of mutations and the low population frequency of some of these mutations, it is often difficult to ascertain the phenotypic consequences of a given mutation and correlations may also give rise to conflicting results (Bercovich et al. 2008). Genotyping more cohorts of PKU patients may therefore be useful to clarify the relationship between genotype and phenotype in these patients.

Mutation screening is also of value in deciding potential treatments for patients. Dietary supplementation of sapropterin, a synthetic form of BH_4, the cofactor of PAH, is efficacious in a subset of *PAH* mutations in lowering blood Phe levels (Kure et al. 1999; Bernegger and Blau 2002). More recently, a novel set of compounds have been developed specifically targeting nonsense mutations and *in vitro* studies have shown that these compounds may be of therapeutic benefit in PKU patients carrying nonsense mutations (Howard et al. 1996; Barton-Davis et al. 1999; Welch et al. 2007; Du et al. 2009; Nudelman et al. 2010; Ho et al. 2013). Mutation screening would identify those patients for whom these approaches, including other mutation-specific therapies such as anti-aggregation compounds for p.G46S (Leandro et al. 2011), may be applicable.

The aim of this study is to screen a cohort of patients at the PKU Clinic at the Children's Hospital at Westmead to identify the mutations prevalent in NSW and to determine which patients would benefit from sapropterin treatment and potentially nonsense-related therapy.

Materials and Methods

One hundred and eleven patients, including seven families with two or more affected individuals, were recruited from the records of the NSW Newborn Screening Programme or from the PKU Clinic at the Children's Hospital at Westmead, Sydney Australia. This research was approved by the Human Ethics Committee of the Children's Hospital at Westmead. Blood samples were collected and genomic DNA was extracted using a salting out extraction protocol (Miller et al. 1988).

The 13 coding exons and the intron–exon boundaries of *PAH* were amplified by polymerase chain reaction (PCR) using a combination of primers previously published (Bräutigam et al. 2003), and new primers designed using Primer3 software (Rozen and Skaletsky 2000). To reduce the cost of screening, a tiered sequencing approach was adopted, whereby patients were screened first for mutations in exons 7 and 12. Screening of exons 2, 3, 5 and 6 were then carried out if less than two pathogenic mutations were identified, and subsequently the remainder of the exons if required.

PCR products were sequenced in both the forward and reverse directions using ABI3730XL (Life Technologies, Carlsbad, CA) by Macrogen Inc. (Seoul, Korea), and the traces were analysed using MutationExplorer™ (SoftGenetics, State College, PA). The genomic DNA reference sequence was NC_000012.11 and the cDNA reference sequence was NM_000277.1, with the A from the ATG translation initiation start site numbered +1. Mutation nomenclature is in accordance with the guidelines from Human Genome Variation Society (den Dunnen and Antonarakis 2000) and also as reported in the *PAH*db Knowledgebase (http://www.pahdb.mcgill.ca).

Novel unreported mutations were first confirmed by an independent PCR and bi-directional sequencing. In addition, a minimum of 300 control alleles were screened for each of the novel mutations to provide additional support for pathogenicity. Detection of the novel mutations was carried by restriction digests: *Bgl* II for c.60+62C>T, *Mnl* I for c.168+1G>T, *Bfa* I for c.169-2A>G, *Rsa* I for c.461A>T (p.Y154F) (all enzymes from New England Biolabs, Ipswich, MA). Control screening for c.510-1G>A was carried out using Custom TaqMan® SNP Genotyping Assay (Life Technologies) according to manufacturer's instructions.

After primary sequence analysis, there were six patients in whom only one heterozygous pathogenic mutation had been identified. There were also 14 samples with apparently homozygous mutations. All 20 samples were then screened for large deletions and duplications using multiplex ligation-dependent probe amplification (MLPA, SALSA® MLPA® kit P055, MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. Fragments were resolved on ABI PRISM® 3100 Genetic Analyzer (Life Technologies). Control samples ($n \geq 4$) were included with each run, and the average of the peak areas of each amplified fragment was calculated using these controls. The ratio of the peak areas of patient samples to the average was used to determine if a deletion or insertion was present. A value of 0.6 or lower was indicative of a deletion and a value of 1.5 or higher was indicative of duplication, as per the manufacturer's recommendations. Samples with an apparent deletion or duplication were confirmed by a second independent MLPA reaction and real-time quantitative polymerase chain reaction (QPCR). QPCR conditions are available upon request.

Results

The coding region of *PAH* (including exon-intron boundaries) was analysed in 111 PKU patients, including seven families with multiple affected sibs (six sibling pairs and one trio) and five consanguineous families, equating to 201 independent chromosomes. The genotypes are summarized in Table 1. Sixty-one different pathogenic mutations, including five previously unreported variations and two large deletions (spanning an entire exon), were identified in 199 out of the 201 independent chromosomes, representing a mutation detection rate of 99%. The majority of patients (excluding consanguineous families and counting only one individual from each family, 93/99, 93.9%) were compound heterozygotes. In all patients with apparently homozygous mutations, the possibility of hemizygosity due to the presence of a large deletion was excluded using MLPA.

The two most common mutations [c.1222C>T (p.R408W) and c.1315+1G>A (IVS12+1G>A)] accounted for 18.6% and 12.1% respectively of the alleles. In all, the nine most common mutations accounted for 57.2% of alleles (Table 2). The most common type of mutations was missense, with 38 distinct mutations in 66.8% of alleles screened. Large deletions, encompassing an entire exon, were identified in four patients using MLPA and confirmed by quantitative PCR. Two patients had a heterozygous deletion of exon 6, while another two had a heterozygous deletion of exon 3. The percentage of alleles with large deletions in this study is 2.0%, a proportion similar to other mutation studies (Mallolas et al. 1999; Kozak et al. 2006; Birk Møller et al. 2007).

Four novel pathogenic mutations were identified: c.168 +1G>T (IVS2+1G>T), c.164-2A>G (IVS2-2A>G), c.461A>T (p.Y154F) and c.510-1G>A (IVS5-1G>A). For each of these patients, all 13 exons of *PAH* were sequenced to exclude the possibility of other pathogenic mutations. In addition, a minimum of 300 chromosomes from a normal (Caucasian) population was screened using the methods described above and none of the variants in question were detected in the normal population, or in dbSNP or 1000 Genomes database. The effect of mRNA splicing for the mutations c.168+1G>T, c.169-2A>G and c.510-1G>A was analysed *in silico* using Splice Site Prediction by Neural Network provided by Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html, Reese et al. 1997). All three mutations were predicted to affect the splice site signal, leading to abnormal splicing. Attempts to extract and amplify *PAH* mRNA from peripheral blood from patients carrying the c.168+1G>T and c.510-1G>A mutations were unsuccessful. The levels of *PAH* transcripts were too low to be detected (data not shown). The classical phenotype in all three patients in whom the mutations were identified was in accordance with the prediction of these mutations affecting splicing of the *PAH* transcript and leading to a null allele.

A previously unreported missense mutation was identified in one patient with moderate PKU (p.Y154F) and a second patient with mild PKU (p.Y154F and p.G103C). The former patient with moderate PKU had the c.1315 +1G>A mutation as the second allele, which results in low amounts of protein and protein activity detected in in vitro systems, and is generally assumed to be a null allele (Waters et al. 1998). The phenotypes of these two patients suggest that the missense mutation p.Y154F retains some levels of residual PAH enzymatic activity.

Ten polymorphisms were identified in the cohort, with nine having been previously reported: c.168G>A (p.E56E, in *cis* with IVS2+1G>A), c.163+19T>C (IVS2+19T>C), c.353-22C>T (IVS3-22C>T), c.441+47C>T (IVS4 +47C>T), c.510-54A>G (IVS5-54A>G), c.696A>G (p.Q232Q), c.735G>A (p.V245V), c.969+43G>T (IVS9 +43G>T), c.1155G>C (p.L385L) and c.1242C>T (p.Y414Y). A novel variant (c.60+62C>T or IVS1+62C>T) in intron 1 was designated a silent polymorphism, as it is not predicted to affect transcript splicing and is also found at a high allele frequency in the controls screened (31% of 300 normal chromosomes) and in dbSNP (rs1522296, minor allele frequency 0.35).

There were seven families in the cohort with more than one affected child. For six of the families, the siblings shared the same two pathogenic mutations. In the last family, only one allele was shared (p.R408W), with the second allele being p.A345S in one sibling and p.S87R in the other two siblings. Parental screening showed that the father was heterozygous for p.R408W and the mother heterozygous for both p.S87R and p.A345S, presumed compound heterozygous. The mother was not initially suspected of having PKU, but Phe testing carried out after genotype was confirmed revealed persistent elevated levels of blood Phe (mean 184 micromol/L), indicative of non-PKU hyperphenylalaninaemia. In addition, the patient with the genotype p.[S87R];[R408W] was classified as having classical PKU compared to his two siblings with p.[A345S];[R408W] who had HPA. Therefore, it would appear that the p.S87R allele is a more severe mutation compared to p.A345S.

A number of patients in this cohort have been previously tested for response to a BH_4-load (Mitchell et al. 2005). These patients were given BH_4 over a period of 7 days, and blood Phe levels were measured at 8 h, 32 h and the 7th day. A decrease of Phe of 30 %, compared to pre-BH_4 level, was deemed to be clinically significant (Mitchell et al. 2005). Patients are classed as 'positive' if they reached a decrease of 30% after 8 h, 'intermediate' if a decrease of 30 % was observed at 32 h or at the 7th day and 'negative' if no decrease of 30% or greater was observed at any point

Table 1 Genotypes of the 111 PKU patients screened

	Allele 1		Allele 2		
ID	cDNA[a]	Protein	cDNA[a]	Protein	Comments[b]
1	c.842C>T	p.P281L	c.1241A>G	p.Y414C	
2	c.194T>C	p.I65T	*(Not detected)*		
3	c.60+5G>T (IVS1+5G>T)		c.1315+1G>A (IVS12+1G>A)		
4	c.331C>T	p.R111*	c.1162G>A	p.V388M	
5	c.473G>A	p.R158Q	c.1315+1G>A (IVS12+1G>A)		
6	c.117C>G	p.F39L	c.1222C>T	p.R408W	
7	c.500A>T	p.N167I	c.1222C>T	p.R408W	
8	c.194T>C	p.I65T	c.434A>T	p.D145V	
9	c.727C>T	p.R243*	c.782G>A	p.R261Q	
10	c.896T>G	p.F299C	c.1222C>T	p.R408W	
11	c.117C>G	p.F39L	*(not detected)*		
12	c.838G>A	p.E280K	c.1042C>G	p.L348V	
13	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
14	c.117C>G	p.F39L	c.1222C>T	p.R408W	
15	c.896T>G	p.F299C	c.1222C>T	p.R408W	
16	c.581T>C	p.L194P	c.1222C>T	p.R408W	
17	c.461A>T	p.Y154F	c.1315+1G>A (IVS12+1G>A)		p.Y154F novel
18	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
19	c.169-?_352+?del (exon 3 deletion)		c.842C>T	p.P281L	
20	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
21	c.782G>A	p.R261Q	c.1315+1G>A (IVS12+1G>A)		
22	c.782G>A	p.R261Q	c.1208C>T	p.A403V	
23	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
24	c.754C>T	p.R252W	c.912+1G>A (IVS8+1G>A)		
25	c.143T>C	p.L48S	c.1222C>T	p.R408W	
26	c.969+5G>A (IVS9+5G>A)		c.969+5G>A (IVS9+5G>A)		Consanguinity
27	c.117C>G	p.F39L	c.896T>G	p.F299C	
28	c.561G>A	p.W187*	c.1315+1G>A (IVS12+1G>A)		
29	c.117C>G	p.F39L	c.1222C>T	p.R408W	
30	c.1222C>T	p.R408W	c.1241A>G	p.Y414C	
31	c.782G>A	p.R261Q	c.842C>T	p.P281L	
32	c.194T>C	p.I65T	c.818C>T	p.S273F	
33	c.266_267insG	p.A90fs	c.266_267insG	p.A90fs	Consanguinity
34	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
35	c.510-?_706+?del (exon 6 deletion)		c.896T>G	p.F299C	
36	c.727C>T	p.R243*	c.912+1G>A (IVS8+1G>A)		Sibling of p37
37	c.727C>T	p.R243*	c.912+1G>A (IVS8+1G>A)		Sibling of p36
38	c.473G>A	p.R158Q	c.1315+1G>A (IVS12+1G>A)		
39	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
40	c.1238G>C	p.R413P	c.1238G>C	p.R413P	
41	c.194T>C	p.I65T	c.782G>A	p.R261Q	
42	c.728G>A	p.R243Q	c.728G>A	p.R243Q	Consanguinity, sibling of p43
43	c.728G>A	p.R243Q	c.728G>A	p.R243Q	Consanguinity, sibling of p42
44	c.163+1G>A (IVS2+1G>A)		c.163+1G>A (IVS2+1G>A)		Consanguinity
45	c.194T>C	p.I65T	c.1222C>T	p.R408W	
46	c.1208C>T	p.A403V	c.1222C>T	p.R408W	

(continued)

Table 1 (continued)

ID	Allele 1		Allele 2		
	cDNA[a]	Protein	cDNA[a]	Protein	Comments[b]
47	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
48	c.721C>T	p.R241C	c.782G>A	p.R261Q	Sibling of p49
49	c.721C>T	p.R241C	c.782G>A	p.R261Q	Sibling of p48
50	c.194T>C	p.I65T	c.912+1G>A (IVS8+1G>A)		
51	c.1066-11G>A (IVS10-11G>A)		c.1241A>G	p.Y414C	Sibling of p110
52	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
53	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
54	c.169-?_352+?del (exon 3 deletion)		c.1241A>G	p.Y414C	
55	c.733G>C	p.V245L	c.1223G>A	p.R408Q	
56	c.896T>G	p.F299C	c.1315+1G>A (IVS12+1G>A)		Sibling of p57
57	c.896T>G	p.F299C	c.1315+1G>A (IVS12+1G>A)		Sibling of p56
58	c.117C>G	p.F39L	c.1222C>T	p.R408W	
59	c.754C>T	p.R252W	c.1315+1G>A (IVS12+1G>A)		
60	c.143T>C	p.L48S	c.1066-3C>T (IVS10-3C>T)		
61	c.782G>A	p.R261Q	c.782G>A	p.R261Q	Consanguinity
62	c.1222C>T	p.R408W	c.1241A>G	p.Y414C	
63	c.653G>T	p.G218V	c.842C>T	p.P281L	
64	c.510-1G>A (IVS5-1G>A)		c.1066-11G>A (IVS10-11G>A)		c.510-1G>A novel
65	c.1066-11G>A (IVS10-11G>A)		c.1222C>T	p.R408W	Sibling of p66
66	c.1066-11G>A (IVS10-11G>A)		c.1222C>T	p.R408W	Sibling of p65
67	c.168+1G>T (IVS2+1G>T)		c.331C>T	p.R111*	c.168+1G>T novel
68	c.194T>C	p.I65T	c.1315+1G>A (IVS12+1G>A)		
69	c.168+1G>A (IVS2+1G>A)		c.745C>T	p.L249F	
70	c.926C>T	p.A309V	c.1222C>T	p.R408W	
71	c.1139C>T	p.T380M	c.1315+1G>A (IVS12+1G>A)		
72	c.311C>T	p.A104D	c.733G>C	p.V245L	
73	1243G>A	p.D415N	c.1315+1G>A (IVS12+1G>A)		
74	c.311C>T	p.A104D	c.806delT	p.I269fs	
75	c.473G>A	p.R158Q	c.842C>T	p.P281L	
76	c.1042C>G	p.L348V	c.1241A>G	p.Y414C	
77	c.441+5G>T (IVS4+5G>T)		c.1184C>G	p.A395G	
78	c.1157A>G	p.Y386C	c.1315+1G>A (IVS12+1G>A)		
79	c.143T>C	p.L48S	c.842+3G>C (IVS7+3G>C)		
80	c.510-?_706+?del (exon 6 deletion)		c.1241A>G	p.Y414C	
81	c.754C>T	p.R252W	c.1066-11G>A (IVS10-11G>A)		
82	c.169-2A>G (IVS2-2A>G)		c.814G>T	p.G272*	c.169-2A>G novel
83	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
84	c.194T>C	p.I65T	c.194T>C	p.I65T	
85	c.1033G>T	p.A345S	c.1222C>T	p.R408W	Sibling of p86 and p109
86	c.261C>A	p.S87R	c.1222C>T	p.R408W	Sibling of p85 and p109
87	c.727C>T	p.R243*	c.1315+1G>A (IVS12+1G>A)		
88	c.194T>C	p.I65T	c.1222C>T	p.R408W	
89	c.1241A>G	p.Y414C	c.1241A>G	p.Y414C	
90	c.1159A>G	p.E390G	c.1241A>G	p.Y414C	
91	c.140C>T	p.A47V	c.838G>A	p.E280K	
92	c.117C>G	p.F39L	c.1315+1G>A (IVS12+1G>A)		

(continued)

Table 1 (continued)

ID	Allele 1		Allele 2		
	cDNA[a]	Protein	cDNA[a]	Protein	Comments[b]
93	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
94	c.1042C>G	p.L348V	c.1315+1G>A (IVS12+1G>A)		
95	c.782G>A	p.R261Q	c.1315+1G>A (IVS12+1G>A)		
96	c.503delA	p.Y168fs	c.842+3G>C (IVS7+3G>C)		
97	c.307G>T	p.G103C	c.461A>T	p.Y154F	p.Y154F novel
98	c.473G>A	p.R158Q	c.781C>T	p.R261*	
99	c.782G>A	p.R261Q	c.1045T>C	p.S349P	
100	c.331C>T	p.R111*	c.1208C>T	p.A403V	
101	c.727C>T	p.R243*	c.1222C>T	p.R408W	
102	c.727C>T	p.R243*	c.1222C>T	p.R408W	
103	c.912+1G>A (IVS8+1G>A)		c.1222C>T	p.R408W	
104	c.194T>C	p.I65T	c.814G>T	p.G272*	
105	c.284_286delTCA	p.I95del	c.1045T>C	p.S349P	
106	c.838G>A	p.E280K	c.1315+1G>A (IVS12+1G>A)		
107	c.117C>G	p.F39L	c.1315+1G>A (IVS12+1G>A)		
108	c.194T>C	p.I65T	c.441+5G>T (IVS4+5G>T)		
109	c.261C>A	p.S87R	c.1222C>T	p.R408W	Sibling of p85 and p86
110	c.1066-11G>A (IVS10-11G>A)		c.1241A>G	p.Y414C	Sibling of p51
111	c.818C>T	p.S273F	c.1222C>T	p.R408W	

[a] Numbering based upon reference sequence NM_000277.1, with the A from the ATG translation initiation start site numbered +1. Protein nomenclature based upon NP_000268.1

[b] 'Novel' indicates mutations not reported in *PAH*db (last accessed 07 August 2013, Scriver et al. 2003)

of the study. The genotypes of these patients were determined as part of this study and are summarized in Table 3. In four patients with a positive BH_4 response, the BH_4-responsive alleles were likely to be p.F39L, p.L48S and p.Y414C since the second alleles were null alleles. The fifth patient with a positive BH_4 response had two missense alleles (p.I65T and p.S273F), both of which may potentially be responsive alleles. From similar deductions in the patients with intermediate responses, other possible BH_4-responsive alleles were p.A104D, p.Y154F and p.R261Q. The mutation p.R261Q has been reported in BH_4-responsive patients (including a homozygous patient reported in Hennerman et al. 2005), whereas the mutation p.A104D has been identified in responsive patients compound heterozygous with a null second allele (Wang et al. 2007). Two of the alleles, p.D145V and p.V245L, have not been reported in BH_4-responsive patients previously. In addition, a nonresponsive patient from our cohort also had the p.V245L mutation, indicating it is less likely to be a BH_4-responsive allele. However, predictions based on genotype may not always give the correct BH_4 phenotype. The identical genotype p.[F39L];[R408W] was observed in three patients (#6, #14, #29), all three of which showed different responses to the BH_4 treatment. Of note, it has been previously reported that, contrary to what one might expect, a patient homozygous for the p.R408W mutation was BH_4 responsive (Leuzzi et al. 2006), but one of our patient's homozygous for this mutation was not responsive (data not shown). The other discrepant results were observed (patients #50 and #55). The former was heterozygous for p.I65T, which has been previously associated with BH_4 responsiveness (www.biopku.org/home/pah.asp). Similarly the p.R408Q allele carried by patient #55 is also considered a BH_4-responsive allele (www.biopku.org/home/pah.asp).

Discussion

The mutation analysis of 111 PKU patients in NSW, Australia, showed a wide spectrum of *PAH* mutations, with 61 distinct mutations present in 201 independent alleles. The two most common mutations, p.R408W and c.1315+1G>A, account for over 30% of the alleles and the nine most common mutations account for 57.2% of alleles. A comparison between the mutation spectra from NSW and Victoria (VIC), Australia (and between other world regions), is shown in Table 2. Interestingly, p.I65T was

Table 2 Allele frequencies (%) of the nine most common mutations from this study (bold) and selected others compared with those in other populations (minimum number of alleles = 100, with the exception of VIC, Australia) and their frequencies as reported in *PAH*db (http://www.pahdb.mcgill.ca)

Population	Number of alleles	Mutations (allele frequency %)														
		p. R408W	**c.1315 +1G>A**	**p. I65T**	**p. Y414C**	**p. R261Q**	**p. F39L**	**p. F299C**	**p. P281L**	**p. R243***	c.1066-11G>A	p. L48S	p. R243Q	Ex6-96A>G	p. R111*	p. R413P
NSW, Australia (this study)	201	18	12	5.5	5.0	5.0	3.5	3.0	2.5	2.5	2.0	1.5	1.0	0.0	1.5	1.0
VIC, Australia (Ramus et al. 1995)	83	19	16	18	0.0	4.8	3.6	2.4	1.2	2.4	6.0	1.2	0.0	0.0	0.0	0.0
England (Zschocke 2003)	124	10	27	8.0	2.0	0.0	3.0	3.0	2.0	0.0	4.0	7.0	0.0	0.0	0.0	0.0
Germany (Aulehla-Scholz and Heilbronner 2003)	438	25	10	0.0	4.6	3.9	0.7	0.9	3.7	2.1	3.0	2.1	0.0	0.0	0.0	1.0
Ireland (O'Donnell et al. 2002)	558	41	2.0	10	1.0	0.0	12	4.0	0.0	3.0	1.0	0.0	0.0	0.0	0.0	0.0
Lithuania (Kasnauskiene et al. 2003)	184	73	0.0	0.0	0.5	0.5	0.0	0.0	1.0	0.0	0.5	0.0	0.0	0.0	0.5	0.0
Denmark (Guldberg et al. 1993)	308	18	37	0.0	10	2.0	1.0	0.0	1.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0
Catalonia, Spain (Mallolas et al. 1999)	198	2.9	0.5	5.6	1.2	5.6	0.0	0.0	0.0	2.2	13	0.5	3.4	0.0	1.1	0.0
Italy (Giannattasio et al. 2001)	289	0.7	1.0	0.0	2.1	14	0.0	0.0	3.1	0.7	19	9.7	0.0	0.0	0.0	0.0
Turkey (Dobrowolski et al. 2011)[a]	1176	5.9	0.0	0.2	0.0	7.7	0.0	0.0	7.7	1.4	23	6.6	0.7	0.0	0.3	0.3
Brazil (Acosta et al. 2001)	230	3.5	1.3	3.5	0.0	12	0.0	0.0	2.1	0.0	17	0.8	1.3	0.0	0.0	0.0
USA (Guldberg et al. 1996)	294	19	7.8	4.1	5.4	2.4	4.1	1.0	2.7	1.0	4.4	1.7	0.0	0.3	0.3	0.0
USA (Enns et al. 1999)	267	15	8.2	4.5	2.6	1.5	2.2	2.2	3.4	0.8	7.2	2.2	1.1	0.4	0.4	0.0
China, northern (Song et al. 2005)	370	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22	11	8.7	7.0
*PAH*db (Scriver et al. 2003)	3206	6.7	2.8	4.1	3.6	2.1	1.3	1.4	2.9	1.1	5.2	2.0	0.9	0.5	0.6	0.4

[a] The number of independent alleles was not clear from the data provided. Allele frequencies are calculated using total number of chromosomes tested

Table 3 Genotypes and BH_4 responsiveness of patients tested in Mitchell et al. (2005)

BH_4 response[a]	ID	Allele 1	Allele 2	Putative BH_4-responsive allele[b]
Positive	#14	p.F39L	p.R408W	p.F39L
	#25	p.L48S	p.R408W	p.L48S
	#30	p.R408W	p.Y414C	p.Y414C
	#32	p.I65T	p.S273F	p.I65T or p.S273F
	#80	Exon 6 deletion	p.Y414C	p.Y414C
Intermediate	#8	p.I65T	p.D145V	p.I65T or p.D145V
	#17	p.Y154F	IVS12+1G>A	p.Y154F
	#29	p.F39L	p.R408W	p.F39L
	#31	p.R261Q	p.P281L	p.R261Q
	#54	Exon 3 deletion	p.Y414C	p.Y414C
	#72	p.A104D	p.V245L	p.A104D
Negative	#6	p.F39L	p.R408W	(p.F39L)
	#33	p.A90fs	p.A90fs	
	#44	p.R243Q	p.R243Q	
	#50	p.I65T	IVS8+1G>A	(p.I65T)
	#53	p.R408W	IVS12+1G>A	
	#55	p.V245L	p.R408Q	(p.R408Q)
	#69	IVS2+1G>A	p.L249F	
	#70	p.A309V	p.R408W	
	#96	p.Y168fs	IVS7+3G>C	

[a] BH_4 response determined by changes in blood Phe levels after BH_4 supplementation (20 mg/kg/day). Positive = more than 30 % decrease in blood Phe after 8 h; intermediate = some decrease in blood Phe during the duration of study (7 days); negative = no decrease in blood Phe

[b] Putative BH_4-responsive allele determined by presence of null allele or homozygosity or hemizygosity in previously reported BH_4-responsive patients; some of these alleles are also found in nonresponsive patients (*in brackets*). Full details of BH_4 loading test can be found in Mitchell et al. (2005)

more common in VIC than in NSW (Ramus et al. 1995), but otherwise the distribution of mutations in the two populations was similar. The p.R408W mutation was the predominant mutation in Ireland, Latvia and Lithuania (O'Donnell et al. 2002; Kasnauskiene et al. 2003; Pronina et al. 2003), whereas c.1315+1G>A had the highest frequency in England, Germany and Denmark (Guldberg et al. 1993; Aulehla-Scholz and Heilbronner 2003; Zschocke 2003). Other examples of region-specific mutations include c.1066-11G>A from Mediterranean regions and p.R243Q and p.R413P from Asia (Chien et al. 2004; Lee et al. 2004; Song et al. 2005). The low incidence of these mutations in our cohort reflects the high cultural diversity in NSW, Australia (Australian Bureau of Statistics 2012).

Pathogenic mutations were not found in only two of the alleles tested. Polymorphisms in the location of the PCR primers may lead to the drop-out of an allele during amplification, as well as complex tertiary structures in the DNA and poor template quality or quantity (Tvedebrink et al. 2009; Saunders et al. 2010). Alternate primer sets or whole exome/genome sequencing may be of use in the identification of the second alleles in these patients, as mutations further into the intron or in the promoter or untranslated regions were not investigated in this study. Also, large complex chromosomal arrangements not affecting copy number, such as gene inversions and balanced translocations, could not be ruled out as a cause of PAH deficiency.

Tetrahydrobiopterin (BH_4) is the natural cofactor of the PAH enzyme, and its synthetic form, sapropterin, has been approved for use as treatment of PKU (Cajigal 2008), although it is currently not widely available in Australia. The treatment is not suitable for all patients, and there is a general observation that patients with mild PKU or HPA are more likely to respond to BH_4 (Bernegger and Blau 2002). Indeed, screening of BH_4-responsive patients found that there are certain mutations associated with BH_4 responsiveness and that these mutations are generally ones previously classed as leading to a mild phenotype (Muntau et al. 2002). Following the assumption (Muntau et al. 2002) that patients with at least one BH_4-responsive mutation would be responsive to BH_4, as many as 47 % of patients in our cohort may benefit from this treatment.

A number of the patients in the NSW cohort have been previously tested for BH_4 responsiveness (Mitchell et al. 2005). The correlation between genotypes and BH_4 responses in these patients is uncertain (Table 3), supporting previous findings that genotype is not an absolute predictor of BH_4 responsiveness (Lindner et al. 2001; Karačić et al. 2009). There are various reasons proposed for the difficulty of correlating BH_4 responsiveness with genotype. First, the methods of ascertaining BH_4 responsiveness have changed with time, and there may also be differences in the interpretation of responsiveness between the centers at which the tests were carried out (Mitchell et al. 2005; Fiege and Blau 2007; Anjema et al. 2011). Individuals may differ in their rate of BH_4-absorption, protein catabolic rate and Phe intake during the test. There is also a suggestion that the combination of the genotypes may be of greater significance than the presence of the individual alleles due to the multimeric nature of the PAH enzyme and the majority of PKU patients being compound heterozygotes for two different mutations (Scriver and Kaufman 2001). On the other hand, improvement in enzymatic activity in certain mutant PAH proteins expressed in BH_4 supplemented media supports the notion that genotype indeed plays a role in determining BH_4 response (Kim et al. 2006). Regardless, genotypic information may allow patients with BH_4-responsive mutations to be prioritized for BH_4 trials ahead of those with only known nonresponsive mutations.

In summary, we have undertaken *PAH* mutation screening in a large clinic-based cohort of PKU patients from NSW and have examined phenotype–genotype correlations, including potential BH_4 responsiveness. Mutation analysis of PKU patients in NSW, Australia, revealed a wide spectrum of mutations present similar to what has been reported in other Caucasian populations. The decreasing costs of direct sequencing or the use of next generation sequencing technology will facilitate widespread genotyping of all PKU patients, as well as carrier testing for family members. These methods are not appropriate for the detection of large exonic deletions, although these remain rare causes of PKU. Mutation screening may assist with decisions relating to disease severity and management, especially with regard to the detection of alleles associated with BH_4 responsiveness and the identification of patients who may benefit from being given access to this treatment and other novel therapies.

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Conflict of Interest

The authors declare no conflict of interest.

Synopsis

Mutation screening of a large cohort of phenylketonuria patients in Australia found a wide spectrum of mutations in this population, with a high proportion of patients likely to benefit from sapropterin treatment.

Compliance with Ethics Guidelines

Gladys Ho, Ian Alexander, Kaustuv Bhattacharya, Barbara Dennison, Carolyn Ellaway, Sue Thompson, Bridget Wilcken and John Christodoulou declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Details of Contributions of Individual Authors

GH

Design of experiments, implementation of experiments, data analysis and manuscript preparation

IA, KB, BD, CE, ST, BW, JC

Design of experiments, clinical information and manuscript preparation

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CASE REPORT

Extraosseous Extension Caused by Epidural Hematoma in Gaucher Disease Mimicking Malignant Bone Tumor

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Abstract Gaucher disease is an inherited autosomal-recessive disorder caused by the defective hydrolysis of glucocerebroside. The resultant hepatosplenomegaly, hematological changes, and orthopedic complications are the predominant symptoms. However, extraosseous manifestation of Gaucher disease, mimicking malignant bone tumor, is supposed to be rare. No reports of extraosseous manifestation of Gaucher disease caused by epidural hematoma were identified in the English literature. A 64-year-old man visited a nearby clinic for low back pain and was referred to our tumor clinic on suspicion of malignant bone tumor on sacral MRI. MRI revealed a demarcated solid lesion extending into the surrounding soft tissues on both sides of the sacral roots. During preoperative examination, he suffered from pathologic fracture in right mid-femur. We performed internal fixation with intramedullary nailing, simultaneously harvesting tissue specimens. Histopathological analysis showed aggregates of Gaucher cells in the right femur and hematoma in the sacrum. Epidural hematoma in Gaucher disease, usually attributed to thrombocytopenia, is a rare manifestation of skeletal complication, mimicking malignant processes.

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Competing interests: None declared

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Introduction

Gaucher disease is an inherited autosomal recessive disorder characterized by a heterogeneous set of signs and symptoms caused by the defective hydrolysis of glucocerebroside. A deficiency in the enzyme glucocerebrosidase (glucosylceramidase, acid β-glucosidase) leads to the accumulation of glucocerebroside in the spleen, liver, and bone marrow. The resultant hepatosplenomegaly, hematological changes, and orthopedic complications are the predominant symptoms. For many years, knowledge regarding the hematological and visceral aspects of this condition has exceeded knowledge regarding its skeletal manifestations. Today bone involvement is known to be frequent. Especially it occurs in approximately 75 % of Gaucher disease type I, the most frequent type of Gaucher disease. Bone lesions range from mild osteopenia, medullary expansion and remodeling defects to osteonecrosis of the femur or humeral heads and spinal cord compression from vertebral collapse (Lutsky and Tejwani 2007; Mikosch and Hughes 2010). Extraosseous manifestation of Gaucher disease, mimicking malignant bone tumor, is supposed to be rare. We here report the first case in which extraosseous manifestation is caused by not Gaucher cell deposits but epidural hematoma.

Case Report

About 20 years previously, a 64-year-old man was diagnosed as having Gaucher disease type I, based on findings of thrombocytopenia associated with hepatosplenomegaly, although he had not received any enzyme therapy. He visited a nearby clinic for low back pain and was referred to our tumor clinic on suspicion of malignant

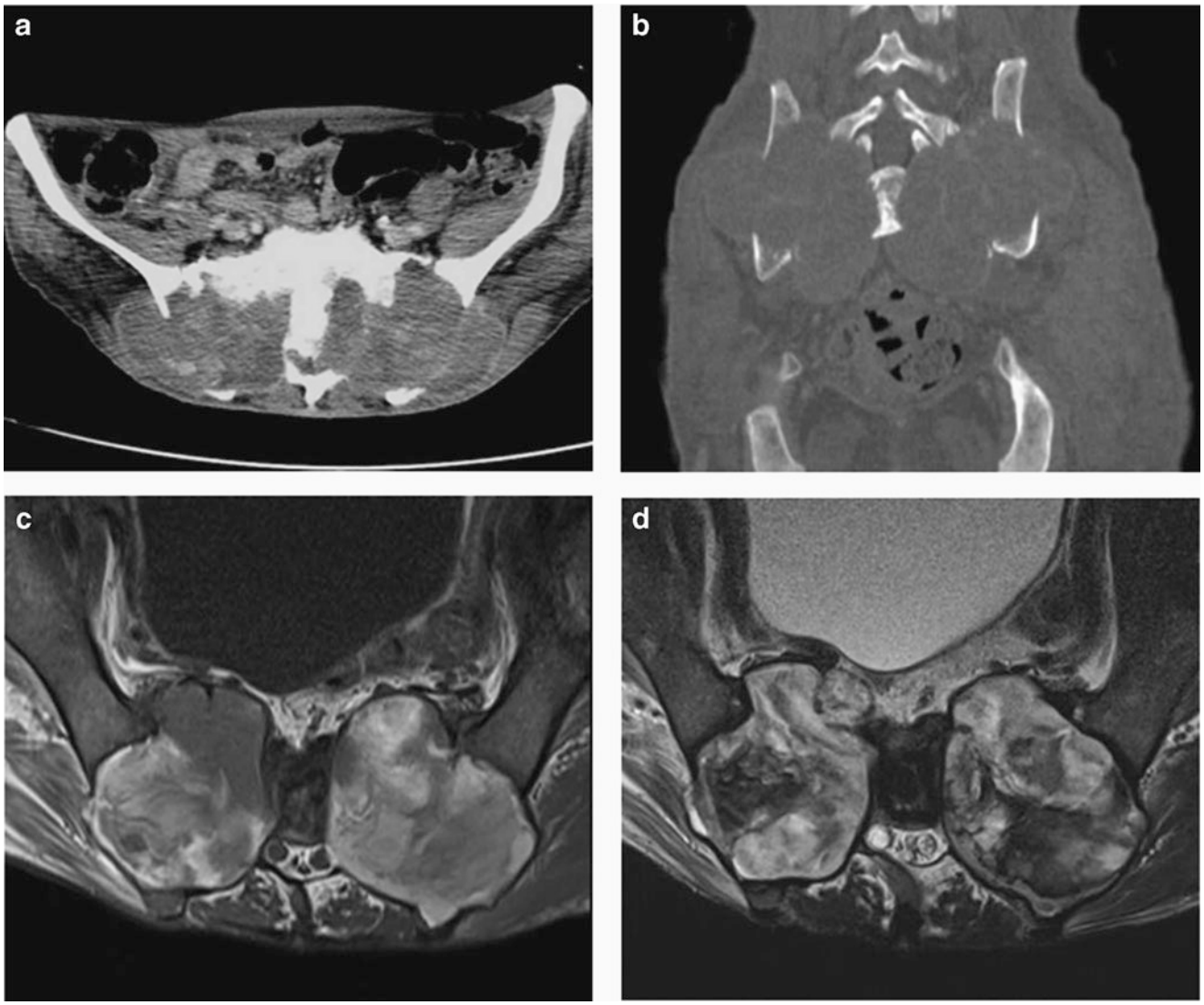

Fig. 1 CT scan demonstrates large bone destruction around both iliosacral joint in axial view (**a**) and frontal view (**b**). (**c**) T1-weighted image shows iso-high-mixed masses extending into the surrounding soft tissues on both sides of the sacral roots, consistent with hematoma. (**d**) T2-weighted image shows the masses become slightly heterogeneous

bone tumor. Physical examination showed a palpable hard mass in both gluteal region and no neurological defects. CT scan demonstrated large bone destruction around both iliosacral joint and MRI revealed a demarcated solid lesion extending into the surrounding soft tissues on both sides of the sacral roots (Fig. 1). The differential diagnoses were malignant bone tumors, Gaucher cell deposits, and chronic hematoma. During preoperative examination for open biopsy, he suffered from a pathologic fracture in the right mid-femur. We performed internal fixation with intramedullary nailing (Fig. 2), simultaneously harvesting tissue specimens of both tumors in the right femur and the sacrum. Histopathological analysis showed aggregates of Gaucher cells in the right femur and hematoma in the sacrum (Fig. 3). Low back pain was improved without further surgical intervention, and he received enzyme replacement therapy.

Discussion

Lysosomal storage occurs in reticuloendothelial cells and leads to hepatomegaly and splenomegaly, which causes hypersplenism and cytopenia. Bleeding is a frequent symptom in Gaucher disease type I. It is usually attributed to thrombocytopenia, although deficiencies of various coagulation factors and platelet dysfunctions have been reported. A spontaneous bleeding etiology as one of the musculoskeletal complications is extremely rare and very few case series have been reported. Six patients with Gaucher disease have been reported to present spontaneous iliopsoas hematoma (Flipo et al. 1992;

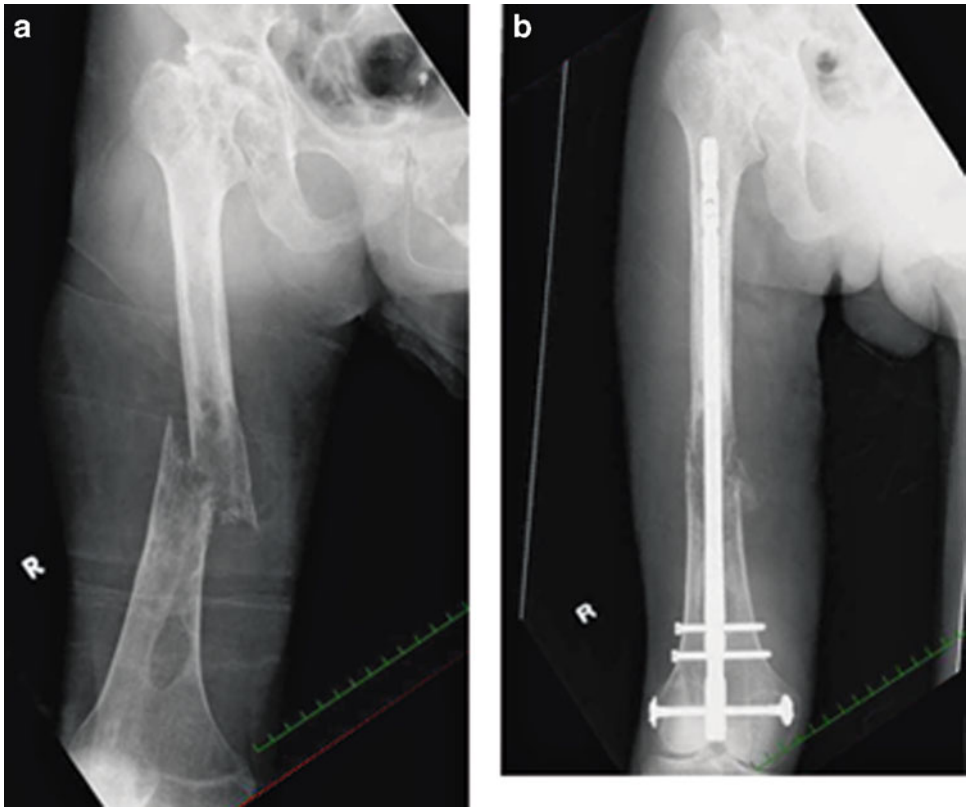

Fig. 2 Radiography of the right femur shows several osteolytic lesions and a pathological fracture (**a**) and intramedullary nailing fixation (**b**)

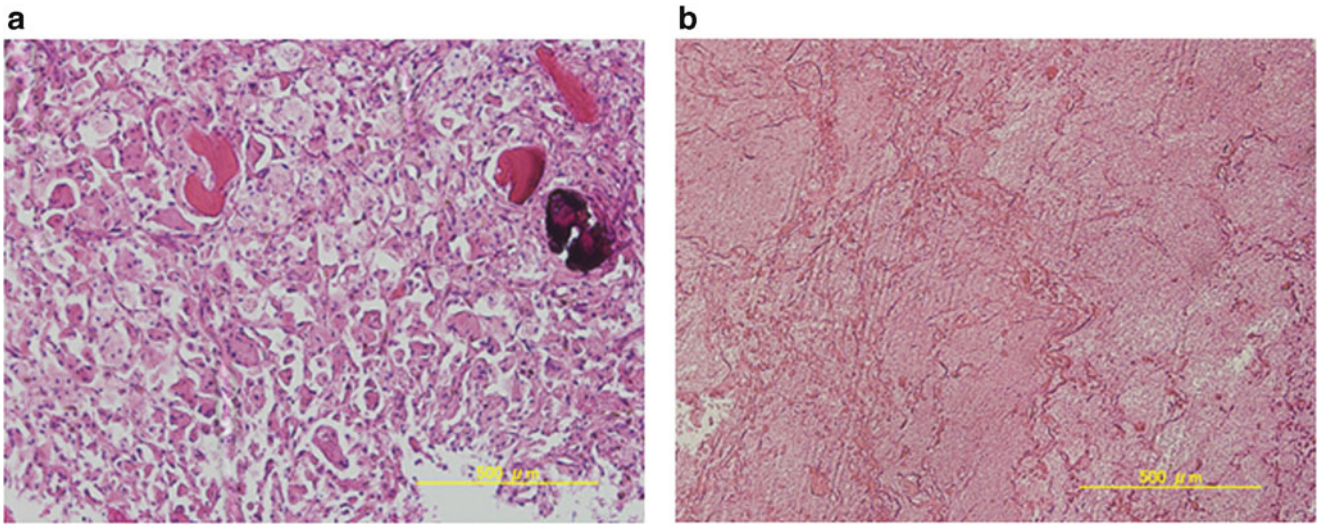

Fig. 3 Histopathological examination shows aggregates of histiocytes with striated cytoplasm, typical of Gaucher cells, in bone marrow of the right femur (**a**) and blood clot (**b**) without any cells in the sacrum (H&E, x300)

Jmoudiak et al. 2003; Lesić et al. 2006; Selton et al. 2011). Horev et al. reported that MRI examination revealed an unexpected high intramedullary as well as subperiosteal signal, suggesting a subacute hemorrhage or hematoma in five Gaucher disease patients (Horev et al. 1991). Grewal et al. described that a patient with Gaucher disease type I suffered from a spontaneous hematomyelia (Grewal et al. 1991). We here report the first case in which Gaucher disease should lead to epidural hematoma. Generally, an epidural hematoma causes serious findings in the lower extremities with neurological deficits and requires emergency evacuation of the hematoma. But this patient did not have severe

physical and neurological problems. The speculated reason is that repetitive bleeding in our case resulted in chronic asymptomatic epidural hematoma, showing gradually progressing bone destruction of the sacrum and slowly growing mass extending into surrounding soft tissues.

Patients with Gaucher disease type I frequently have diffuse hypergammaglobulinemia or monoclonal gammopathy of undetermined significance, and many case reports have documented the cooccurrence of Gaucher disease type I with hematological and nonhematological malignancies including primary malignant bone tumors (Shiran et al. 1993; Böhm et al. 2001; Choy and Campbell 2011). In Bohn's review (Böhm et al. 2001) of the literature, approximately 33 % of the malignancies in patients with Gaucher disease originated from the bone, which indicates a higher relative risk of developing malignant disorders of the bone to patients with Gaucher disease than to the general population. Extraosseous manifestation of Gaucher disease is supposed to be rare, but when cortical destruction with coexisting soft tissue mass is observed in patients with Gaucher disease, this benign process is difficult to differentiate from malignant bone tumors (Hermann et al. 1994). To the best of our knowledge, all reported cases described that extraosseous manifestations of Gaucher disease were caused by Gaucher cell deposits in the spine or extremities (Katz et al. 1993; Hermann et al. 1994; Kenan et al. 1996; Poll et al. 2000; Barone et al. 2000). There have been no previous descriptions that cortical destruction is due to epidural hematoma.

In conclusion, epidural hematoma in Gaucher disease is a rare manifestation of skeletal complication, mimicking malignant processes.

Conflict of Interest

Tadahiko Kubo, Shoji Shimose, Jun Fujimori, Ryo Shimizu, and Mitsuo Ochi declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from the patient for being included in the study.

Contributions of Individual Authors

Tadahiko Kubo contributed to the conception and design.

Shoji Shimose contributed to the analysis and interpretation of data.

Jun Fujimori contributed to the analysis and interpretation of data.

Ryo Shimizu contributed to drafting the article.

Mitsuo Ochi declares contributed to revising it critically.

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CASE REPORT

Thirteen Patients with MAT1A Mutations Detected Through Newborn Screening: 13 Years' Experience

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Abstract

Background: Methionine adenosyltransferase I/III (MATI/III) deficiency is the most common genetic cause of persistent isolated hypermethioninemia.

Patients and Methods: This is a retrospective data analysis of 62 newborns with elevated methionine detected by newborn screening between January 2000 and June 2013. The clinical, biochemical, and molecular findings of a subset of these children with *MAT1A* mutations associated with MATI/III deficiency are presented.

Results: Of the 62 newborns with elevated methionine, 12 were identified as having classical homocystinuria; 37 were false-positives; and 13 were found to have isolated persistent hypermethioninemia in the absence of biochemical markers of homocystinuria, abnormal liver function studies, or other causes of elevated methionine. These 13 individuals underwent genetic testing for changes in the *MAT1A* gene, associated with MATI/III deficiency. Three of 13 were found to have the common autosomal dominant R264H mutation, one was found to be a compound heterozygote for two novel pathogenic mutations, and three were found to be heterozygotes for previously reported mutations shown to cause autosomal recessive MATI/III deficiency when present in homozygous or a compound heterozygous configuration. The remaining six patients had variants of unknown clinical significance or novel mutations. For the majority of individuals, methionine persisted above the normal range but trended downward over time. None of these 13 individuals was started on a low-methionine diet, and all have age-appropriate growth and development.

Conclusion: These cases show that individuals with even single changes in the *MAT1A* gene may have elevations in methionine identified by newborn screening, which may persist for months after birth without any clinical consequences.

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Competing interests: None declared

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Introduction

Methionine adenosyltransferase I/III (MATI/III) deficiency is the most common genetic cause of persistently elevated methionine (Couce et al. 2008). Following the introduction of methionine into newborn screening programs to detect newborns with homocystinuria due to cystathionine beta-synthase (CBS) deficiency, a subset of children with persistently elevated methionine but with normal homocysteine levels were detected. No known molecular reason for their metabolic abnormalities was evident (Mudd et al. 1995). Subsequent investigation identified the cause of elevated methionine to be due to a deficiency of methionine adenosyltransferase, expressed in the liver, which is encoded by the *MAT1A* gene (Ubagai and Lei 1995). Following the discovery of this enzyme deficiency and gene, it was noted that there were two main inheritance patterns for this condition: (1) a less common autosomal recessive form, which in addition to persistently elevated methionine, in some cases, is also characterized by developmental delay and progressive brain demyelization, and (2) an autosomal dominant form, characterized by the R264H mutation, which is clinically benign (Mato et al. 2001; Ubagai and Lei 1995; Chamberlin et al. 1997, 2000).

Since 1995, 37 mutations in the *MAT1A* gene have been described in patients with MATI/III deficiency (Mudd 2011).

In this report, we discuss 13 newborns with elevated methionine levels detected through newborn screening, subsequently found to have mutations in the *MAT1A* gene. This represents the largest report of trends among individuals identified through newborn screening in the United States who have *MAT1A* mutations, many without the common R264H autosomal dominant mutation.

Patients and Methods

A retrospective chart review was conducted from January 2000 to June 2013 to identify all newborns referred to our center for elevated methionine identified on newborn screening. Eligible patients' records were reviewed, and biochemical laboratories, developmental status, and genetic test results were compiled for comparison. Patients are indicated by letter and number, the number corresponding to the order of which individuals in a single family were referred for evaluation.

Results

Sixty-two newborns with positive newborn screenings for elevated methionine came to the Children's Hospital of Philadelphia from January 2000 to June 2013. Our referral area includes the newborn screenings from the states of Pennsylvania, Delaware, and New Jersey. Methionine cut-offs for these states are <0.80 mg/dL (<53.6 umol/L), <45.0 umol/L, and <0.80 mg/dL (<53.6 umol/L), respectively.

All 62 newborns were evaluated at our center within the first 6 weeks of life to assess for an inborn error of metabolism responsible for the observed elevated methionine. Of the 62, 37 (59.7 %) were found to be false-positives and had no further elevation of methionine or other metabolites outside of the normal range. Twelve (19.3 %) were found to have CBS deficiency and were subsequently managed. The remaining 13 (20.9 %) were found to have persistently elevated methionine with normal or modest levels of total homocysteine and free homocystine, normal liver function studies, and no other observed cause for elevated methionine. Of note, the modest elevations in methionine were lower than the expected values for an infant with CBS deficiency. Of the 13, three sibling pairs were identified (patients B1, B2; C1, C2; D1, D2).

Of the 13 cases with persistently elevated methionine, the initial methionine level identified on newborn screening was not predictive of MATI/III deficiency, false-positives, or classical homocystinuria. All of the infants were either receiving breast milk, standard formula for infants, or a combination of the two. No infants received any specialized metabolic formulas. Eleven of 13 infants also had liver enzymes analyzed at their first clinical evaluation to assess for liver dysfunction as a cause for elevated methionine. Liver enzymes showed clinically unremarkable findings in all cases. See Table 1 for the initial screening and clinical labs.

Subsequent to the identification of persistently elevated methionine, genetic testing was performed to determine if MATI/III mutations could be identified to explain their biochemical profile. In all patients, at least one variant or mutation was identified in the *MAT1A* gene. Table 2 presents a summary of these findings.

Following the identification of elevated methionine and mutations/variants in the above patients, it was recommended that these patients continue to follow-up in our clinic for regular monitoring of methionine levels. A summary of the number of clinical evaluations and average methionine levels for these 13 patients can be seen in Table 1. All patients described in this series had average methionine levels higher than the normal range. However, for the majority of patients, these levels have fallen over time. The trends of the patients' methionine levels can be seen in Fig. 1.

To date, all patients described have had normal development by parental report and are meeting milestones at an age-appropriate level. All patients are eating a diet typical for a child their age.

Discussion

The majority of individuals with MATI/III deficiency have been identified through newborn screening programs. Several papers report their experience in the identification and treatment of these individuals, although the majority of these patients have been found to have the R264H autosomal dominant mutation or known autosomal recessive mutations (Couce et al. 2013; Chien et al. 2005; Couce et al. 2008; Martins et al. 2012). In 2013, Couce et al. described 18 patients identified in newborn screening programs in Spain with MATI/III deficiency, 15 of which had the R264H mutation. Follow-up data were also reported for these patients and similarly shows that even in individuals without clinical features, methionine levels continue to remain elevated. Mean values of methionine are only presented, however, and trend data is not available (Couce 2013). In 2005, Chien et al. described 16 individuals with hypermethioninemia identified through neonatal screening in Taiwan over a 12-year time period. Of these, nine had mutations in the *MAT1A* gene, four had

Table 1 Initial screening and biochemical values and mean methionine values for patients and with persistently elevated methionine first identified on newborn screening

Patient	State	Age at first visit (weeks)	Breast milk/Formula	Met levels – NBS (nmol/mL)	Met levels – First visit (nmol/mL) 8–49	Initial tHcy (umol/L) 0–14	Initial ALT (U/L) 7–50	Initial AST (U/L) 22–71	Mean Met (nmol/mL) 8–49	Age at last Eval (months)
A1	PA	3	Formula	42.9	80.8	4.7	33	64	80.8 (1)*	0.67
B1	NJ	6	Formula (Enfamil with Iron)	195.0	218.9	15.04 (H)	35	25	218.9 (1)*	1.3
B2	NJ	2	Breast Milk, Formula Supplement (Enfamil with Iron)	126.0	232.8	7.8	30	25	258.76 (11)*	37.2
C1	PA	3.5	Formula (Carnation Good Start)	99.9	181.1	10.64	<6	43	87.2 (3) *	29.0
C2	PA	6	Formula (Carnation Good Start)	42.9	523.2	18.8 (H)	7	47	285.6 (2)*	10.6
D1	PA	5	Formula (Enfamil with Iron)	64.3	209.8	9.71	n/a	n/a	138.8 (5)*	16.2
D2	PA	4	Formula (Carnation Good Start)	64.3	112.8	n/a	n/a	n/a	88.75 (2)*	5.1
E1	NJ	2	Breast Milk	86.5	406.4	13.57	35	91	298.5 (4)*	8.4
F1	PA	2	Formula (Similac/Carnation Good Start)	70.4	88.9	5.76	39	28	63.4 (5) *	24.3
G1	PA	2	Breast Milk, Formula Supplement	52.9	84.8	6.23	22	56	76.2 (5)*	16.1
H1	DE	4	Formula (Similac Advance)	71.5	235.5	9.36	8	106	244.94 (5) *	6.9
I1	PA	3.5	Breast Milk	64.3	80.00	6.95	33	61	63.1 (3)*	4.8
J1	PA	2.7	Formula (Carnation Good Start Gentle)	47.6	114.3	4.32	23	91	97.5 (2)*	2.5

Met Methionine, *tHcy* total homocysteine, *ALT* Alanine transaminase, *AST* Aspartate aminotransferase

( )* number of measurements of plasma methionine

Table 2 Variant/Mutations observed in 13 patients with persistently elevated methionine

	Genotype	Genotype information
A1	R219L	Novel – Variant of Unknown Clinical Significance
B1	R264H	Reported Previously – Autosomal Dominant (Mato et al. 2001)
B2	R264H	Reported Previously – Autosomal Dominant (Mato et al. 2001)
C1	A55D	Reported Previously – Autosomal Recessive (Ubagai and Lei 1995)
C2	A55D	Reported Previously – Autosomal Recessive (Ubagai and Lei 1995)
D1	A259V	Reported Previously – unclear inheritance pattern (Fernandez-Irigoyet et al. 2010)
D2	A259V	Reported Previously – unclear inheritance pattern (Fernandez-Irigoyet et al. 2010)
E1	I37T/G91S	Novel – Suspected Pathogenic Novel – Suspected Pathogenic
F1	R177W	Novel – Suspected Pathogenic
G1	M138V	Novel – Variant of Unknown Clinical Significance
H1	R264H	Reported Previously – Autosomal Dominant (Mato et al. 2001)
I1	P357L	Reported Previously – Autosomal Recessive (Ubagai and Lei 1995)
J1	R177W	Reported Previously (see patient F1) – unclear inheritance pattern

at least one copy of the known autosomal recessive R264H mutation, two were compound heterozygotes for known autosomal recessive mutations, and the remaining three were heterozygotes or compound heterozygotes for novel mutations that were suspected to be pathogenic. The majority of patients were put on a restricted diet, and all individuals were reported to have normal growth, development, and neurological examinations (Chien et al. 2005). In 2008, Couce et al. described five individuals with MATI/III deficiency identified over 7 years' time through newborn screening in Galicia, Spain. All five patients were heterozygotes for the R264H autosomal dominant mutation and were kept on diets not exceeding recommended protein requirements for their age. At the time of publication, with a mean of 2.5 years of clinical follow-up, all individuals were asymptomatic (Couce et al. 2008). Finally, in 2011, Martins et al. reported 11 cases of MATI/III deficiency identified through the Portuguese Newborn Screening Laboratory. All cases reported were heterozygotes for the R264H autosomal dominant mutation. All individuals had normal development; however, one patient was found to have myelination abnormalities of unknown clinical significance (Martins et al. 2012).

Our report is the first that focuses on the trend of methionine levels in individuals with *MAT1A* mutations other than the common R264H autosomal dominant mutation. The majority of patients in this case report are heterozygotes for known autosomal recessive mutations, variants of unknown clinical significance, or reported mutations with unclear inheritance patterns. The majority of individuals in this study presented with methionine levels that trended downward upon follow-up. None of the individuals reported in this series have had any symptoms of MATI/III deficiency. All patients have normal development and tolerate diets typical for other infants and children their age.

Of note, three sibling pairs were identified in this study, B1 and B2, C1 and C2, and D1 and D2. B1 and B2 are heterozygotes for the common R264H mutation and are difficult to compare because B1 has only had one clinical evaluation to date. Patients C1 and C2, heterozygotes for the A55D autosomal recessive mutation, exhibit a similar trend of methionine, with both falling substantially over time and eventually falling within the normal range. The values, however, differ drastically. As observed in Table 1 and Fig. 1, C2's initial methionine level is nearly double that of C1. Similarly, siblings D1 and D2, despite having identical mutations, have varying patterns of methionine. As shown in Fig. 1, D1's levels fell sharply following initial presentation, rose again, and have relatively leveled off, still above the normal threshold for methionine levels. Patient D2, however, showed a sharp decrease and is near the upper limit of normal for methionine levels.

Thus, we conclude the following from this report: (1) Our data shows that newborns with single *MAT1A* mutations other than the common R264H mutation can present with elevated methionine levels and be detected by newborn screening and, if followed over time, will continue to have methionine elevations outside the normal range. (2) Although increased methionine levels persisted for months after birth, none of the patients had any clinical symptoms or developmental delay. (3) Siblings with identical mutations or variants in the *MAT1A* gene do not have identical trends of methionine levels, which may reflect different methionine intake in diet or could suggest that there may be other modifier genes that influence methionine values. (4) Clinicians who evaluate patients following positive newborn screenings for methionine should be aware that

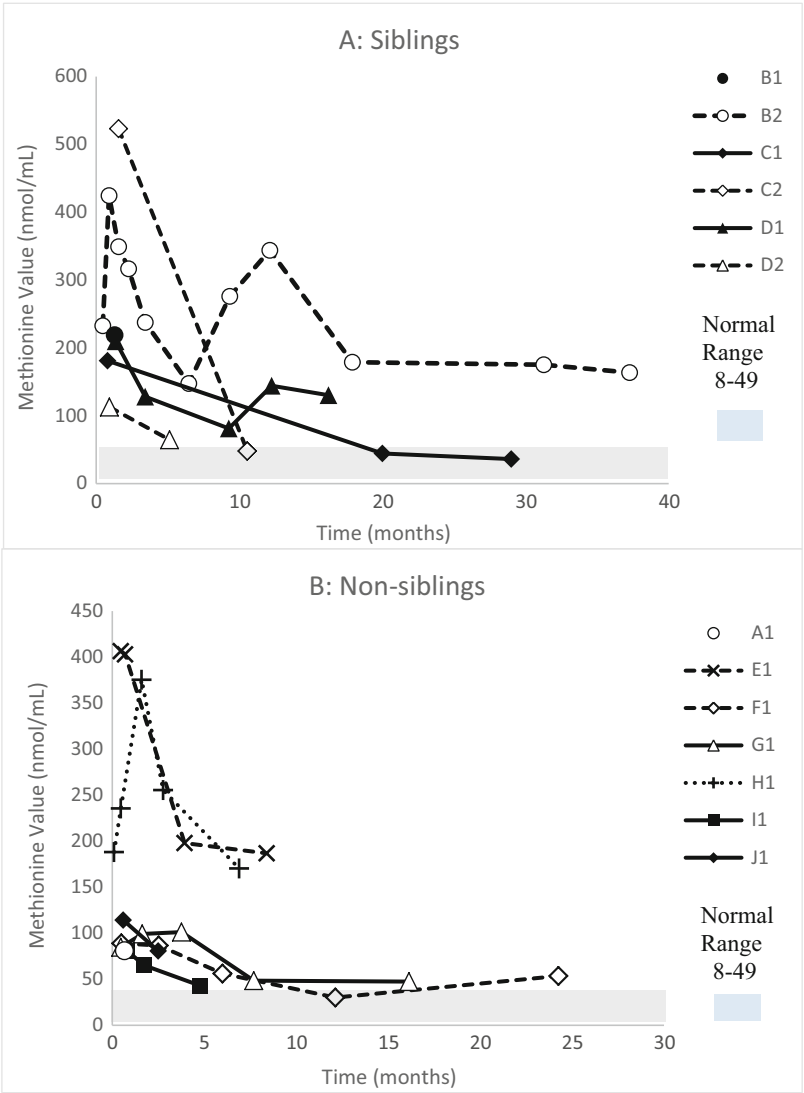

Fig. 1 Methionine trends in patients with MATIA mutations. (**a**) Data for sibling pairs. (**b**) Data for non-siblings

isolated persistent elevation of methionine could be caused by single mutations in the *MAT1A* gene. (5) Increasing cut-off values for methionine may impede detection of newborns carrying benign mutations in the *MAT1A* gene. However, to ensure that no cases of CBS deficiency are missed, homocysteine should also be introduced into newborn screening panels.

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ORIGINAL ARTICLE

AGC1 Deficiency Causes Infantile Epilepsy, Abnormal Myelination, and Reduced *N*-Acetylaspartate

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Abstract Background: Whole exome sequencing (WES) offers a powerful diagnostic tool to rapidly and efficiently sequence all coding genes in individuals presenting for consideration of phenotypically and genetically heterogeneous disorders such as suspected mitochondrial disease. Here, we report results of WES and functional validation in a consanguineous Indian kindred where two siblings presented with profound developmental delay, congenital hypotonia, refractory epilepsy, abnormal myelination, fluctuating basal ganglia changes, cerebral atrophy, and reduced *N*-acetylaspartate (NAA).

Methods: Whole blood DNA from one affected and one unaffected sibling was captured by Agilent SureSelect Human All Exon kit and sequenced on the Illumina HiSeq2000. Mutations were validated by Sanger sequencing

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in all family members. Protein from wild-type and mutant fibroblasts was isolated to assess mutation effects on protein expression and enzyme activity.

Results: A novel *SLC25A12* homozygous missense mutation, c.1058G>A; p.Arg353Gln, segregated with disease in this kindred. *SLC25A12* encodes the neuronal aspartate-glutamate carrier 1 (AGC1) protein, an essential component of the neuronal malate/aspartate shuttle that transfers NADH and H^+ reducing equivalents from the cytosol to mitochondria. AGC1 activity enables neuronal export of aspartate, the glial substrate necessary for proper neuronal myelination. Recombinant mutant p.Arg353Gln AGC1 activity was reduced to 15% of wild type. One prior reported *SLC25A12* mutation caused complete loss of AGC1 activity in a child with epilepsy, hypotonia, hypomyelination, and reduced brain NAA.

Conclusions: These data strongly suggest that *SLC25A12* disease impairs neuronal AGC1 activity. *SLC25A12* sequencing should be considered in children with infantile epilepsy, congenital hypotonia, global delay, abnormal myelination, and reduced brain NAA.

Introduction

Whole exome sequencing (WES) is gaining wide recognition as a powerful approach to rapidly and efficiently evaluate for coding mutations that cause a wide range of inherited monogenic diseases. In particular, there is a growing utilization of WES to evaluate individuals presenting for diagnostic evaluation of highly heterogeneous conditions, such as suspected mitochondrial disease (McCormick et al. 2013). In this study, a consanguineous Indian family presented in the Mitochondrial-Genetics Diagnostic Clinic for evaluation of a pathogenic etiology for two siblings with intractable epilepsy, global developmental delay, and congenital hypotonia in whom a mitochondrial disorder had previously been suspected but not confirmed by muscle-based enzymatic analysis. An extensive array of clinical diagnostic genetic testing performed to evaluate for known mitochondrial DNA and nuclear DNA causes of early infantile epileptic encephalopathy or mitochondrial diseases was unrevealing. Indeed, it is well known that muscle biopsies can be unrevealing of a specific histologic or biochemical abnormality in children even with known mitochondrial diseases (Haas et al. 2008) and that sequencing of all currently known mitochondrial disease genes is likely to identify a specific genetic etiology for only up to one-half of individuals with clinical manifestations of possible mitochondrial disease (Calvo et al. 2012). Following completion of mitochondrial DNA (mtDNA) genome sequencing and mtDNA deletion analysis in the proband's muscle, an autosomal recessive monogenic cause of disease was assumed to be present based on a similarly severe presentation in the proband's brother and known consanguinity. The family was therefore enrolled in a research study to pursue family-based whole exome sequencing to identify the specific genetic cause of their suspected mitochondrial disease.

Methods

Study participant description. Written informed consent for participation in The Children's Hospital of Philadelphia (CHOP) Institutional and Ethical Review Board–approved research studies, both in the CHOP Center for Applied Genomics (HH, PI) and in the CHOP Metabolism Division to evaluate the metabolic consequences of suspected mitochondrial disease (MJF, PI) was obtained from all immediate members of the study family, including two affected siblings, one healthy sister, and both unaffected parents (Fig. 1a). The two affected siblings were enrolled for research-based whole exome analysis based on having highly similar phenotypes that most notably included global developmental delay, epilepsy, hypotonia, and multiple dysmorphic features. The proband was a consanguineous Indian girl (Fig. 1a, individual II-1) who presented at age 6.7 years to the Mitochondrial-Genetics Diagnostic Clinic at The Children's Hospital of Philadelphia for diagnostic evaluation of refractory epilepsy with both focal and generalized seizures onset at 10 months controlled with topiramate and phenobarbital, global developmental delay without significant regression, absent speech, inability to follow commands, inability to sit unassisted or walk, and congenital profound hypotonia. Brain magnetic resonance imaging (MRI) performed at age 16 months (Fig. 1b–c) was significant for bilateral, symmetric abnormal signal in the putamina, consistent with suspected metabolic disease. Delayed myelination was also noted, as was prominence of the subarachnoid spaces and sulci that was consistent with cerebral volume loss. Brain magnetic resonance spectroscopy (MRS) at that time was significant for increased choline and myoinositol peaks and decreased *N*-acetylaspartate (NAA) peak, as well as increased lactate peaks in both the parenchyma and cerebrospinal fluid (CSF). Repeat brain MRI/MRS at age 5 years, 11 months (Fig. 1d–e), revealed normal-appearing basal ganglia with resolution of the signal abnormality previously seen in the putamen, and prominent ventricles and sulci consistent with cerebral volume loss versus hypoplasia. Brain MRS was consistent with previous findings including decreased NAA and increased lactate through the brain parenchyma and increased lactate in the CSF. Choline also appeared elevated in some areas (Fig. 1f). Extensive metabolic and genetic diagnostic evaluations, including muscle biopsy, were

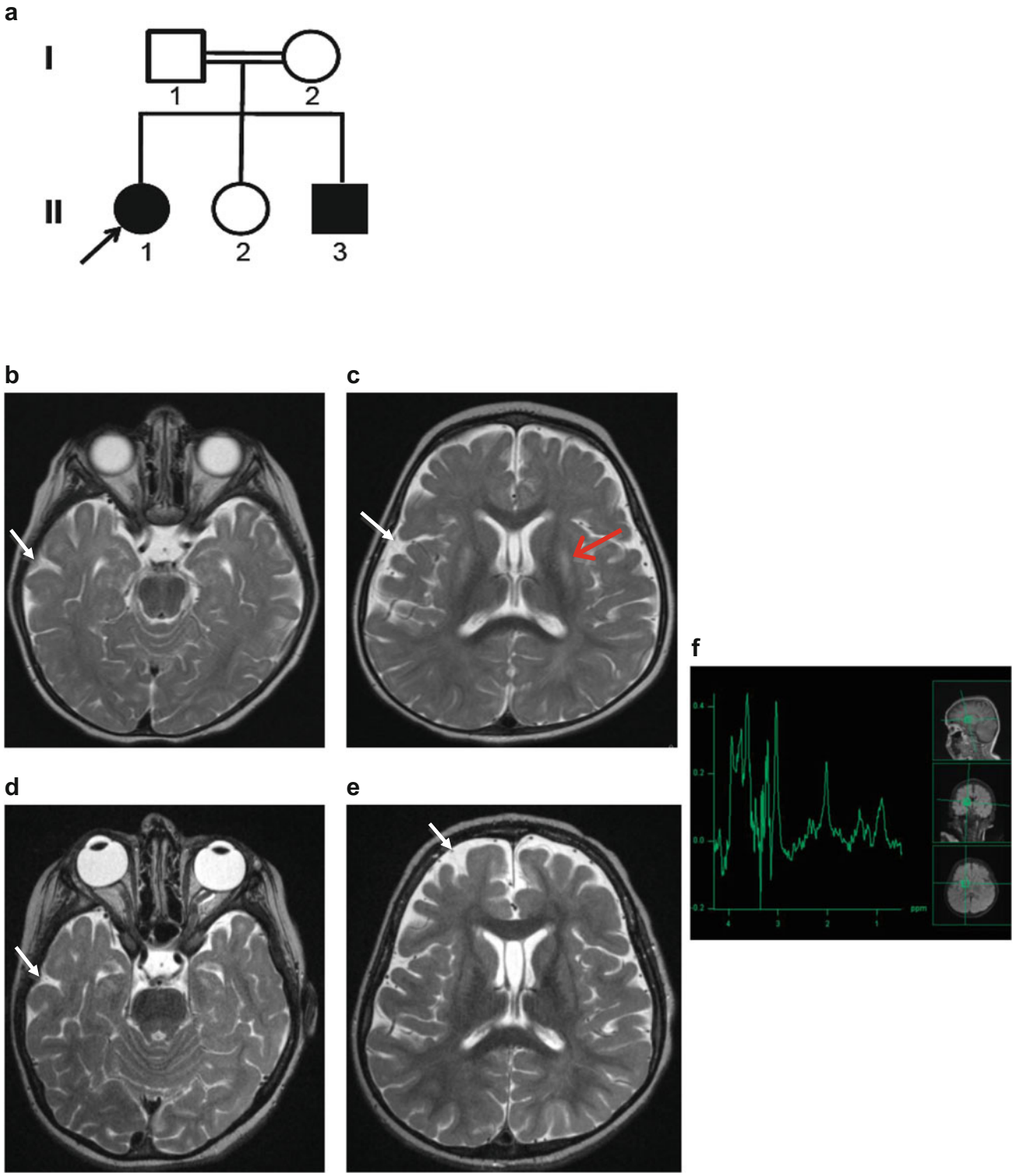


Fig. 1 Pedigree and neuroimaging findings of consanguineous Indian kindred. (a) Family pedigree. The parents are first cousins with two affected children (individuals II-1 and II-3) and one healthy daughter (individual II-2). **(b** and **c) Brain MRI of individual II-1 at age 16 months.** T2 axial images show increased extra-axial cerebrospinal fluid spaces, large sulci (white arrows), bright signal in putamen (red line arrow), and delayed myelination. **(d** and **e) Brain MRI of individual II-1 at age 5 years, 11 months.** As seen on the same sequences and cuts as shown at age 16 months, there is persistence of the signs of atrophy (white arrows), resolution of the T2 signal change in the putamena, and improved but still decreased myelination. **(f) Brain MRS of individual II-1 at age 5 years, 11 months** shows low *N*-acetylaspartate (NAA) peak at approximately 2 parts per million in a voxel placed over the basal ganglia

unrevealing of an underlying metabolic or genetic etiology in the proband. Her similarly affected younger brother (Fig. 1a, individual II-3) presented with global developmental delay at age 13 months, epilepsy onset at age 10 months, and had normal metabolic screening laboratory studies. No brain imaging or tissue biopsies were performed. Detailed descriptions of both children's clinical presentations, including their family history and prior diagnostic evaluations, are presented in the Supplemental File.

AGC1 activity analysis. Human wild-type and mutant AGC1 proteins were overexpressed as inclusion bodies in the cytosol of *Escherichia coli* C0214, solubilized, and purified as described (Palmieri et al. 2001; Fiermonte et al. 2009). The transport activities of the recombinant purified proteins were assayed as described (Palmieri et al. 1995, 2001). The amount of both wild-type and mutant AGC1 incorporated into liposomes was about 20 % of the protein added to the reconstitution mixture. Western blots were performed using the AGC1 B-2 monoclonal antibody (from Santa Cruz Biotechnology, Inc., USA) and porin monoclonal antibody (from MitoScience, USA).

Results

Family-based exome sequencing and bioinformatics analysis. We conducted whole exome sequencing on the affected boy (individual II-3) and his unaffected sister (individual II-2) (Fig. 2a and Supplemental File). We considered the exome variant profile under the assumption of an autosomal recessive model due to known consanguinity. An average coverage of 69-fold was established, where 89 % of the target region was covered at least 10-fold. Variants were filtered to exclude synonymous variants, variants having minor allele frequency exceeding 1%, variants with other occurrences in our in-house exome database, or benign variants, which collectively left variants in only two genes as possible disease-causing candidates (Fig. 2b). Additional whole exome sequencing analysis performed on the entire nuclear pedigree provided improved depth of coverage (Supplemental Table 1) but did not reveal any additional shared homozygous variants by both affected siblings. Both substitution variants were selected for Sanger validation (Supplemental File), with only the *SLC25A12* (solute carrier family 25 member 12) mutation showing complete segregation with the disease phenotype in this kindred (Fig. 2c). *SLC25A12* encodes the mitochondrial aspartate-glutamate carrier isoform 1 (AGC1). The specific *SLC25A12* homozygous mutation identified is c.1058G>A, which results in an Arg353Gln substitution in the 678 amino acid AGC1 protein (Palmieri et al. 2001). Moreover, *SLC25A12* is located in one of the large regions of homozygosity shared by both affected children that we had demonstrated by genome-wide SNP microarray analysis (Supplemental Table 2). The *SLC25A12* c.1058G>A mutation identified in this family appeared to be novel, as it was not detected in the 1000 Genomes Project, the NHLBI Exome Variant Server, or an additional 1200 exome samples previously analyzed in our in-house database.

The arginine at amino acid position 353 in the AGC1 protein is highly conserved in the mitochondrial carrier subfamily of the aspartate glutamate carrier (Fig. 2d), the glutamate carrier, and also in many other mitochondrial carrier subfamilies, thereby confirming its importance in the function and/or structure of mitochondrial carriers rather than in substrate binding (Palmieri 2013). The Arg353Gln mutation occurs in a different domain of the AGC1 transporter from the Gln590Arg mutation that was previously reported in one subject (Wibom et al. 2009). The Gln590Arg mutation involves an amino acid residue that protrudes into the internal cavity of the AGC1 carrier immediately above the substrate binding site (Wibom et al. 2009). In contrast, the Arg353Gln mutation involves an amino acid residue that is located just below the m-gate of the carrier and is thought to participate in closing and opening the carrier on the matrix side through an interaction with a highly conserved glutamate at residue 384 (Fig. 2d) (Pierri et al. 2014).

Functional validation of aberrant AGC1 activity. This carrier transports only L-aspartate and L-glutamate, and its physiological role is to catalyze the exchange of intramitochondrial aspartate with cytosolic glutamate plus a proton (Palmieri 2004). To investigate whether the R353Q mutation of AGC1 affects protein function, we overexpressed the wild-type and mutant AGC1 proteins in *Escherichia coli*, purified the proteins, and reconstituted these purified proteins into phospholipid vesicles (liposomes). We then followed the time courses of [^{14}C] aspartate/aspartate or [^{14}C]glutamate/aspartate exchange by adding radioactive aspartate or glutamate to proteoliposomes containing unlabeled aspartate and reconstituted with either the recombinant wild-type or mutant AGC1 (Fig. 3a). The proteoliposomal uptake of either [^{14}C]aspartate or [^{14}C]glutamate was much lower when the vesicles were reconstituted with the mutant AGC1 than with wild-type AGC1, although the amount of protein inserted in the liposomal membrane was the same for both recombinant proteins. Eighty-five percent inhibition of the initial transport rates of aspartate/aspartate and glutamate/aspartate exchanges was caused by the AGC1 mutation. Therefore, the transport activity of the R353Q mutant AGC1 was dramatically decreased but not completely abolished. Similar amounts of AGC1 and porin (a mitochondrial protein of the outer mitochondrial membrane) were seen in the mitochondrial protein fraction isolated from fibroblasts

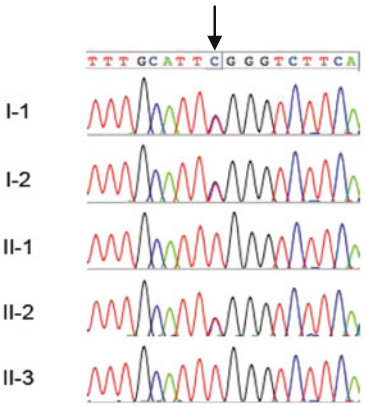
a

Filter	Homozygous	Compound heterozygous	All
NS/SS/I with at least 5X coverage	3149	1274	4065
MAF<0.01 in 1000 Genomes and ESP6500SI	105	63	162
Not present in in-house exome data*	14	28	42
Conserved and damaging	2	0	2

b

Gene	Mutation location (hg19)	cDNA alteration	Protein alteration
SLC25A12	chr2:172669962	c.1058G>A	p.R353Q
ALMS1	chr2:73777400	c.8075A>G	p.N2692S

c



d

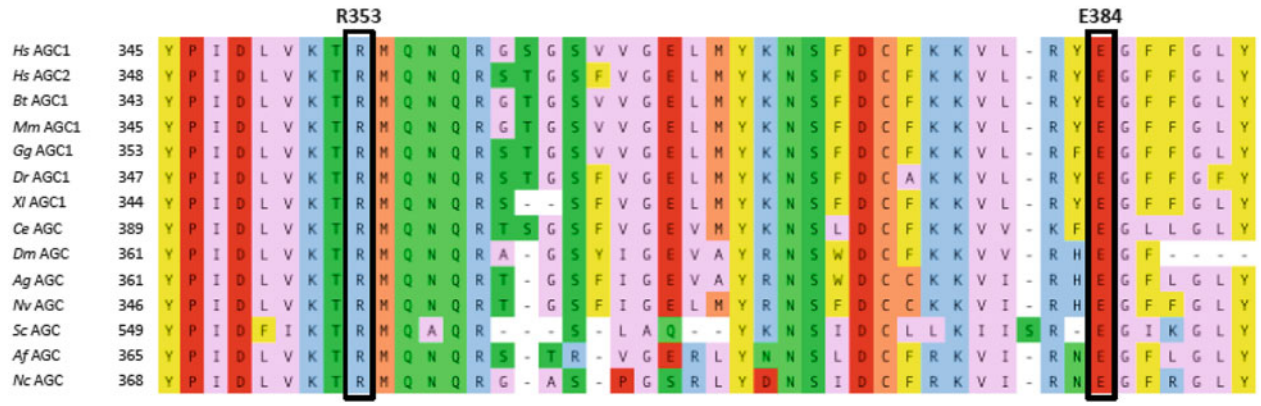


Fig. 2 (**a**) **Overview of candidate genes with potentially pathogenic alleles identified by whole exome sequencing in one affected sibling (individual II-3).** NS, non-synonymous variant. SS, acceptor or donor splice site variants. *I* coding indels, *MAF* minor allele frequency. ESP6500SI, 6503 exomes data from the NHLBI Exome Sequencing Project. *, Neither homozygous nor compound heterozygous variants were present in over 1,200 exome datasets that we had previously sequenced. (**b**) **Details of two candidate genes in which homozygous missense variants were identified by whole exome sequencing in individual II-3.** (**c**) **Mutation validation by Sanger sequencing** confirmed the *SLC25A12* mutation (c.1058G>A, black arrow) segregates with disease in individuals II-1 and II-3. Their healthy sibling and parents are each heterozygous carriers, consistent with autosomal recessive disease. (**d**) **Evolutionary conservation of the Arg353 residue in the AGC1 protein.** The Arg353 residue is located just below the m-gate of the AGC1 carrier, where it participates in closing and opening of the carrier on the mitochondrial matrix side through an interaction with a highly conserved glutamate at residue 384. *Hs Homo sapiens*, *Bt Bos taurus*, *Mm Mus musculus*, *Gg Gallus gallus*, *Dr Danio rerio*, *Xl Xenopus laevis*, *Ce Caenorhabditis elegans*, *Dm Drosophila melanogaster*, *Ag Anopheles gambiae*, *Nv Nasonia vitripennis*, *Sc Saccharomyces cerevisiae*, *Af Aspergillus fumigatus*, *Nc Neurospora crassa*

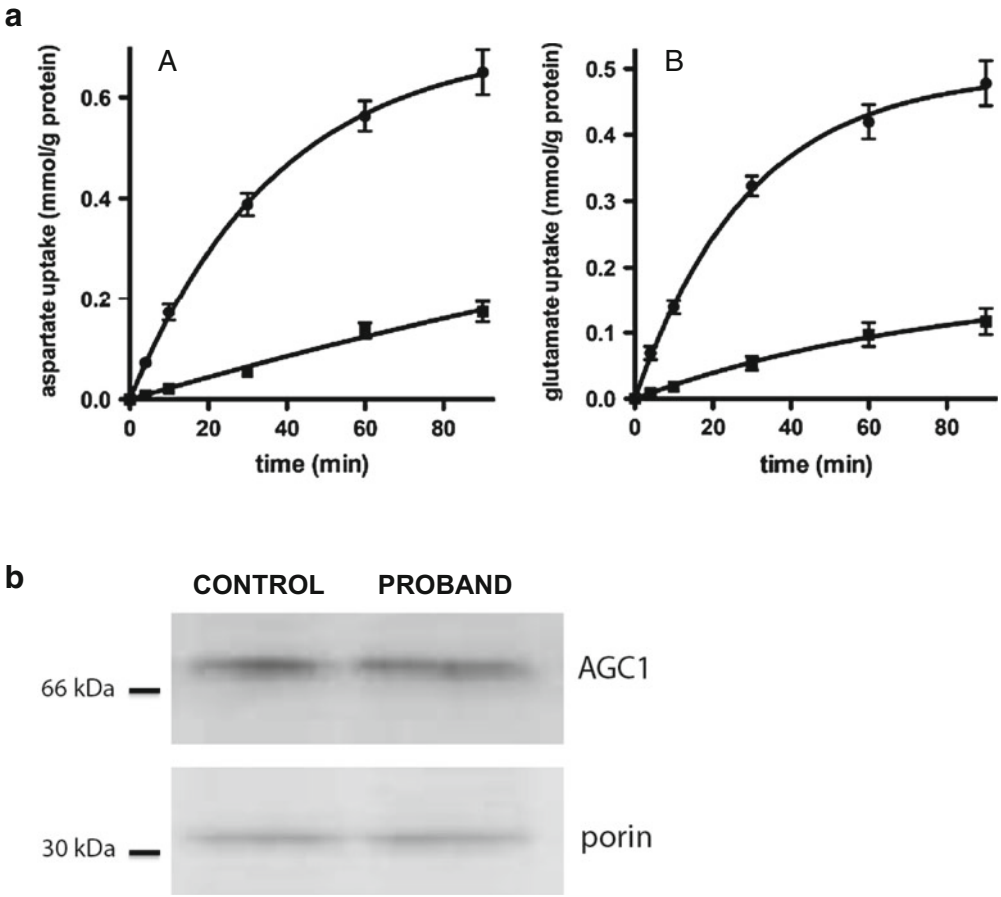


Fig. 3 Functional validation of AGC1 mutation in study proband's cells. (a) Transport assays of wild-type and mutant AGC1. Time courses of [^{14}C]aspartate/aspartate (*panel A*) and [^{14}C]glutamate/aspartate (*panel B*) exchanges in proteoliposomes reconstituted with the recombinant wild-type (*filled circles*) or R353Q mutant AGC1 from individual II-1 (*filled squares*) are shown. At time zero, 50 μM [^{14}C]aspartate (*A*) or 200 μM [^{14}C]glutamate (*B*) was added to proteoliposomes containing 20 mM aspartate. At the indicated times, the uptake of the labeled substrate was stopped by the addition of 15 mM pyridoxal 5′-phosphate and 10 mM bathophenanthroline. Data shown indicates mean and standard deviation of four independent experiments that were each performed in duplicate. **(b) Expression analysis of AGC1 in fibroblasts from AGC1 proband and unrelated healthy control.** Mitochondrial proteins (40 μg) were separated on 15 % SDS-PAGE, transferred onto nitrocellulose membrane, and immunodecorated with anti-AGC1 (*upper panel*) or anti-porin (*lower panel*) antibodies. Densitometry analysis revealed similar content of AGC1 and porin in the investigated mitochondrial extracts in three independent experiments

of the proband (individual II-1) and an unrelated healthy control that were immunodecorated with specific antibodies against AGC1 and porin (Fig. 3b), indicating that the R353Q mutation does not impede mitochondrial localization of AGC1. Finally, fluorescence microscopy of the proband's fibroblasts treated with 25 nmol MitoTracker Red (a mitochondria-specific fluorescent dye) revealed no alterations of the mitochondrial morphology as compared to control fibroblasts (data not shown).

Discussion

Whole exome sequencing analysis identified a novel homozygous c.1058G>A missense mutation in *SLC25A12* that segregated with disease in a consanguineous Indian kindred in which a brother and sister were similarly affected with severe global developmental delay, hypotonia, and intractable epilepsy with both focal and generalized seizures. Identification of *SLC25A12* as the disease gene highlighted the diagnostic relevance of the presumed nonspecific decrease in NAA and delayed myelination that had been identified on the proband's brain magnetic resonance spectroscopy and imaging studies. Indeed, this is only the second report of autosomal recessive *SLC25A12* mutations causing severe neurologic disease. The initial 2009 report linking *SLC25A12* to neurologic disease involved a Swedish girl who was found by candidate gene sequencing to harbor a homozygous AGC1 mutation that caused infantile-onset severe psychomotor retardation,

hypotonia, epilepsy, global cerebral hypomyelination, and dramatically decreased NAA (Wibom et al. 2009).

SLC25A12 encodes a neuronal-specific mitochondrial aspartate-glutamate carrier (aspartate-glutamate carrier isoform 1, AGC1). AGC1 is an important component of the neuronal malate/aspartate shuttle (Palmieri 2004), which is a crucial system to export intramitochondrial aspartate and to transfer the reducing equivalents of NADH from cytosol to mitochondria and hence to support oxidative phosphorylation (Lasorsa et al. 2003). A distinct disorder, AGC2 deficiency, results from *SLC25A13* mutations that reduce aspartate-glutamate carrier isoform 2 (AGC2) function with impaired malate/aspartate shuttle activity in the liver (Saheki and Kobayashi 2002). The prior report in the Swedish infant demonstrated complete loss of recombinant mutant AGC1 activity, resulting in no transport of aspartate or glutamate even after a 60 min incubation period (Wibom et al. 2009). In contrast, the homozygous c.1058G>A missense mutation identified in this consanguineous Indian family reduces recombinant mutant AGC1 activity to approximately 15 % of the initial wild-type rate, as determined when measuring either aspartate transport or glutamate transport. Delayed myelination and profound developmental delay were similarly observed both in our family and in the initially reported Swedish subject, where all three children now reported with AGC1 deficiency have had essentially no motor development through mid-childhood. The myelination defect primarily stems from neuronal loss attributable to the energy deficit caused by a lack of cellular reducing equivalents generated by the glutamate-aspartate shuttle (Wolf and van der Knaap 2009). The brain insult is possibly compounded by a lack of neuronal-generated NAA that is required as a precursor by oligodendrocytes to synthesize myelin and would lead to the secondary hypomyelination also observed in these patients (Wibom et al. 2009). As reduced NAA can be indicative of either decreased NAA production or neuronal loss, it is not possible to discern which mechanism predominates in AGC1 deficiency.

The complex, early-onset, and intractable epilepsy phenotype observed in the AGC1-deficient patients may be a feature present in individuals with many different primary and secondary mitochondrial disorders that involve decreased energy production in neurons (McCormick et al. 2013). Over the last few years, a growing number of genes involved in diverse cellular functions have been associated with early-onset intractable epilepsy together with global developmental delay. Recognized genes function as ion channels (*SCN1A*, *SCN2A*, *SCN8A*, *KCNQ2*, *KCNT2*), transcription factors (*ARX*, *ARHGEF9*, *CDKL5*, *PLCB1*), synaptic proteins (*PNKP*, *PCDH19*, *SPTAN1*, *STXBP1*), a mitochondrial glutamate symporter that transports glutamate and a hydrogen ion across the inner mitochondrial membrane (*SLC25A22*), and now another gene associated with inner mitochondrial membrane glutamate transport (*SLC25A12*). While *SLC25A22* mutation patients manifest both early myoclonic epilepsy and ohtahara-type early epileptic encephalopathy (Molinari et al. 2005, 2009; Poduri et al. 2013), the *SLC25A12* mutation patients' epilepsy resembles instead the non-myoclonic variant. Overall, this report establishes *SLC25A12* as a likely early-onset epileptic encephalopathy gene and emergence of altered glutamate handling as a functional subclass. While the precise cellular mechanism underlying seizures in these patients is not known, we conjecture that their seizures result from a combination of cellular injury and, possibly, although unproven, a cytosolic accumulation of glutamate that could impair synaptic uptake and trigger a hyperexcitable state. Interestingly, the AGC1 knockout mouse shows drastically reduced brain aspartate and NAA levels, global hypomyelination, and progressive failure of neuronal glutamatergic signaling (Ramos et al. 2011).

Should a primary energy deficiency be found to play a pathologic role in the etiology of AGC1-deficient seizures, use of the ketogenic diet might potentially offer a viable therapeutic strategy in these patients to reduce neuronal injury and improve epilepsy outcome. Although the role of the ketogenic diet for treatment of epilepsy in mitochondrial respiratory chain disorders is debatable, specifically relevant to the pathogenesis of neurologic disease in AGC1 deficiency is that although respiratory chain capacity is presumably normal, the reduction of the malate/aspartate shuttle results in a decrease of mitochondrial reducing equivalents that effectively generates an energy-deficient state despite normal respiratory chain capacity. Based on this consideration, the ketogenic diet might conceivably offer a viable therapeutic option for AGC1 deficiency through direct provision of acetyl CoA to the tricarboxylic acid cycle while bypassing the NAD^+-dependent glycolytic and pyruvate metabolism pathways.

This study also emphasizes the high heterogeneity in mitochondrial disease of both etiology and common biomarkers indicative of disease status. In these cases, clinical phenotypes and screening assays in blood, urine, and even muscle fail to provide diagnostic certainty for definitively categorizing an individual as having a primary mitochondrial disease. Indeed, while lactate was reported to be increased in the Swedish AGC1 proband in blood at 6 mmol and in CSF at 2.6 mmol each on one occasion, they were otherwise reportedly normal (Wibom et al. 2009). Similarly, concentrations of lactate and amino acids in blood in both affected siblings, and in CSF in our proband, were essentially normal, as is commonly true in known cases of mitochondrial disease (Haas et al. 2007). Interestingly, brain lactate was globally increased in our proband on brain spectroscopy performed both at 1 and 5 years of

age, which may result from the primary AGC1 deficiency or be a nonspecific finding in epilepsy, although the Swedish AGC1 proband did not have elevated lactate signal on MRS at 2 years, 9 months (Wibom et al. 2009). The muscle biopsy performed after the time of symptomatic onset in our proband at age 1 year showed normal histology and mitochondrial proliferation. Mitochondrial proliferation is a nonspecific finding that can be seen in the setting of reduced mitochondrial oxidative phosphorylation capacity, as is commonly seen in individuals with mitochondrial DNA (mtDNA) mutations involving mitochondrial tRNA genes or with mtDNA deletions (Wong 2010). While it is not clear what the basis for mitochondrial proliferation is in this case, as she had no evident reduction in muscle mitochondrial electron transport chain enzyme activity, it is possible that this was a response to a decrease in anaplerotic substrates due to the AGC1 defect reducing glutamate import into the mitochondria. While AGC1 does have neuronal expression, it is also known to be expressed in skeletal muscle and heart (Iijima 2001). The muscle biopsy was most useful in this case by facilitating demonstration that the children's disease was not due to a maternally inherited mitochondrial DNA genome mutation or deletion, nor due to a nuclear disorder that causes muscle-based mtDNA depletion. Thus, attention and diagnostic energy could be properly focused at that point on identifying the likely novel nuclear gene mutation underlying their now proven autosomal recessive disease. Ultimately, the recognition that *SLC25A12* mutations cause deficiency of a tissue-specific isoform of the mitochondrial AGC carrier explains the absence of more generalized multisystemic symptoms and metabolic findings in these patients.

In conclusion, it is clear that whole exome sequencing analysis facilitates diagnosis of individually rare causes of monogenic disease both in nuclear genes and, potentially, in the mitochondrial genome if it is targeted for analysis and the level of heteroplasmy is sufficient in the target tissue tested (Falk et al. 2012). Here, we have applied this approach to identify the second-ever reported cases of an autosomal recessive *SLC25A12* mutation that causes neuronal AGC1 deficiency. Differences in the three children affected in two unrelated kindreds demonstrates that AGC1 deficiency can variably lead to abnormal brain myelination, cortical atrophy, and/or neuronal energy deficiency with epilepsy and fluctuating basal ganglia involvement. Overall, AGC1 deficiency manifests as a severe, infantile-onset epilepsy syndrome with global developmental delay, congenital hypotonia, abnormal myelination, and reduced cerebral *N*-acetylaspartate levels, which could potentially be used as a diagnostic finding specific to this disease.

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One Sentence Synopsis

SLC25A12 mutations impair neuronal AGC1 activity and should be considered in children with infantile epilepsy, congenital hypotonia, global delay, abnormal myelination, and reduced brain *N*-acetylaspartate.

Compliance with Ethics Guidelines

Author disclosure statement. Marni J. Falk, Dong Li, Xiaowu Gai, Elizabeth McCormick, Emily Place, Francesco M. Lasorsa, Frederick G. Otieno, Cuiping Hou, Cecilia E. Kim, Nada Abdel-Magid, Lyam Vazquez, Frank D. Mentch, Rosetta Chiavacci, Jinlong Liang, Xuanzhu Liu, Hui Jiang, Giulia Giannuzzi, Eric D. Marsh, Yiran Guo, Lifeng Tian, Ferdinando Palmieri, and Hakon Hakonarson have no conflicts of interest to disclose.

Informed consent. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Animal studies. This article does not contain any studies with animal subjects performed by any of the authors.

Author contributions. MJF and HH designed and supervised all aspects of the study. MJF, EM, EP, and EDM clinically evaluated the subjects. MJF performed the skin biopsy to facilitate fibroblast analyses. FML, GG, and FP performed AGC1 activity studies and AGC1 expression analysis in fibroblasts. RC, FDM, YG, and EP coordinated research study subject enrollment. EM coordinated clinical results validation. FGO, CH, CEK, NA, LV, JL, HL, and XJ performed DNA sample extraction and handling, library preparation, whole exome sequencing, and data transfer. DL, LT, and XG performed bioinformatic analyses. MJF, DL, FP, EDM, and HH wrote the manuscript.

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RESEARCH REPORT

Mutational Spectrum of the CTNS Gene in Egyptian Patients with Nephropathic Cystinosis

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Abstract Background: Nephropathic cystinosis is a rare autosomal recessive disorder caused by mutations in the *CTNS* gene, encoding for cystinosin, a carrier protein transporting cystine out of lysosomes. Its deficiency leads to cystine accumulation and cell damage in multiple organs, especially in the kidney. In this study, we aimed to provide the first report describing the mutational spectrum of Egyptian patients with nephropathic cystinosis and their genotype–phenotype correlation.

Methods: Fifteen Egyptian patients from 13 unrelated families with infantile nephropathic cystinosis were evaluated clinically, biochemically, and genetically. Screening for the common 57-kb deletion was performed by standard multiplex PCR, followed by direct sequencing of the ten coding exons, exon-intron interfaces, and promoter region.

Results: None of the 15 Egyptian patients had the 57-kb deletion. Twenty-seven mutant alleles and 12 pathogenic mutations were detected including six novel mutations: two frameshift (c.260_261delTT; p.F87SfsX36, c.1032del-CinsTG; p.F345CfsX19), one nonsense (c.734G>A; p.W245fsX), two missense (c.1084G>A; pG362R, c.560A>G; p.K187R), and one intronic splicing mutation (IVS3+5g>t). A novel promoter region mutation (1-593-41C>T) seemed to be detected but was excluded as a pathogenic mutation by quantitative real-time PCR analysis.

Conclusions: This study could be the basis for future genetic counseling and prenatal diagnosis of patients with nephropathic cystinosis in Egyptian and surrounding populations. The screening for the 57-kb deletion is not recommended anymore outside its geographical distribution, especially in the region of the Middle East. A common Middle Eastern mutation (c.681G>A; E227E) was pointed out and discussed.

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Introduction

Nephropathic cystinosis is one of the leading hereditary causes of renal Fanconi syndrome in children worldwide. It is an autosomal recessive lysosomal storage disorder caused by mutations of the *CTNS* gene located on chromosome 17p13 (Town et al. 1998). The *CTNS* gene is made up of 12 exons, the last ten encoding for cystinosin, a protein that

facilitates cystine transport out of lysosomes. Defective cystinosin-mediated cystine transport leads to the accumulation and crystallization of cystine in cells of different organs, particularly the kidney, cornea, and thyroid (Nesterova and Gahl 2013). Patients with infantile nephropathic cystinosis (OMIM 219800), comprising about 95% of all cases, usually develop renal Fanconi syndrome in the first year of life and renal failure in the first decade if not treated. Other less common types are the juvenile (OMIM 219900) and the non-nephropathic ocular cystinosis (OMIM 219750) (Nesterova and Gahl 2013).

The aminothiol cysteamine is the only specific therapy for cystinosis so far. It helps in the depletion of cystine from lysosomes through different transporter mechanisms (Butler and Zatz 1984); however, it does not completely stop the progression of the disease and does not restore the lost renal function. Early diagnosis and management of nephropathic cystinosis is of utmost importance to delay renal deterioration (Gahl et al. 1990) and other systems' affection (Kimonis et al. 1995) and, hence, improves the patient's survival and quality of life.

The diagnosis of nephropathic cystinosis is confirmed by elevated cystine concentration in white blood cells (WBC), or even better in granulocytes (Levtchenko et al. 2004), which is the diagnostic cornerstone. Molecular analysis of the *CTNS* gene confirms the diagnosis and offers the advantage of prenatal diagnosis (Nesterova and Gahl 2013). Detection of corneal cystine crystals by a slit lamp examination is another confirmatory sign, but a rather late one as it is reasonably sensitive close to the second year of life (Soliman et al. 2009).

Almost 100 *CTNS* mutations have been reported so far in the literature (www.hgmd.cf.ac.uk). The most prevalent is the 57-kb deletion removing the first ten exons of the *CTNS* gene with its upstream sequence. It constitutes approximately 75% of mutated alleles in cystinotic patients from Northern European descent (Touchman et al. 2000). However, *CTNS* gene mutations have been rarely reported outside Europe and North America.

In this study, we evaluated *CTNS* mutations in 15 Egyptian pediatric patients with infantile nephropathic cystinosis and commented on their genotype–phenotype correlation. This would be the first report on the mutational spectrum of nephropathic cystinosis in Egypt and in the region of North Africa.

Patients and Methods

Patients

DNA samples of 15 Egyptian nephropathic cystinosis patients (nine males/six females, 3–16 years) from 13 different families of pure Egyptian background were obtained. Patients were recruited from the Center of Pediatric Nephrology and Transplantation (CPNT), Cairo University Children Hospitals, Cairo, Egypt, over the period from November 2010 to June 2012; however, some of them have been diagnosed and treated in the center for several years. DNA was extracted from either EDTA blood by the salting out technique or blood spots on filter paper by QIAamp, DNA mini kit (Qiagen). The study was approved by the institutional review board, and written informed consents were obtained from subjects' parents/legal guardians.

Methods

Molecular analysis was performed at the laboratory of pediatrics/pediatric nephrology at UZ Leuven, K.U. Leuven. All patients were first screened for the 57-Kb deletion by a standard multiplex PCR technique using LDM1 and D17S829 primer sets as previously described (Heil et al. 2001), and then direct sequencing of the ten coding exons and exon–intron interfaces of the *CTNS* gene (ENSG00000040531, ENST00000046640) (www.ensembl.org) was performed (ABI 3100, Applied Biosystems). Data were analyzed using SEQUENCE Pilot (JSI Medical Systems). Promoter region sequencing was performed for patients with unidentified mutant alleles (Phornphutkul et al. 2001). Sequences of all used primers are available upon request.

Mutation prediction analysis was performed for newly detected variants using the following pathogenicity software programs: PMut, Mutation Taster, PolyPhen2, Spliceman, BDGP, and Automated Splice Site Analysis.

Total RNA was isolated from leukocytes of patient 2 (probable splicing mutation) and patient 12 (probable promoter mutation) using RNeasy MiniKit (Qiagen). Reverse transcription-PCR (RT-PCR) was performed using Superscript III Reverse-Transcriptase (Life Technologies) according to the manufacturer's protocol. In patient 2, cDNA amplification for the *CTNS* gene was performed compared to a healthy control using the primers previously described [forward: 5′-CCTCTTCCAGTAACATTGAGG-3′ and reverse 5′-CGCGTGCAGGCTGAAGAAGA-3′] (AlcÄntara-Ortigoza et al. 2008) crossing the exonic boundaries and giving a product extending from exon 2 to exon 9 (722 bp in normal individuals); the resulting amplification product was directly sequenced.

In patient 12, cDNA was used for quantitative real-time PCR (qPCR) (Rotor-Gene Q, Qiagen) together with platinum Sybr Green PCR mix (Life Technologies), *CTNS* cDNA primers mentioned above, and primers for *GAPDH* as a reference gene. Three healthy control samples were

assayed in the same run by qPCR. The patient and controls were assayed each in triplicate. Cycling parameters were as follows: 95°C, 30 s; 60°C, 60 s; 72°C, 60 s for 40 cycles; and then extension at 72°C for 5 min. Resulting data were expressed as percent of reduction in gene expression compared to normal controls after normalization with *GAPDH* using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001).

Cystine assay in WBC was recently established and performed at the Inherited Metabolic Disorder Laboratory (IMDL), Center of Social and Preventive Medicine (CSPM), Cairo University Children Hospitals. Cystine assay was performed by LC-MS/MS (Micromass, Waters), as previously described (Chabli et al. 2007), using the internal standard d,l-cystine-2,2′,3,3,3′,3′-d6 (C/D/N isotopes), L-cystine calibration curve, and a 3.0 x 50 mm, 3.5 μ particle size Xterra C18 HPLC column.

Results

Fifteen Egyptian patients with infantile nephropathic cystinosis were evaluated clinically, biochemically, and genetically. Consanguinity was reported in ten out of 13 families, and corneal cystine crystals were detectable in all patients. Age at presentation ranged from 4 to 12 months, and age at diagnosis ranged from 5 months to 9 years. Patients were already on cysteamine therapy at the time of sampling for a period ranging from 2 to 84 months, on a dosage ranging from 15 to 45 mg/kg/day. All patients were below the 3rd percentiles for weight and height in their age groups. One patient died (patient 8) at the age of 8 years before completion of the study. One patient underwent renal transplantation (patient 4) at the age of 14 years. Three patients have ESRD, and four patients have hypothyroidism and are on L-thyroxin replacement therapy. Cystine levels in WBC ranged from 1.5 to 15.3 nmol ½ cystine/mg proteins (Table 1).

None of the 15 Egyptian patients had the common European 57-Kb deletion. Twelve pathogenic mutations were identified representing 27 discovered mutant alleles (Table 2). Ten out of 15 patients had homozygous mutations. Six previously reported *CTNS* mutations were detected in our study: c.829dup; p.T277NfsX19 (Besouw et al. 2012), c.922G>A; G308R (Shotelersuk et al. 1998), 809_811delCCT; p.S270del (Attard et al. 1999), c.15G>A; p.W5X (Kalatzis et al. 2002), c.681G>A; E227E (Aldahmesh et al. 2009), and c.1015G>A; p.G339R (Shotelersuk et al. 1998). All these mutations were previously associated with infantile nephropathic cystinosis in various populations. Figure 1 presents the worldwide geographical distribution of the previously reported *CTNS* mutations detected in Egyptian patients.

Newly detected *CTNS* variants include two frameshift mutations (c.260_261del; p.F87SfsX36 and c.1032del-CinsTG; p.F345CfsX19), one nonsense mutation (c.734G>A; p.W245fsX), two missense mutations (c.1084G>A; pG362R and c.560A>G; p.K187R), and one intronic splicing mutation (IVS3+5g>t) (Fig. 2). A novel promoter region variant was also detected in a homozygous state in patient 12 (1-593-41C>T) corresponding to the important specificity protein-1 (Sp-1) binding motif (GGCGGCG) that was reported to extend from 1-593-46 to 1-593-40 in the *CTNS* promoter area (Phornphutkul et al. 2001) (Fig. 2).

The newly detected variants were tested with different predictive software programs. Table 3 provides a summary for all prediction results. The frameshift and the nonsense variants led to truncated proteins and were easily predicted pathogenic. c.1084G>A in patient 10 replacing glycine with arginine at position 362, which is a highly conserved residue among different species, was also detected as being pathogenic. The other missense variant (c.560A>G) in patient 8 replacing lysine with arginine at position 187 was expected to be neutral based on the amino acid change; however, this mutation occurs at the second base before exon–intron 8 interface (Fig. 2) and could be an exonic splicing mutation as expected by three splicing evaluation software programs. The intronic mutation (IVS35g>t) in patient 2 was more strongly suspected to have a splicing damaging effect during transcription (Table 3).

The splicing mutation (IVS3+5g>t) was confirmed by demonstrating the skipping of exon 3 evidenced by sequence analysis after RT-PCR (Fig. 3). On the other hand, the promoter region mutation in patient 12 (1-593-41C>T) led to a modest reduction (27 ± 6.5%) in *CTNS* RNA gene expression after normalization to *GAPDH* and comparison with three healthy controls. To our regret, RNA samples from patient 8 (c.560A>G) or his parents were unobtainable.

Discussion

We here present the first report on the mutational spectrum of Egyptian patients with nephropathic cystinosis. Thirteen unrelated families and 15 patients representing a wide geographical distribution within Egypt were screened for the *CTNS* gene mutations.

Most of the Egyptian patients had a severe phenotype as evident in Table 1, but this phenotypic severity could be attributed to many factors. The *CTNS* genotype is definitely an important one (Attard et al. 1999); however, delayed diagnosis in many of our patients, underdosage due to financial or logistical problems, and differences in response

Table 1 Clinical features of Egyptian patients with nephropathic cystinosis

Proband	Age in years	Sex	Onset of symptoms in months	Age at diagnosis in months	F.S	M.A	R	L.D	HT	SDS Weight/ Hight	Creatinine (mg/dl) at diagnosis/ last visit	Cysteamine (mg/kg/ day)	WBC Cystine (nmol ½ cys/ mg Protein)	Remarks
1	3	M	6	20	+	–	+	+	–	–2.8/-5.5	1.0/1.3	30	1.7	
2	5	F	8	14	+	+	+	–	–	–1.7/–2.8	0.3/1.2	30	3.8	
3	3	M	9	22	+	–	+	–	–	–2.9/–3.6	0.5/0.7	30	5.1	
4	15	F	12	108	–	–	-	+	–	–3.7/–8.2	4.8/0.7	25	4.0	RTX one year ago and on GH
5a	16	F	6	60	+	+	+	–	+	–4.5/–7.6	2.7/6.1	25	4.9	On regular HD
5b	6	M	5	6	+	–	+	–	–	–2.3/–5.0	0.7/0.6	30	10.6	
6	7.5	M	5	9	+	–	+	-	–	–3.5/–6.8	0.6/0.9	30	1.5	
7	5	M	6	14	+	+	+	–	–	–2.9/–5.4	1.7/4.9	40	9.9	On regular HD and prepared for RTX
8	8	M	6	9	+	+	+	-	-	–2.7/–5.4	0.8/5.6	–	–	Deceased
9	4	M	7	15	+	–	+	+	–	–2.7/–3.7	0.4/0.3	35	4.7	
10	4.5	F	10	30	+	–	–	–	–	–	–	30	–	
11a	11	F	5	30	+	+	+	–	+	–2.7/–5.0	0.7/5.6	15	–	On regular HD
11b	6.5	F	4	5	+	+	+	–	+	–2.9/–8.5	0.3/1.5	45	–	
12	3.5	M	8	14	+	+	+	–	–	–4.1/–3.9	0.5/0.6	45	15.4	
13	4	M	6	36	+	+	+	+	+	–4.0/–5.9	1.4/1.7	45	7.5	

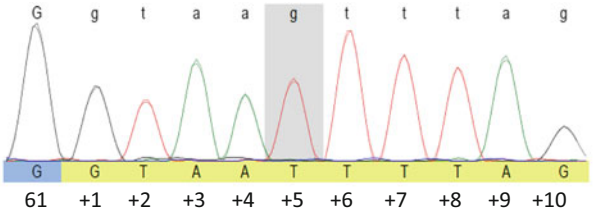
F.S Fanconi syndrome, *GH* Growth hormone, *HD* Hemodialysis, *HT* Hypothyroidism, *L.D* Limb deformity, *M.A* Metabolic acidosis, *R* Rickets, *RTX* Renal transplantation, *SDS* Standardized score, Families 5 and 11 have 2 siblings each (a) and (b)

Table 2 Genotype of Egyptian patients with nephropathic cystinosis

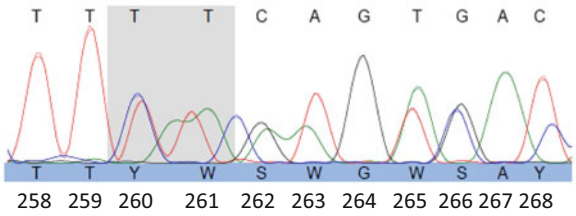
Proband	Mutant allele1	Mutant allele2	Location	Protein effects	Consequence of mutation	Reference
1	c.829dup	c.829dup	Exon 10	p.T277NfsX19	Truncated protein at AA296	(Besouw et al 2012)
2	IVS3+5g>t	IVS3+5g>t	Intron 3	–	Skipping of exon 3	This study
3	c.922G>A	c.922G>A	Exon 11	G308R	AA change at TM6	(Shotelersuk et al 1998)
4	809_811del	809_811del	Exon 10	p.S270del	AA deleted from TM5	(Attard et al 1999)
5a,5b	c.829dup	c.829dup	Exon 10	p.T277NfsX19	Truncated protein at AA296	(Besouw et al 2012)
6	c.15G>A	c.15G>A	Exon 3	p.W5X	Truncated protein at AA5	(Kalatzis et al 2002)
7	c.681G>A	c.681G>A	Exon 9	E227E	Alternative splicing	(Aldahmesh et al 2009)
8	260_261delTT	c.560A>G	Exons 6 and 8	p.F87SfsX36, p. K187R	Truncated protein at AA123, Alternative splicing	This study
9	c.1015G>A	c.1015G>A	Exon 12	p.G339R	AA change at TM7	(Shotelersuk et al 1998)
10	c.1084G>A	ND	Exon 12	pG362R	AA change at cytosolic LTM	This study
11a,11b	c.734G>A	c.1032delCinsTG	Exons 10 and 12	p.W245fsX, p. F345CfsX19	Truncated protein at AA245, Truncated protein at AA364	This study
12	ND	ND	–	–	–	–
13	c.829dup	c.829dup	Exon 10	p.T277NfsX19	Truncated protein at AA296	(Besouw et al 2012)

AA, Amino acid; LTM, Lysosomal targeting motif; ND, Not detected; TM, Transmembrane domain; Families 5 and 11 have 2 siblings each (a) and (b).

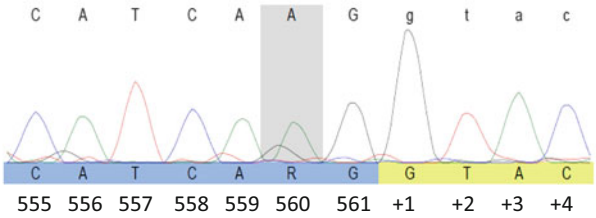
Fig. 1 Worldwide geographical distribution of 57-kb deletion and previously reported *CTNS* mutations detected in the Egyptian population. *Oval*: geographical distribution of 57-kb deletion. *Circle*: geographical distribution of the Middle Eastern mutation c.681G>A



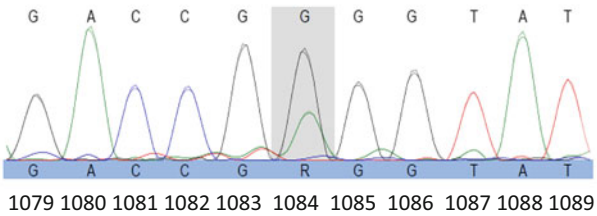
Proband 2: IVS3+5g>t (H)



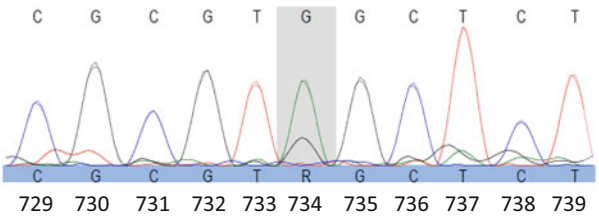
Proband 8: c.260_261delTT; p.F87SfsX36 (h)



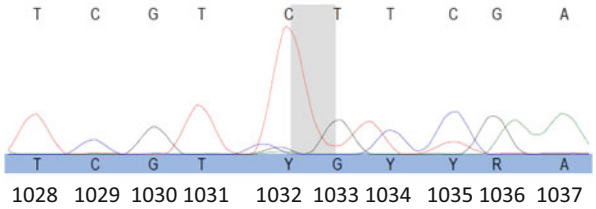
Proband 8: c.560A>G; p.K187R (h)



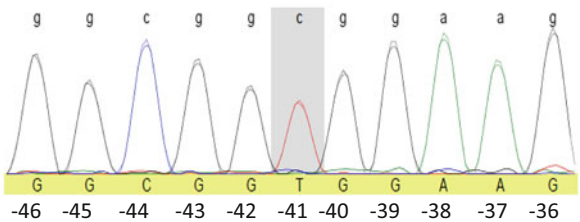
Proband 10: c.1084G>A; p.G362R (h)



Probands 11a, 11b: c.734G>A; p.W245fsX (h)



Probands 11a, 11b: c.1032delCinsTG; p.F345CfsX19 (h)



Proband 12: 1-593-41C>T (H)

Fig. 2 Newly discovered *CTNS* variants in Egyptian patients. *H* homozygous, *h* heterozygous

to cysteamine therapy may all be implicated (Soliman et al. 2013).

In our series of patients, growth retardation was a striking feature in almost all patients; however, more severe cases were usually associated with truncating mutations (Table 2). The recent introduction of growth hormone in the therapeutic panel of Egyptian nephropathic cystinosis patients hopefully will improve the overall growth pattern. Although all therapeutic measures were taken to oppose the effects of hypocalcemia and hypophosphatemia, still most of our patients are complaining of rickets and some already show skeletal deformities at relatively young ages. The only patient who underwent renal transplantation (patient 4) is doing well with normally functioning graft and is showing increased annual height velocity post transplantation. Not surprisingly though, she is still suffering remarkable growth

Table 3 Predicted effects of new *CTNS* variants

Variant	Prediction						
Deletions or insertions							
c.260-261delTT; p.F87SfsX36	Frame shift						
c.1032delCinsTG; p.F345CfsX19	Frame shift						
Nonsense							
c.734G>A; p.W245fsX	Nonsense						
Missense	**PMut (AA change)**			**Mutation taster (AA change/splice)**		**PolyPhen2 (AA change)**	
	NN output	Reliability	Result	Probability	Result	Score	Result
c.1084G>A; p.G362R	0.8395	6	Pathogenic	0.999	Pathogenic	0.647	Possibly damaging
c.560A>G[a]; p.K187R	0.0145	9	Neutral	0.952	Pathogenic	0.002	Benign
Probable splicing variants	**Spliceman (splice)**			**BDGP (splice)**		**Automated splice site analysis (splice)**	
	Ranking	Result		Score	Result	Final/initial binding	Result
IVS3+5g>t	0.85	Pathogenic		1.0>0.67	Pathogenic	6.6%	Pathogenic
c.560A>G[a]	0.55	Pathogenic		1.0>0.99	Neutral	20.4%	Pathogenic

AA Amino acid

PMut: http://mmb2.pcb.ub.es:8080/PMut/; **Mutation taster:** www.mutationtaster.org/; **PolyPhen2:** genetics.bwh.harvard.edu/pph2/; **Spliceman:** fairbrother.biomed.brown.edu/spliceman/; **BDGP:** www.fruitfly.org/seq_tools/splice.html; **Automated splice site analysis:** https://splice.uwo.ca/

[a] A missense mutation and a probable splicing mutation

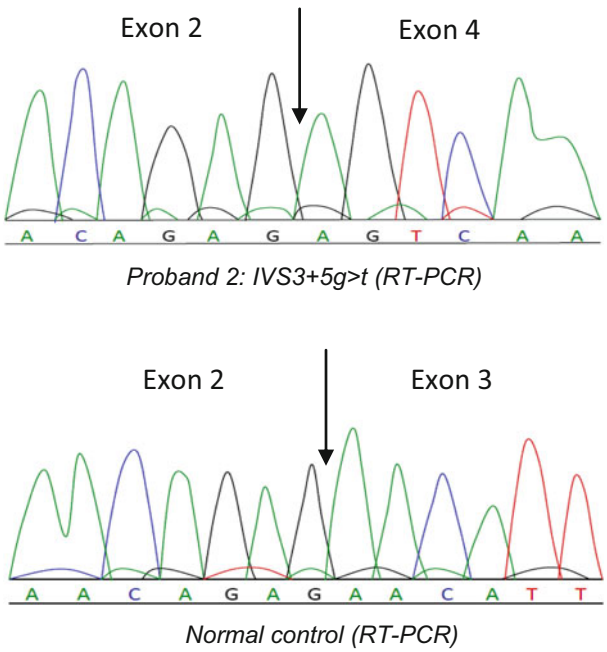


Fig. 3 RT-PCR/sequence analysis of the mutation (IVS3+5g>t) of patient 2 in which skipping of exon 3 of the *CTNS* gene is evident compared to a healthy individual

retardation and residual genu valgum given the significantly late diagnosis in this particular patient (at 9 years of age) and the consequent therapeutic delay.

The detection of six novel mutations and six mutations previously reported in 13 families denotes the marked genetic heterogeneity of Egyptian patients with nephropathic

cystinosis, which is different from many populations in which a single mutation may constitute over 50% of causative mutations. Also the high rate of homozygous mutations in our study (67%) sheds light on the gravity of the role of consanguineous marriage in elevating the incidence of nephropathic cystinosis and other autosomal recessive disorders in Egypt and in other Arab countries with similar and even higher rates of consanguineous marriages (Tadmouri et al. 2009).

The novel mutations found in this study include the frameshift mutation (c.260_261delTT; p.F87SfsX36) detected in patient 8, converting phenylalanine at position 87 to serine and resulting in a truncated protein at AA 123, completely abolishing the protein function. Likewise, the other novel frameshift mutations detected in sisters 11a and 11b (c.1032delCinsTG; p.F345CfsX19) resulted in the conversion of phenylalanine to cysteine at position 345 and a truncated protein at AA 364, disrupting the seventh and final transmembrane domain of cystinosin (Attard et al. 1999). The nonsense mutation (c.734G>A; p.W245fsX) also detected in patients 11a and 11b led to the conversion of tryptophan at position 245 into a stop codon (TAG), thus immediately stopping the protein translation.

The missense mutation (c.1084G>A; pG362R) detected in patient 10 is the first reported mutation in the lysosomal targeting motif of cystinosin (GYDQL) extending from AA 362 to 366. This mutation replaced the highly conserved glycine at position 362 at the C-terminus of the protein with the basic amino acid arginine. This charge alteration is expected to disrupt the cytosolic lysosomal targeting motif (Attard et al. 1999). Although previous intentional site-directed mutagenesis of C-terminus lysosomal targeting motif led to the redirection of most of cystinosin to the plasma membrane, still partial localization to lysosomes occurred, denoting the presence of a second lysosomal targeting signal, which was identified in the third cytoplasmic loop (YFPQA) extending from AA 281 to 285 (Cherqui et al. 2001). This could be the reason of the relatively milder phenotype of patient 10 (no metabolic acidosis, rickets, limb deformity, or hypothyroidism) (Table1).

The other missense mutation was detected in patient 8 (c.560A>G; p.K187R), and although exonic, it is highly likely to cause alternative splicing, as it was detected as being pathogenic by three different splicing software programs: Mutation Taster, Spliceman, and Automated Splice Site Analysis (Table 3). Unfortunately, the patient with this mutation died recently at the age of 8 years from intracranial hemorrhage complicating hemodialysis before we could confirm the splicing nature of his mutation.

The intronic splice site mutation (IVS3+5g>t) detected in patient 2 led to the skipping of exon 3 after RT-PCR/sequencing analysis as the similar splicing mutation at the same position (IVS3+5g>a), which was also demonstrated to lead to exon 3 skipping in a Mexican patient with nephropathic cystinosis (AlcÄntara-Ortigoza et al. 2008), confirming the importance of the guanine base at the IVS+5 position for *CTNS* exon 3 transcription.

Promoter region mutations reported in the *CTNS* gene are rare. Only three different mutations were reported in eight patients (Phornphutkul et al. 2001, Mason et al. 2003). The most commonly reported is the mutation causing infantile type (1-593-42G>C) which was discovered in six patients, one from the USA and five from Italy. The other two reported mutations (1-593-50G>T and 1-593-50insT) resulted in ocular cystinotic phenotypes. All three mutations were located at or near a highly critical region in the promoter area corresponding to the Sp-1 binding motif essential for gene transcription (Phornphutkul et al. 2001). We here report the fourth promoter region mutation at the Sp-1 binding motif in the *CTNS* gene (1-593-41C>T) detected in patient 12 in our study in a homozygous state. qPCR analysis of this patient's RNA sample showed only a modest reduction in *CTNS* gene expression when compared to three control individuals (27 ± 6.5 %). This indicates that this variant is not the mutation responsible for the patient phenotype (Table 1); however, reducing *CTNS* gene expression in the first place is also indicative of the importance of the Sp-1 binding motif for *CTNS* gene transcription. Caution during the interpretation of promoter region mutations is highly recommended.

In addition to the 13 Egyptian cystinotic families, the absence of the 57-kb deletion has been previously reported in the region of the Middle East in 13 families from Saudi Arabia (Aldahmesh et al. 2009), 10 from Turkey (Topaloglu et al. 2012), and 24 from southwestern Iran (Shahkarami et al. 2013). A single study in the Far East (Thailand) also reported the absence of the 57-kb mutant allele in six patients of Thai and Cambodian origins (Yeetong et al. 2012). Apparently, this common mutation is restricted to the Northern European/American populations and, to a lesser extent, to countries of possible genetic contact as in Italy (Mason et al. 2003) and Mexico (AlcÄntara-Ortigoza et al. 2008). This supports the theory that this founder mutation originated very recently during human evolution, perhaps less than 2,000 years ago somewhere in Northern Europe (Kalatzis and Antignac 2002); so it has not got the chance to spread to remote ethnicities. Based on these observations, we do not recommend anymore the routine screening for the 57-kb deletion before *CTNS* sequencing in populations outside its geographical distribution, at least in the region of the Middle East.

The most common mutation detected in Egyptian patients is c.829dup; p.T277NfsX19, discovered in a homozygous state in four patients belonging to three

unrelated families (6/26 or 23.1% of Egyptian familial mutant alleles). This mutation is a frameshift mutation starting at codon 277, leading eventually to a truncated protein at amino acid 296. It was associated with increased creatinine levels in two Egyptian unrelated patients (1 and 13) at the time of diagnosis (2 and 3 years, respectively), and the latter patient is already complaining of hypothyroidism at 4 years (Table 1). This mutation has been reported only once before in a heterozygous state in a European patient (Besouw et al. 2012). It is apparently a hot spot in Egypt and could be the focus for further investigations. Apart from this mutation, all other detected *CTNS* mutations in Egyptian patients were only present in a single family.

In similar studies in the Middle East, only one mutation was detected in all populations. This mutation was the exonic splice site mutation c.681G>A; p.E227E, completely abolishing the adjacent donor site at exon 9 and replacing it with a cryptic donor site (Aldahmesh et al. 2009). It comprises 39.5% of Iranian (Shahkarami et al. 2013), 20% of Turkish (Topaloglu et al. 2012), and 15.4% of Saudi familial mutant alleles (Aldahmesh et al. 2009). This mutation was not detected previously in European or American populations (a pure Middle Eastern mutation), and it was present in the Egyptian population in our study in a homozygous state in a single patient (7.7% of familial mutant alleles); so its prevalence decreases gradually upon heading to the west (Fig. 1). This could suggest that the origin of this founder mutation is Iran or perhaps a place further to the east.

Other previously reported *CTNS* mutations detected in our study include c.15G>A; p.W5X, which is a nonsense mutation leading to truncated protein at AA 5 with complete loss of function. c.922G>A; p.G308R and c.1015G>A; p.G339R are two missense mutations that were tested on the level of cystinosin protein carrier function and led to absent carrier capability (Kalatzis et al. 2004). Likewise, the small deletion c.809_811del; p.S270del was tested functionally and resulted in almost complete loss of function (Kalatzis et al. 2004). The most widely distributed among the six previously reported mutations in Egyptian patients was c.1015G>A; p.G339R, as it was reported in Turkey (Topaloglu et al. 2012), Italy (Mason et al. 2003), Germany (Kiehntopf et al. 2002), France (Attard et al. 1999), Spain (Macías-Vidal et al. 2009), the USA (Shotelersuk et al. 1998), and Canada (Rupar et al. 2001), followed by c.922G>A; p.G308R reported in Saudi Arabia (Aldahmesh et al. 2009), Italy (Mason et al. 2003), France (Attard et al. 1999), Spain (Macías-Vidal et al. 2009), and the USA (Shotelersuk et al. 1998). The mutation c.809_811del; p.S270del was also relatively widespread, as it was reported in France (Attard et al. 1999) and India (Tang et al. 2009) (Fig. 1).

The most common Egyptian mutation (c.829dup; p.T277NfsX19) was completely absent in other studies from the Middle East. Likewise, the most common mutation in the Saudi population (1013 T>G; L338R), representing 34.6 % of familial mutant alleles, was not detected in the three surrounding populations, and apart from the founder mutation (c.681G>A; E227E), there were no other mutations detected in common among the Saudi, Turkish, and Iranian patients. This is quite remarkable considering the long history of commercial relations, invasions, and genetic contact between these four close countries over the last few thousand years.

The newly detected mutations in our study may not be restricted to the Egyptian population, as the cystinotic genotypes of most Arab and all African populations are still largely obscure. However, our study helps in the understanding of the genetic basis of this orphan disease and its correlation with phenotypic features in this part of the world where consanguinity is the rule, not the exception. It also paves the way for future family counseling and prenatal diagnosis in the region.

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Synopsis

This study summarizes the Egyptian experience in the diagnosis and management of patients with nephropathic cystinosis over the past few years and reports six novel and six recurrent mutations in the *CTNS* gene.

Compliance with Ethics Guidelines

Conflict of Interest

Neveen A. Soliman, Mohamed A. Elmonem, Lambertus van den Heuvel, Rehab H. Abdel Hamid, Mohamed Gamal, Inge Bongaers, Sandrine Marie, and Elena Levtchenko declare that they have no conflict of interest regarding the contents of the current study.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consents were obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Neveen A Soliman was involved in the conception and design of the study, recruitment and management of patients, interpretation of data, and drafting the manuscript.

Mohamed A Elmonem was involved in the conception and design of the study, biochemical and genetic analysis of patients, interpretation of data, and drafting the manuscript.

Lambertus van den Heuvel was involved in the conception and design of the study, genetic analysis of patients, interpretation of data, and revising the manuscript critically for intellectual content.

Rehab H. Abdel Hamid was involved in the design of the study, recruitment and management of patients, interpretation of data, and revising the manuscript critically for intellectual content.

Mohamed Gamal was involved in the design of the study, recruitment and management of patients, interpretation of data, and revising the manuscript critically for intellectual content.

Inge Bongaers was involved in the design of the study, genetic analysis of patients, interpretation of data, and revising the manuscript critically for intellectual content.

Sandrine Marie was involved in the design of the study, biochemical analysis of patients, interpretation of data, and revising the manuscript critically for intellectual content.

Elena Levtchenko was involved in the conception and design of the study, genetic analysis of patients, interpretation of data, and revising the manuscript critically for intellectual content.

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RESEARCH REPORT

Enzyme Replacement Therapy in Mucopolysaccharidosis II Patients Under 1 Year of Age

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Abstract Mucopolysaccharidosis (MPS) II, or Hunter syndrome, is a lysosomal storage disease characterized by multi-systemic involvement and a progressive clinical course. Enzyme replacement therapy with idursulfase has been approved in more than 50 countries worldwide; however, safety and efficacy data from clinical studies are currently only available for patients 1.4 years of age and older. Sibling case studies of infants with MPS I, II, and VI who initiated ERT in the first weeks or months of life have reported no new safety concerns and a more favorable clinical course for the sibling treated in infancy than for the later-treated sibling. Here we describe our experiences with a case series of eight MPS II patients for whom idursulfase treatment was initiated at under 1 year of age. The majority of the patients were diagnosed because of a family history of disease. All of the infants displayed abnormalities consistent with MPS II at diagnosis. The youngest age at treatment start was 10 days and the oldest was 6.5 months, with duration of treatment varying between 6 weeks and 5.5 years. No new safety concerns were observed, and none of the patients experienced an infusion-related reaction. All of the patients treated for more than 6 weeks showed

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improvements and/or stabilization of some somatic manifestations while on treatment. In some cases, caregivers made comparisons with other affected family members and reported that the early-treated patients experienced a less severe clinical course, although a lack of medical records for many family members precluded a rigorous comparison.

Abbreviations

CT	Computed tomography
ERT	Enzyme replacement therapy
GAG	Glycosaminoglycan
HSCT	Hematopoietic stem cell transplantation
I2S	Iduronate-2-sulfatase
IRR	Infusion-related reaction
IV	Intravenous
LSD	Lysosomal storage disorder
MPS	Mucopolysaccharidosis
MRI	Magnetic resonance imaging
uGAG	Urinary glycosaminoglycan

Introduction

Mucopolysaccharidosis II (MPS II or Hunter syndrome; OMIM 309900) is an X-linked lysosomal storage disorder caused by a deficiency in the lysosomal enzyme iduronate-2-sulfatase (I2S), leading to impaired glycosaminoglycan (GAG) catabolism (Neufeld and Muenzer 2001). The storage materials, heparan sulfate and dermatan sulfate, accumulate within lysosomes and are present in plasma, urine, and cerebral spinal fluid (Neufeld and Muenzer 2001; Tomatsu et al. 2005). Although patients with MPS II appear normal at birth, the disease is progressive and signs and symptoms usually become apparent during early childhood (Wraith et al. 2008). Early signs and symptoms of the disease may include dysmorphic facial features, airway obstruction, chronic rhinorrhea, recurrent respiratory infections, recurrent ear infections, hearing loss, hernia, hepatosplenomegaly, chronic watery diarrhea, and joint stiffness and contractures (Martin et al. 2008).

Clinical heterogeneity is a hallmark of MPS II, with patients presenting on a wide spectrum of severity. Not all patients display all associated signs and symptoms (Young et al. 1982a). About two-thirds of patients have cognitive impairment and/or behavioral abnormalities (Froissart et al. 1998, Vafiadaki et al. 1998). These patients are often described as having the severe phenotype, and they typically only survive into the second and occasionally the third decade of life (Jones et al. 2009, Young and Harper 1983; Young et al. 1982b). Patients with the attenuated phenotype do not have cognitive impairment; however, they can experience all of the somatic signs and symptoms of the disease, although often with a more gradual onset. This includes neurological manifestations such as communicating hydrocephalus and carpal tunnel syndrome (Martin et al. 2008). Such patients may live into the fifth or sixth decade of life or longer (Jones et al. 2009).

In 2006, enzyme replacement therapy (ERT) with recombinant human idursulfase (Elaprase®, Shire Human Genetic Therapies, Inc., Lexington, MA) was approved for the treatment of patients with MPS II based on phase II/III clinical trial data from patients aged 5 years and older (Muenzer et al. 2006). Some evidence reported since that time has suggested that earlier treatment before irreversible organ damage occurs may improve clinical outcomes and growth (Muenzer et al. 2012; Muenzer et al. 2009, Schulze-Frenking et al. 2011). A recent 53-week, open-label, safety trial of 0.5 mg/kg weekly idursulfase in children aged 1.4–7.5 years ($n = 28$) has been completed (Giugliani et al. 2013). Adverse events experienced by the younger patients were similar to those experienced by patients aged 5 years and older in the pivotal phase II/III trial of idursulfase (Muenzer et al. 2006). The most common adverse events (57 %) were infusion-related reactions (IRRs). The most common serious adverse reactions occurring in at least 10 % of patients (≥3 patients) included bronchopneumonia/pneumonia (18 %), ear infection (11 %), and pyrexia (11 %). Available genotype data from 27/28 patients demonstrated that patients with a complete gene deletion, large gene rearrangement, nonsense, frameshift, or splice site mutation were more likely to experience IRRs and serious adverse events after idursulfase administration than were patients with missense mutations (idursulfase [Elaprase] prescribing information, Shire 2013).

There are currently no clinical trial data addressing the treatment of children under 1 year of age. Benefits with early ERT in MPS I and MPS VI have been suggested by sibling case reports (Gabrielli et al. 2010; McGill et al. 2010). Tylki-Szymańska and colleagues published an unusual case study of twin boys, one of whom has MPS II (Tylki-Szymanska et al. 2012). The twins are the younger siblings of an older affected sister. MPS II in the sister was caused by the heterozygous *IDS* gene mutation c.1568A>G in association with almost totally skewed X-chromosome inactivation of the X chromosome carrying the wild-type *IDS* allele of paternal origin. The affected twin was treated with idursulfase from 3 months of age with no significant adverse events, and after 3 years of treatment, was reported to have normal somatic development. The only somatic sign of the disease is that the affected twin has a mild deformity of one vertebra. Although comparisons are difficult, the older affected sister

at the age of 3 experienced the following somatic manifestations: mild coarse facial features; decreased range of motion in elbow, hip and ankle joints; slight hepatomegaly; and a small umbilical hernia.

Tajima and colleagues have very recently published a case study of two Japanese brothers with MPS II caused by an inversion mutation (Tajima et al. 2013). The older brother initiated treatment with idursulfase at 3.0 years of age, while the younger sibling initiated treatment at 4 months. At the start of treatment, the older brother showed typical somatic features of MPS II, including mitral valve regurgitation, gibbus deformity, joint stiffness, umbilical hernia, coarse facies, short stature, hepatomegaly, and cognitive impairment. After 2 years of treatment, the older brother's somatic disease was stable or improved, while cognitive decline continued. By comparison, after 32 months of ERT, the younger brother remained free from most of the somatic features that had already appeared in his brother at the same age. His only apparent disease manifestations were otitis media with effusion, slight findings of dysostosis multiplex, and mild cognitive impairment.

Here, we present data about the treatment of very young patients with MPS II in a case series of eight patients who received ERT with idursulfase beginning before 1 year of age, including a patient who began therapy at 10 days of age. All of the patients had non-consanguineous parents. A summary of patient demographics; birth weight, height, and head circumference; signs/symptoms at diagnosis, and details of treatment with enzyme replacement therapy are presented in Table 1.

Case 1

Patient 1, a Brazilian male, has a family history of an affected older half-brother and an affected maternal uncle, both with severe MPS II. He was diagnosed at 16 weeks 5 days gestation by an I2S enzyme activity assay that revealed undetectable I2S activity in amniotic fluid cells. A molecular genetic analysis revealed the familial p.R88H mutation, confirming the diagnosis. The prenatal course was uneventful and no abnormalities were noted on ultrasound. At 37 weeks 2 days gestation, Patient 1 was delivered via cesarean section due to labor failure to progress. Physical examination of the infant revealed a very subtle lumbar gibbus. A lumbar X-ray was notable for L3-L5 abnormality (Fig. 1a). An echocardiogram was normal. I2S enzyme activity was measured in plasma [1.2 nmol/4 h/mL (ref = 122–463 nmol/4 h/mL)] and in leukocytes [4.3 nmol/4h/mg/ptn (ref = 31–110 nmol/4 h/mg/ptn)], reconfirming the diagnosis of MPS II. Placental analysis by electronic microscopy revealed discrete lysosomal storage in endothelial cells and in pericytes, as has been previously reported (Baldo et al. 2011).

ERT with 0.5 mg/kg idursulfase weekly infused over 3 h via a peripheral IV line was initiated when the patient was 10 days old. Infusions were given weekly in the hospital setting and were continued for a total of six infusions, at which point ERT was stopped in preparation for hematopoietic stem cell transplantation (HSCT). At 19 days of age, the infant presented with a fever of 39°C, significant nasal obstruction, and yellow nasal discharge. He was admitted to the hospital and a 10-day course of intravenous antibiotics was prescribed. The fever and respiratory infection resolved without sequelae. ERT was not discontinued during this episode, as the child was not febrile on the day of the scheduled infusion.

The response to ERT was evaluated with a uGAG analysis. The baseline uGAG level at 5 days of age was 2,000 mg/g creatinine (upper limit of normal: 170 mg/g creatinine), which fell to 1,300 mg/g creatinine after 6 infusions of ERT. No adverse events were noted.

At 70 days of age, the patient underwent hematopoietic stem cell transplantation (HSCT) with umbilical cord blood from an unrelated donor. After transplantation, no additional ERT was given. His development has continued to be followed, and the patient's mother reports that he is doing much better than his older half-brother at the same age. A detailed account of the patient's post-transplant development will be published elsewhere.

Case 2

Patient 2 is an American male of Caucasian race. An ultrasound performed in the second trimester of the pregnancy revealed ventriculomegaly in the fetus. An amniocentesis was then performed; the chromosome analysis was normal. A fetal magnetic resonance imaging (MRI) study of the brain revealed mild prominence of the right ventricle and a midline cystic structure of the cavum vergae. Neurosurgical consultation was obtained, and it was felt these were likely benign findings. No prenatal testing for MPS II was performed because the older brother was not diagnosed until the mother's seventh month of gestation with Patient 2. There was no other family history of the disorder. Patient 2 was born at 39 weeks gestation via a planned cesarean section.

Six hours after the birth, the infant experienced respiratory distress and was transferred to the neonatal intensive care unit. The infant was initially given supplemental oxygen, then was intubated and given surfactant. After 24 h, the infant was extubated and eventually weaned to room air. Laboratory testing revealed elevated C-reactive protein levels, but cultures of blood, urine, and cerebral

Table 1 Summary of patient demographics and details of treatment with enzyme replacement therapy (ERT)

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Consanguinity	N	N	N	N	N	N	N	N
Positive MPS II family history (Relationship to Case)	Sibling, maternal uncle	Sibling	Maternal second cousin	Sibling	Maternal half-uncle, maternal great-uncles, maternal first cousin once removed	None	Maternal uncles, maternal first cousin once removed	Maternal great-uncle
Gestational ultrasound findings	Normal	Ventriculomegaly	ND	ND	Polyhydramnios	Normal	Normal	Large for gestational age
Apgar score 1′/5′	9/10	8/9	8/10	9/9	6/9	9/10	ND	ND
Birth weight in g, (percentile)	3,320 (54)	3,856 (92)	3,050 (27)	3,884 (93)	3,005 (24)	3,920 (95)	2,900 (16)	4,366 (>99)
Birth length in cm, (percentile)	51 (72)	52 (86)	49 (31)	52 (89)	48 (16)	51 (73)	47 (7)	53 (96)
Birth head circumference in cm, (percentile)	34 (35)	36 (89)	33 (12)	37 (98)	33 (12)	36 (89)	33.6 (24)	ND
Age at diagnosis	Prenatal	1 week	6 weeks	1 day	4 weeks	11 weeks	1 week	5.5 months
Genotype	p.R88H	p.R95G	p.P86L	p.R493P	c.1270insCC	p.G336E	c.1133A>G	c.1362-1365dup
Signs and symptoms at ERT baseline	Lumbar gibbus	Mild frontal bossing, hepatomegaly, lumbar kyphosis, pectus excavatum	Diastasis recti abdominis, hepatosplenomegaly, umbilical hernia	Mild coarse facies, hepatosplenomegaly, hearing loss, lumbar kyphosis	Coarse facies, Diastasis recti abdominis, hepatomegaly, umbilical hernia	Hydrocele, inguinal hernia, hepatomegaly, congestive heart failure	Mild frontal bossing, chronic otitis media with effusion, scapular flaring with right shoulder abduction	Mild coarse facies, small thickened ears, hepatomegaly, bilateral syndactyly of the second and third toes, gibbus; bilateral foot adduction, frequent upper respiratory infections, recurrent episodes of acute otitis media

(continued)

Table 1 (continued)

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
X-Ray/MRI skeletal findings at ERT baseline	Lumbar gibbus at L3-L5	Lumbar kyphosis, pectus excavatum, mild broadening of the ribs	ND	Mild focal kyphosis (apex at L2), anterior beaking of the caudal aspect of L2	Normal	ND	ND	Thoracolumbar gibbus with the apex at L2, bilateral forefoot adductus
Echocardiography at ERT baseline	Normal	Mildly dilated left ventricle with low-normal systolic function and increased apical trabeculations	Normal	ND	Small atrial septal defect or stretched patent foramen ovale	Abnormal (See Table 2)	Normal	Normal
Age at ERT start	1.5 weeks	6 weeks	8 weeks	10 weeks	11 weeks	12 weeks	6 months	6.5 months
Dose in mg/kg/ weekly infusion	0.5	1.5 at first, reduced to 0.5 as the patient gained weight[b]	0.6 mg for the first 8 infusions, and 0.5 mg for 9th and 10th infusions[b]	1.4 at first, reduced to 0.5 as the patient grew[b]	0.5	0.5	0.5	0.66 at first, reduced to 0.5 as the patient gained weight[b]
Total duration of treatment	6 weeks	2 years[a]	10 weeks	3 years[a]	5.5 years[a]	20 months[a]	4 years[a]	3.5 years[a]
HSTC	Yes	No	Yes	No	No	No	No	No

ERT enzyme replacement therapy, *HSTC* Hematopoietic stem cell transplantation, *MPS II* mucopolysaccharidosis II, *ND* no data

[a] Duration of treatment to date of last follow-up examination; patient currently continues on treatment

[b] Off-label use of the medication. The approved dose is 0.5 mg/kg weekly (Elaprase® prescribing information 2011)

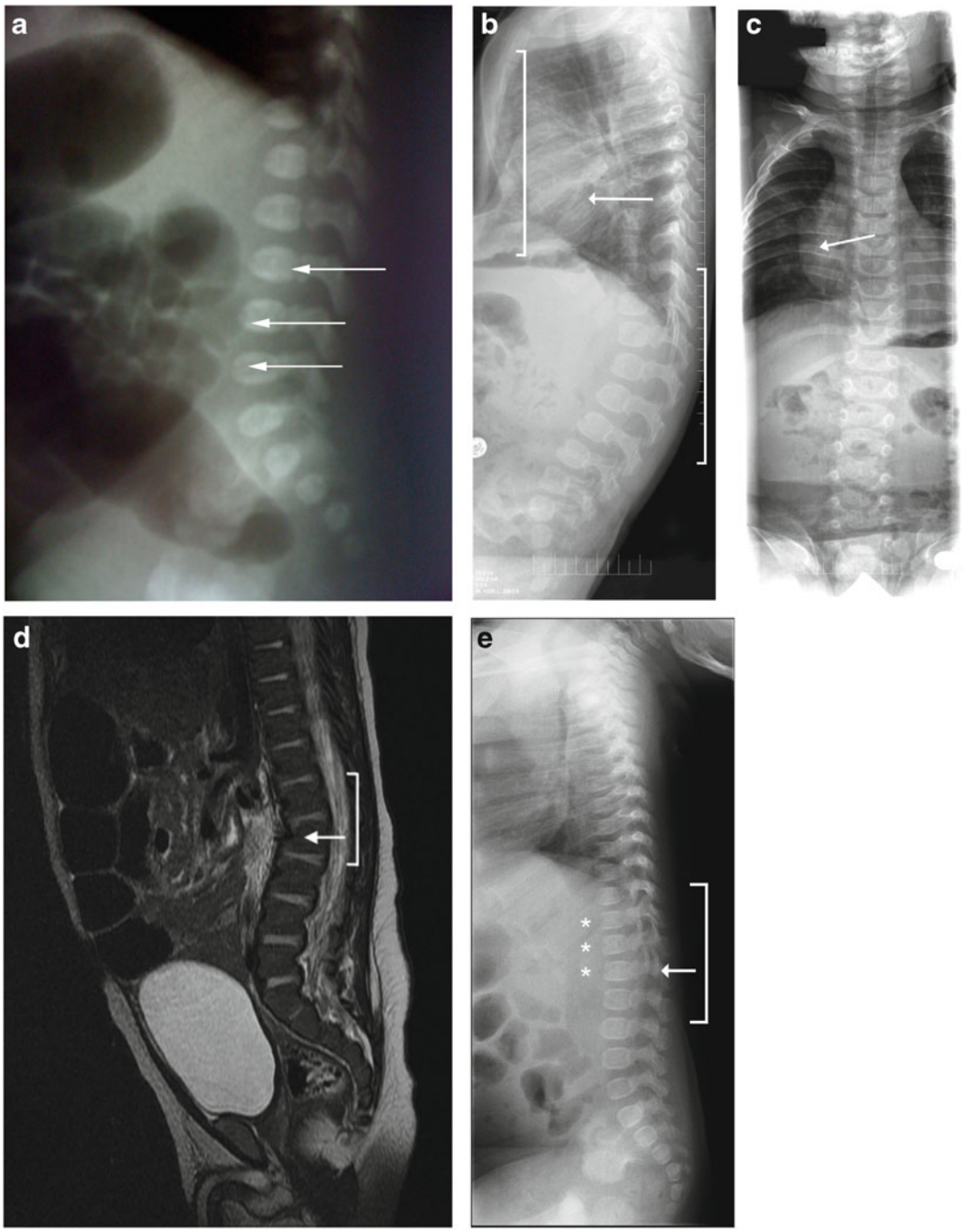

Fig. 1 Skeletal abnormalities in infants with MPS II. (**a**) Skeletal X-ray image of Patient 1 at 1 day of age showing L3-L5 abnormality (*arrows*). **b** and **c**) Skeletal X-ray images of Patient 2 at 6 months of age showing pectus excavatum (*panel **b**, left bracket*), mild lumbar kyphosis (*panel **b**, right bracket*), and mild broadening of the ribs (*panels **b** and **c**, arrows*). (**d**) A computed tomography scan of Patient 4's abdomen performed at 8 weeks of age showing upper lumbar kyphosis (*bracket*) with mild inferior beaking at L2 (*arrow*). (**e**) Skeletal X-ray images of Patient 8 at 5.5 months of age showing a thoracolumbar gibbus (*bracket*) with the apex at L2 (*arrows*) and beaked vertebrae (*stars*)

spinal fluid were negative. Nonetheless, the infant received antibiotic treatment for 7 days. The infant was discharged from the hospital at 2 weeks of age.

The diagnosis of MPS II was suspected while the infant was in the hospital due to mild frontal bossing (Fig. 2a) and hepatomegaly (liver edge palpable 4 cm below the right costal margin). A skeletal survey showed mild lumbar kyphosis, pectus excavatum, and mild broadening of the ribs (Fig. 1b, c). The child also failed the initial newborn hearing screen, but the follow-up assessment with an

Fig. 2 Mild coarse facial features and frontal bossing characteristic of MPS II. (**a**) Patient 2 at 7 months of age. **b** and **c**) Patient 4 at 6 weeks of age. Images used with permission

audiologist was normal. An echocardiogram performed at 2 days of age showed a non-obstructive band across the left ventricle. A follow-up echocardiogram at 1 month of age revealed a mildly dilated left ventricle with low-normal systolic function and increased apical trabeculation, possibly compatible with mild left ventricular non-compaction.

A uGAG analysis was performed at 6 days of age, revealing an elevated level of 151.6 mg/mmol creatinine (upper limit of normal: 53 mg/mmol creatinine). An I2S enzyme activity assay in leukocytes revealed no activity at 7 days of age, confirming the diagnosis. A molecular genetic analysis revealed a p.R95G mutation.

ERT was initiated when the child was 6 weeks of age. The starting dose was 1.5 mg/kg idursulfase given over 3 h via peripheral IV line; a full 6 mg vial was completely used in order not to waste medication, an off-label usage of the medication (Elaprase prescribing information 2011). As the infant gained weight, the dose per kilogram body weight decreased until a 0.5 mg/kg dose was achieved. He received weekly infusions in the infusion center at the hospital until 7 months of age, when he was transitioned to home therapy. At 10 months of age, a central line was placed. He remains on treatment at 2 years of age, and no adverse events have been seen.

At 7 months of age, the physical exam was essentially within normal limits except for mild frontal bossing and pectus excavatum (as noted at birth) and the palpable liver edge 2–3 cm below the right costal margin. The uGAG level, tested in a different hospital from the birth hospital, was 185 mg/g creatinine (normal $<$ 148 mg/g creatinine). When last examined at 2 years of age, the physical examination was completely normal except for a somewhat broad forehead with mild frontal bossing. Development was appropriate for age.

Case 3

Patient 3 is a German male. The infant was delivered spontaneously at 38 weeks' gestation after an uncomplicated pregnancy. The presence of diastasis recti abdominis was noted on physical examination at birth. No complications were reported in the postnatal period. When the infant was 6 weeks of age, the mother requested I2S testing because her maternal cousin's son (the infant's second cousin) had just been diagnosed with MPS II at 5 years of age. The mother also recalled that her maternal great-aunt had a son who had died at the age of 15 years from presumed Hurler syndrome (severe MPS I). As severe MPS I may appear clinically very similar to MPS II, it seems likely that this boy was also affected by MPS II and was incorrectly diagnosed. The uGAG level at 6 weeks of age was 55.80 mg/mmol creatinine (upper limit of normal: 27.20 mg/mmol creatinine), and an I2S activity assay revealed an activity of 8.2 nM/4 h/mL (normal range: 300–800 nM/4 h/mL). Molecular genetic testing revealed a p.P86L mutation, which was also shared by the infant's second cousin.

On clinical examination at 6 weeks of age, the boy appeared healthy and active. Diastasis recti abdominis was still present and a small umbilical hernia was seen. The liver was palpable 1 cm below the costal arch, but the spleen was not palpable. Reflexes were normal. The boy showed no paresis or contractures and had normal muscle tone and joint range of motion. Echocardiogram and electrocardiogram findings were both normal. An abdominal ultrasound revealed spleen enlargement and marginal liver enlargement. A cranial ultrasound was normal. Cognitive testing using the Bayley Scales of Infant Development was within normal ranges.

ERT was begun when the infant was 2 months of age. A total of 10 doses were given by peripheral IV infusion over 3.5 h. Idursulfase was used off-label at a dosage of 0.6 mg/kg weekly for the first eight infusions (Elaprase prescribing information 2011). During this time the infant's weight increased, so a dose of 0.5 mg/kg was used for the ninth and tenth infusions. No adverse events were seen during ERT, and no premedication was given. Antibody testing for IgE and IgG antibodies to idursulfase was negative throughout. After 10 doses of idursulfase, ERT was discontinued, and bone marrow transplantation with a matched donor was performed.

At 18 weeks of age, after 10 weeks on idursulfase treatment, the uGAG level was 27.61 mg/mmol creatinine, which was within normal range. Growth was appropriate for age. The liver remained palpable 1 cm below the costal arch. There were no evident developmental delays. An MRI of the spinal column revealed mild left convex scoliosis but no dysostosis multiplex. No hearing loss was found on audiological exam.

Case 4

Patient 4, a Hmong male born in the USA, has a family history of an older affected brother who was diagnosed at 2 years and 9 months of age. There were no prenatal complications during the mother's pregnancy with Patient 4, other than a known MPS II carrier status for the mother, and no prenatal testing for MPS II was performed. The infant was delivered vaginally at 40 weeks' gestation. At the time of delivery, umbilical cord blood was obtained and sent for genetic testing for MPS II. The enzyme activity assay on the umbilical cord blood measured an I2S activity level of 36 cpm/h/ml plasma (normal value: >14,376 cpm/h/ml/plasma), confirming the diagnosis of MPS II. The molecular genetic analysis revealed a p.R493P mutation, which is also found in the mother and the older affected brother.

The infant failed the routine newborn hearing test bilaterally. At 2 weeks of age, mild frontal bossing and slightly coarse facial features were present (Fig. 2b and c). A skin examination revealed several areas of dermal melanocytosis on his right ankle, left thigh, and buttocks. A computed tomography scan of the abdomen performed at 8 weeks of age demonstrated hepatosplenomegaly with a liver volume of 244 mL (normal: 125 mL) and spleen volume of 36 mL (normal: 10 mL) as well as upper lumbar kyphosis with mild inferior beaking at L2 (Fig. 1d). The kyphosis was only observed upon imaging and was not evident clinically. An MRI of the spine at 10 weeks of age revealed mild focal kyphosis with apex at L2 and anterior beaking of the caudal aspect of L2. An MRI of the brain at 10 weeks of age was normal.

ERT was initiated at 10 weeks of age with a dose of 0.5 mg/kg rounded to the nearest whole vial amount (a practice that is off label) and infused over 4 h via central IV line. The infant was prophylactically pre-medicated with acetaminophen, diphenhydramine, and hydrocortisone. The child has remained on ERT, which as of the time of publication is greater than 3 years of treatment. During this time, no IRRs have been seen, and the child is currently only pre-medicated with acetaminophen. The infusion time has been reduced to 2 h. Urinary GAG levels have improved, falling from a baseline level of 1856.7 μg/mg creatinine to 272.0 μg/mg creatinine (normal: < 127 μg/mg creatinine) at last measurement after 10 months of treatment. No antibodies to idursulfase have been detected at any point during treatment.

At 7 months of age, the otolaryngologist recognized bilateral hearing loss and placed tympanostomy tubes. Some mild hearing loss persisted after placement, and hearing aids were prescribed. At 14 months of age, electromyography and nerve conduction velocity testing for carpal tunnel syndrome were both normal. An echocardiogram at 16 months of age was normal. The last physical examination was performed at 18 months of age. Mild coarse facial features, including some mild frontal bossing, were present as was some minor joint stiffness in the elbows and wrists. The rest of the examination was unremarkable. The child's parents had no concerns for him at the time of the examination and said that he is developing much better than his older affected brother at the same age. He is a well-established walker. The orthopedist continues to monitor the spinal malformation but no further changes have been noted.

A formal developmental assessment was undertaken for the patient at 18 months of age. According to the Bayley Scales of Infant and Toddler Development, his gross motor and expressive language skills were age appropriate. His self-help, fine motor, receptive language, and verbal comprehension skills showed a 6- to 9-month delay.

Case 5

Patient 5 is an American male with an extensive positive family history of MPS II. The patient's mother had a maternal half-brother, two maternal uncles, and a maternal male first cousin who were all affected and died in the second to third decade of life. The pregnancy was unremarkable, and no prenatal testing was performed. Two routine prenatal ultrasounds were within normal parameters, but at 36 weeks gestation, a third ultrasound revealed polyhydramnios. The patient was delivered vaginally after 38 weeks and 2 days gestation. He was noted to be hypotonic on first exam and was given blow by oxygen.

At 3 h of life, he was noted to have difficulty in breathing and so was transferred to the neonatal intensive care unit (NICU) where he was treated for pneumonia and given surfactant. The infant was released after 13 days.

Enzyme testing for MPS II was ordered while the patient was in the NICU given the family history of MPS II. At 4 weeks of age, his I2S enzyme activity was found to be 0 cpm/h/mL/plasma (normal value: >64,147 cpm/h/mL/plasma). Molecular genetic testing revealed the c.1270insCC mutation in the *IDS* gene. On examination at 11 weeks of age, the patient was noted to have a normal growth pattern with normal head circumference. He presented with mild coarse facies (round face with periorbital fullness), diastasis recti, and fingertip umbilical hernia. The liver edge was palpable at 2 cm below the right costal margin. The rest of the examination was unremarkable. Echocardiogram findings reported either a stretched patent foramen ovale or a small secundum atrial septal defect measuring 4.5 mm, while an electrocardiogram suggested possible left ventricular hypertrophy. The skeletal survey was normal.

ERT was initiated when the infant was 11 weeks of age at a dose of 0.5 mg/kg weekly via peripheral IV initially; the patient later received infusions using a central IV line. The patient received infusions in the hospital for 15 months and then was transitioned to home therapy. He has now been receiving ERT for approximately 6 years and has not become positive for anti-idursulfase antibodies. His uGAG levels have fallen from 88.9 mg/mmol creatinine at baseline (normal: 10.9–22.3 mg/mmol creatinine) to 15.4 mg/mmol creatinine at last measurement in 2011. He has had his central line replaced twice, once due to a device malfunction and once due to an infection. One episode of hypertension was reported by a home health nurse during ERT administration in the home setting; however, this could not be replicated in the emergency room. No other adverse events to ERT have been observed.

The last follow-up examination occurred when the boy was 5.5 years of age. The patient had normal growth [height: 116.1 cm (69th percentile); weight: 21.6 kg (69th percentile)] and very minor joint range-of-motion restrictions. Cardiac echocardiogram found mild aortic valve stenosis, trivial pulmonary valve insufficiency, and normal left ventricular function. A bronchoscopy found mild glossoptosis, pharyngomalacia with posterior pharyngeal nodularity and cobble stoning, and a mild nodular appearance of the tracheal mucosa. Electromyography and nerve conduction velocity testing showed mild right median mononeuropathy at the wrist without evidence of active denervation. Cervical flexion-extension, abdomen, and chest X-rays revealed only subtle bony changes in the spine and hips. In the previous 2 years he has undergone placement of two sets of myringotomy tubes, with only one ear infection in that time. He currently wears a left hearing aid for mild hearing loss.

The patient has not undergone formal developmental testing, but at age 5.5 years his teacher reported that he had typical cognitive ability, which was evident during the last examination. None of his extended family members with MPS II had any history of cognitive impairment, and the affected individuals, who died at 18, 19, and 21 years of age, all were in or completed high school at the time of death without reports of academic problems. An MRI of the brain and spine performed at 5.5 years of age revealed some scattered foci of increased signal in the brain white matter and soft tissue thickening at the level of the foramen magnum without cord compression.

Case 6

Patient 6 is a Brazilian male with a family history of MPS, as the patient's father has five cousins with MPS I. No prenatal genetic testing was undertaken. The child was born vaginally after 37 weeks and 5 days gestation. The physical examination revealed a scrotal hydrocele. There were no complications in the postnatal period.

At 9 weeks of age, the infant presented with wheezing, abnormal cardiac auscultation, hepatomegaly (liver palpable 2 cm below the right costal margin), and inguinal hernia. A chest X-ray revealed an increased cardiac silhouette. He was diagnosed with viral cardiomyopathy and was hospitalized. The following day, an echocardiogram revealed dilated cardiomyopathy and a ventricular ejection fraction of 25 %. The patient was transferred to the intensive care unit. Soon after, the treating physician requested enzyme activity assays in dried blood spots for alpha-L-iduronidase (MPS I), I2S (MPS II), and arylsulfatase B (MPS VI). The diagnosis of MPS II was established at 11 weeks of age, and a molecular genetic analysis revealed the p.G336E mutation. As the child's cardiac function had continued to deteriorate, he was immediately transferred to the intensive care unit of a specialized cardiac care hospital with a diagnosis of severe congestive heart failure secondary to MPS II. A cardiac MRI performed at this hospital revealed substantial left ventricle dysfunction without myocardial fibrosis and no signs of inflammatory cardiomyopathy.

ERT with weekly infusions of 0.5 mg/kg idursulfase in 50 mL administered over 3 h and 30 min via central line was begun in the cardiac care hospital when the child was 12 weeks of age. The patient's uGAG level at diagnosis was 27.62 mg/mmol creatinine (upper limit of normal:

35.8 mg/mmol creatinine). After five weekly infusions, the child was discharged from the cardiac care hospital due to progressive improvement in cardiac function (Table 2). Concomitant medications included captopril, furosemide, spironolactone, and carvedilol.

The patient continues to receive ERT in the clinic via peripheral IV line and has been on treatment for about 20 months to date. No IRRs have been observed during ERT, and serum IgG and IgE antibody assays have been negative. After 5 months of ERT, the cardiologist noted a worsening of ejection fraction and recommended cardiac transplantation, which has not yet been performed. The physical examination at 1 year of age showed above-average growth [weight: 11,860 g (97th percentile), length: 80.5 cm (97th percentile), head circumference: 48.5 cm (97th percentile)], normal motor development, absence of hepatosplenomegaly, and normal joint range of motion. An echocardiogram performed when the child was 16 months of age, after approximately 13 months of ERT, showed sustained dilated cardiomyopathy, with severe impairment of left ventricle systolic function (Table 2).

Case 7

Patient 7 is an American male of Caucasian race. The patient's mother had two brothers and a maternal first cousin with MPS II. Her maternal great-aunt had a son and a grandson with MPS II as well. All of the affected males lived into their fourth decade of life. No complications were reported during the pregnancy with Patient 7. The infant was born vaginally at 36 weeks gestation. In the postnatal period, the patient experienced respiratory distress, and continuous positive airway pressure was maintained for 48 h after the birth.

At 1 week of age a blood sample was sent for enzyme activity testing because of the family history of MPS II, and no I2S activity was detectable. Molecular genetic analysis revealed a 78-base-pair insertion in the *IDS* transcript that has been previously reported and has been associated with an attenuated phenotype (Rathmann et al. 1996). Weekly ERT with 0.5 mg/kg idursulfase administered via a central intravenous line over 4 h was begun when the child was 6 months of age. At baseline, an abdominal ultrasound and echocardiogram were normal. No joint restrictions were noted, although scapular flaring with right shoulder abduction was identified. A tympanogram was consistent with the presence of middle ear fluid on the right, but hearing was normal. The Mullen Scales for Early Learning and Scales of Independent Behavior-Revised, Early Development form, was used to evaluate the patient developmentally. He demonstrated skills within the average or above average in all domains without correcting for gestational age.

The child has been receiving ERT for the past 4 years with no adverse events reported. His uGAG level at baseline was 200 μg GAG/mg creatinine. After 1 year of treatment, his uGAG levels had not decreased (240.8 μg GAG/mg creatinine); no more recent uGAG data are available. At age 2 years, a formal developmental evaluation was performed, and the patient scored in the average or above average range in all domains. At the last physical examination at the age of 3 years 9 months, his growth parameters were all normal, with height and weight at the 95th percentile and head circumference at the 75th percentile. He had slightly coarse facies, with frontal bossing, a receding anterior hairline, and slight puffiness of the upper eyelids. He also exhibited tapering of the fingers with slightly reduced extension of the digits at the distal interphalangeal joint. No evidence of macroglossia, organomegaly, spine deformities, hearing loss, or hernias was seen. At the time, he was growing and developing normally. He had not had a more recent formal developmental evaluation, but he was keeping up with his peers.

Case 8

Patient 8 is an American male of Caucasian race with a positive family history for MPS II, as his mother's uncle (his maternal great-uncle) has an attenuated form of the disease and is currently alive at the age of 42 years. No complications were noted during the pregnancy. A prenatal ultrasound was significant for the fetus being large for gestational age, but otherwise was unremarkable. The infant was born at 38 weeks' gestation. He was transferred to the NICU due to respiratory distress and was discharged after 6 days. There was no documentation of any abnormalities in the immediate postnatal period.

At 2 months of age, the patient was hospitalized for 1 week for the treatment of bronchiolitis. At 5.5 months of age, the patient's grandmother noticed a lump on his back; subsequent evaluation by orthopedics and a skeletal survey revealed a thoracolumbar gibbus with the apex at L2 (Fig. 1e) as well as bilateral forefoot adductus. The astute pediatric orthopedist also noted macrocephaly; a saddle-shaped nose; and small, thick ears, which triggered his suspicion of a metabolic disorder. Upon questioning the mother about a family history, he referred the patient to medical genetics for a possible diagnosis of MPS II.

On examination in the metabolic genetics clinic at 6 months of age, the patient showed mild coarse facies; small, thickened ears; hepatomegaly; and bilateral syndactyly of the second and third toes, in addition to the gibbus and bilateral foot adduction. The mother reported frequent upper respiratory infections, snoring, and recurrent episodes of acute otitis media. A uGAG analysis revealed a level of

Table 2 Echocardiogram findings for Patient 6

Parameter	Since start of ERT										
	−6 days	3 days	7 days	14 days	24 days	Normal range	171 days	293 days	Normal range	391 days	Normal range
Right ventricle diameter (mm)	8.2	ND	5.8	12	4.5	**<11.9**	ND	9.3	**<13.4**	4.8	**<15.2**
Diastolic septum thickness (mm)	5.4	ND	4.2	4.5	5	**<4.2**	59	3.7	**<5.6**	6	**<5.7**
Left ventricle diastolic diameter (mm)	37.6	43.7	41.5	41	40.6	**<21.7**	ND	58	**<30.8**	62.8	**<35.9**
Diastolic wall thickness (mm)	4.4	ND	5.1	4.5	4.3	**<4.2**	ND	3.2	**<5.4**	4	**<6.1**
Systolic septum thickness (mm)	6	ND	5	4.9	5.7	**NA**	ND	5.5	**NA**	7.5	**NA**
Systolic left ventricle diameter (mm)	34.9	ND	38.6	37	36.9	**NA**	ND	50.7	**NA**	54.2	**NA**
Systolic wall thickness (mm)	5	ND	6.8	4.1	6.6	**NA**	ND	ND	**NA**	6.8	**NA**
Aortic diameter (mm)	15	ND	ND	11.5	ND	**<11.6**	ND	14	**<15.3**	14	**<18.6**
Left atrium diameter (mm)	17	ND	ND	16	ND	**<15.7**	ND	30	**<21.2**	30	**<23.4**
Left ventricle shortening fraction (%)	7.2	ND	7.1	19	9.1	**28–42**	ND	12.6	**28–42**	13.7	**28–42**
Left ventricle ejection fraction (%)	12	ND	16	ND	20.3	**56–75**	ND	26.9	**56–75**	28.5	**56–75**
Left ventricle ejection fraction – Simpson's method (%)	ND	21	24	23	ND	**NA**	23	26	**NA**	28	**NA**

ND not done, *NA* not available

106.4 mg/mmol creatinine (normal range: 6.7–16.5 mg/mmol creatinine). A molecular genetic analysis revealed a small, 4-base-pair duplication (c.1362–1365dup) in the *IDS* gene. Findings on echocardiogram and MRI of the brain were normal.

ERT was initiated when the patient was 6.5 months of age. Initially an entire 6 mg vial of idursulfase was infused in order not to waste medication, yielding a dose of 0.66 mg/kg weekly (an off-label dosage). As the patient grew, the dose was adjusted to 0.5 mg/kg weekly. At the time of last follow-up at 4 years of age, he had not experienced any adverse events to ERT. His uGAG levels had fallen from 112.4 mg/mmol creatinine (normal: 6.2–13.6 mg/mmol creatinine) at baseline to 19.4 mg/mmol creatinine. Overall, the patient has done well on ERT. His liver size normalized, and echocardiogram results have remained normal. Myringotomy tubes were placed concurrently with a tonsillectomy and adenoidectomy at age 19 months, and this procedure was repeated at age 3.5 years. The gibbus deformity progressed during his second year of life, but has stabilized since 3 years of age. He developed slight contractures of the joints in the upper extremities by 3.5 years of age. A bilateral carpal tunnel decompression surgery was performed at 3.5 years of age. A formal neurodevelopmental evaluation was conducted at the age of 3 years 8 months using the Capute Scales. The patient's score on the Cognitive Adaptive Test was the age equivalent of 30 months. Given his chronological age, that yields a visual-motor problem solving quotient of 68. On the Clinical Linguistic and Auditory Milestone Scale, his age equivalent was 33 months, which is a language quotient of 75.

Discussion

We have presented a case series of eight patients with MPS II whose treatment with idursulfase was begun under the age of 1 year. The age at initiation of treatment was between 10 days and 6.5 months, and the duration of treatment was between 6 weeks and 5.5 years. No new safety concerns were identified and none of the patients experienced an IRR. Our experience here suggests that the development of disease manifestations occurs rapidly after birth, or perhaps even begins prenatally. All of the patients showed improvements and/or stabilization of some somatic manifestations while on treatment.

The use of ERT in very young patients with MPS II has generated much recent interest, both in the safety profile in this age group and in the ability of ERT to modify the natural history of the disease in younger versus older patients. The pivotal phase II/III trial for idursulfase in MPS II only enrolled patients over the age of 5 years due to the need for patients to participate in the forced vital capacity and 6-min walk tests that made up the composite primary endpoint (Muenzer et al. 2006). Reassuring results from a recent open-label clinical trial of idursulfase in 28 patients aged 1.4–7.5 years indicated no new safety concerns in this population, with improvements seen in liver size and uGAG levels (Giugliani et al. 2013). In addition, there are non-controlled patient registry data suggesting that the safety profile of idursulfase is similar in younger and older patients in clinical practice. A retrospective study using data from the Hunter Outcome Survey (HOS), a voluntary patient registry for patients with MPS II regardless of treatment status (Wraith et al. 2008), evaluated the safety and effectiveness of idursulfase in 124 patients younger than 6 years and compared these findings with those seen in 289 older patients (Muenzer et al. 2011a). The mean age at start of ERT in the younger group was 3.6 ± 1.6 years. There were no new safety concerns in the younger group as compared with the older group. In our experience of using ERT in eight patients under 1 year of age, no new safety concerns were seen and none of the patients experienced IRRs after a treatment duration of between 6 weeks and 5 years. This is encouraging given that the majority of IRRs appear to occur during the first 3 months of therapy (Burton and Whiteman 2011).

The ability of ERT to change the natural history of the disease when begun in very young patients has not been systematically evaluated to date. This is in part because identifying and diagnosing very young patients without a family history of the disease generally requires newborn screening, which has not yet been generally instituted for MPS II (Nakamura et al. 2011). In the HOS study of patients under 6 years of age, ERT effectiveness as assayed by reductions in liver size and uGAG levels was found to be similar between both the younger and older groups of patients, but the clinical significance in altering the long-term disease course could not be evaluated using the available registry data (Muenzer et al. 2011a). There are sibling-pair case study data for MPS I, II, and VI suggesting that the initiation of ERT in infancy results in a better long-term clinical outcome (Gabrielli et al. 2010; McGill et al. 2010; Tajima et al 2013; Tylki-Szymanska et al. 2012). The most current recommendations from the Hunter Syndrome European Expert Council state that the relationship between progressive GAG storage and clinical manifestations in MPS II provides a strong argument for the initiation of ERT as early as possible following diagnosis (Scarpa et al. 2011). Indeed, our experience with these eight patients shows that disease manifestations are present from a few days or weeks of age, or even prenatally in some cases, upon careful examination and imaging. Six out of eight patients displayed organomegaly, five out of eight displayed mild coarse facies, and three out of eight had an umbilical and/or inguinal hernia. Among the five patients who underwent a skeletal survey before starting ERT, four

had spinal malformations (kyphosis or gibbus), as seen in Fig. 1a–e.

In this report, we provide evidence that early ERT has resulted in somatic improvements among our patients, although two of the eight patients (Patient 1 and Patient 3) only received ERT for a short time before HSCT. For Patient 1, ERT plus HSCT was tried based on an extrapolation of the results seen with MPS I (de Ru et al. 2011); such an approach is not the standard of care for MPS II. Early marked decrease in uGAGs was seen in Patients 1 and 3, but the short duration of treatment makes interpretations of longer-term ERT effectiveness impossible for these patients. Among the patients who did not undergo HSCT, Patient 2 has been treated for 2 years; during that time, no new disease manifestations have been seen, and the mild frontal bossing and pectus excavatum which were noted at birth remained stable. His hepatomegaly improved on treatment. Patient 4 has been treated for about 3 years to date. His uGAG levels have significantly improved, he has no respiratory complaints, and his spinal malformation and facial features have remained stable. His hearing loss has progressed, and he has minor joint stiffness in his elbows and wrists, although he has no trouble with ambulation. His parents subjectively report that he is doing much better than his older affected brother at the same age. Patient 5 has been receiving ERT for approximately 5.5 years and has experienced improvements in uGAG level and hepatomegaly. His signs and symptoms include very mild joint restrictions, mild aortic stenosis, mononeuropathy of the right median nerve, subtle bony changes in the spine and hips, umbilical hernia (repaired at 5 years of age), mild hearing loss in the left ear, and otitis media leading to the placement of ventilation tubes. He is a healthy appearing child who is active and doing very well in school. Other affected maternal relatives were reported to have somatic manifestations that led to death at the ages of 13, 18, 19, and 21 years. The patient's mother reported that the other affected family members had obvious short stature at that same age, although their records are not available.

Patient 6 is somewhat unusual in that there was no family history of the disease. He presented at 9 weeks of age with dilated cardiomyopathy and a reduced ventricular ejection fraction, and his cardiac function deteriorated rapidly over the course of the next 3 weeks. After five infusions of ERT, a progressive improvement in cardiac function was seen, and the boy was discharged from the hospital. He has received treatment for the past 20 months; however, his ejection fraction worsened after 5 months on ERT and he is now a candidate for cardiac transplantation. The cardiologist has speculated that the patient may have had both viral myocarditis and cardiac disease due to MPS II in the neonatal period. Interestingly, Patient 2 also displayed very early cardiac signs with a mildly dilated left ventricle at 1 month of age. Cardiac involvement is a well-documented, characteristic feature of MPS II, reported for about two-thirds of patients in HOS (Kampmann et al. 2011). Because cardiologists may be unaware of the signs and symptoms of the MPS, it would be worthwhile to include these disorders in listings of the differential diagnoses for cardiomyopathy in the young child.

Patient 7 began treatment at the age of 6 months and has been receiving ERT for about 4 years. Currently at age 4.5 years, his disease manifestations are limited to slightly coarse facies and tapering of the fingers with slightly reduced extension of the digits at the distal interphalangeal joint. Patient 8, who in this case series initiated ERT the latest, at 6.5 months, experienced improvements in uGAG level and normalization of liver size on treatment. His echocardiogram results have remained normal. However, some disease features have shown progression during the 3.5 years that he has been on treatment, including hearing loss, airway disease, slight joint contractures of the upper extremities, and carpal tunnel syndrome. In addition, his gibbus deformity progressed for about a year before stabilizing.

In summary, in our experience the use of ERT in MPS II patients under 1 year of age did not produce any new safety concerns, and all of our patients showed improvements and/or stabilization of some somatic manifestations while on treatment. We note with interest that, in our cohort of early-treated patients, the effectiveness of ERT does not appear to have decreased over time, even after 3–5 years of treatment in some cases. In addition, in some of the cases, caregivers made comparisons with older affected siblings or other affected family members and subjectively reported that the early treated patients experienced a less severe clinical course, consistent with other case reports (Tajima et al. 2013; Tylki-Szymanska et al. 2012). Unfortunately, the limited nature of the medical records available for other family members precluded a more rigorous comparison of phenotypes. Long-term follow-up of these and other MPS II patients who began therapy within the first months of life will provide valuable information on the ability of ERT to possibly prevent or delay the development of certain disease manifestations. Such efforts would be greatly helped by the implementation of newborn screening programs for this disorder (Nakamura et al. 2011).

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Synopsis

In a case series of eight MPS II patients treated with idursulfase enzyme replacement therapy started under 1 year of age, treatment was well tolerated and produced some somatic improvements, with no new safety concerns seen.

Compliance with Ethics Guidelines

Individual Contributions

The planning, writing, and content decisions for the manuscript were performed by all the authors equally.

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Conflict of Interest Statements

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Informed Consent for Identifying Information

Consent to publish identifying information and facial images was obtained from all parents/guardians of patients for whom identifying information is included in this report.

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RESEARCH REPORT

Fructose-1,6-Bisphosphatase Deficiency: A Case of a Successful Pregnancy by Closely Monitoring Metabolic Control

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Abstract In patients with fructose-1,6-bisphosphatase (FBPase) deficiency, maternal and fetal neurodevelopmental abnormalities can be induced by severe hypoglycemia and lactic acidosis, which is triggered by pregnancy. The patient was a 24-year-old and primipara. Via pre-pregnancy education and management including self-monitoring of blood glucose and prompt medical intervention with glucose infusions, she never experienced hypoglycemia with impaired consciousness. She delivered a 2,406 g male infant at 38 weeks of gestation by cesarean section. No neurological abnormalities in either the mother or the child appeared during the following 2 years. This is the first report with detailed descriptions of the management of a pregnant woman with FDPase deficiency. This report suggests that for a patient with FBPase deficiency, the maternal and fetal complications can be reduced by pre-pregnancy education and self-monitoring of blood glucose to prevent hypoglycemia.

Introduction

Fructose-1,6-bisphosphatase (FBPase) deficiency is a rare metabolic disorder that is caused by a reduction in activity of FBPase, the rate-limiting enzyme of gluconeogenesis. Because of a deficiency of this enzyme, the inability to convert lactic acid or glycerol into glucose leads to hypoglycemia and lactic acidosis; it was first recognized in 1970 by Baker and Winegrad (1970). The disease is caused by mutations in the FBPase gene and is transmitted as an autosomal recessive (the frequency is 1–9/100,000) (Douillard et al. 2012). Patients with FBPase deficiency have limited amounts of glycogen stores. The disease is diagnosed by an enzymatic assay of FBPase activity from hepatic specimens and peripheral cultured monocytes (Kikawa et al. 2002); mutational analysis is useful for the differential diagnosis. By instituting appropriate treatment for hypoglycemia and dietary education, the long-term neurological prognosis is generally good.

The literature contains only two reports of the management of a pregnancy with FBPase deficiency (Table 1); one of the patients suffered recurrent lactic acidosis and hypoglycemia during pregnancy, and subsequently developed sensorineural hearing loss and early-onset cognitive impairment (Krishnamurthy et al. 2007). Hyperemesis, glucose metabolism during pregnancy, and fasting before and after vaginal delivery or cesarean section often lead to hypoglycemia. Furthermore, there is a possibility of not only a maternal cognitive disorder but also deterioration in the development and neurological function of the infant. In this report, we describe a woman with FBPase deficiency in whom hypoglycemic episodes were prevented by pre-partum education and strict glycemic control with self-monitoring of blood glucose throughout the pregnancy; as a result, neither maternal nor fetal cognitive disorders manifested.

Case Report

Since infancy, the patient experienced two episodes of hypoglycemia and metabolic acidosis with vomiting and

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Table 1 Literature review of pregnancies complicated with FBPase deficiency

Authors	Management of pre-pregnancy	Management during pregnancy	Maternal prognosis	Newborn prognosis
Krishnamurthy et al. 2007	None (recurrent episodes of lactic acidemia and hypoglycemia)	Strict compliance with dietary management, including nocturnal uncooked cornstarch	Sensorineural hearing loss and early-onset cognitive impairment	No complications
Asberg et al. 2010	Ingesting some food just before retiring	None (uneventful pregnancy)	No complications	No complications
Current	Instruction regarding the risk of hypoglycemia during pregnancy	Self-monitoring of blood glucose and prompt medical intervention with glucose infusions	No complications	No complications

impaired consciousness. She was diagnosed with FBPase deficiency at age 1 via the lack of FBPase activity in peripheral cultured monocytes (0 nmol/min per mg protein; reference: 4.1–15.3). This assay was conducted by pediatricians at the University of Fukui who specialized in inherited metabolic diseases. Mutation analysis was not performed. Subsequently, the patient received medical treatment including administration of intravenous glucose and sodium bicarbonate two to three times a year because of episodes of fasting hypoglycemia. She had normal growth and attended a regular school without problems.

At age 24, she wanted to achieve a pregnancy; therefore, she was instructed regarding the risk of hypoglycemia during pregnancy. At 6 weeks of gestation, she presented at our medical center with a complaint of hyperemesis. She was in good health, and the physical examination was within normal limits. During a fasting state, the serum glucose level was 112 mg/dl (reference: 75–105) and the lactate level was 15 mg/dl (reference: 4–16). The venous blood gases were as follows: pH, 7.38 (reference: 7.32–7.40); pCO2, 44.9 mmHg (reference: 42–51); HCO3, 25.8 mmol (reference: 21–27); pO2, 29.6 mmHg (reference 35–45); and base excess, 0.8 mmol (reference: −2 to 2). She received intravenous fluids of 1,000–2,000 ml including glucose 100–200 g/day during hospitalization because she experienced difficulty with oral intake. We instructed her regarding self-monitoring of blood glucose at home to prevent hypoglycemia and avoidance of overeating; blood glucose was measured three times daily before meals and if she felt unwell. In addition, we advised her regarding oral ingestion or intravenous administration of glucose when her blood glucose level was below 86 mg/dl or she could not tolerate oral intake.

After her discharge, when her blood glucose level dropped, she immediately orally ingested glucose, including candy, chocolate, and juice. When she could not tolerate oral intake, she immediately presented at our hospital. Although she was hospitalized three times, the prompt infusion of glucose 100–200 g/day avoided hypoglycemia or metabolic acidosis, and she did not experience hypoglycemia with impaired consciousness. Weight gain during pregnancy was 6 kg. A 75 g oral glucose tolerance test (OGTT) at 28 weeks of gestation revealed a normal pattern: fasting serum glucose level, 83 mg/dl; 1-h level, 148 mg/dl; and 2-h level, 106 mg/dl. The pregnancy course was uneventful.

An elective cesarean section was performed at 38 weeks, 3 days of pregnancy because of a breech presentation. Via measurement of glucose and blood gases as well as 200–220 g/day glucose administration when fasting before and after surgery, we maintained strict glycemic control. As a result, she never experienced hypoglycemia or metabolic acidosis. A 2,406 g male infant (−1.4 standard deviation) was delivered with an Apgar score of 8 and 10 at 1 and 5 min. The umbilical arterial blood gases were as follows: pH, 7.37 (reference: 7.32–7.40); glucose level, 112 mg/dl (reference: 75–105). The postoperative course was uneventful, and she had no neurological abnormalities during the following 2 years. Her child's developmental milestones were normal and were equivalent to age 2 years.

Discussion

Patients with FDPase deficiency exhibit severe fasting hypoglycemia and lactic acidosis. The primary treatment is to prevent hypoglycemia by the administration of exogenous glucose, either by frequent meals or by glucose infusion. If the treatment for hypoglycemia is properly administered, the prognosis is good. However, hypoglycemia with severe neurological complications has been reported following the administration of fructose or glycerin (Hasegawa et al. 2003) or during pregnancy. The literature contains only two case reports regarding the management of pregnancy with FBPase deficiency. When the patient was admitted due to recurrent severe hypoglycemic attacks and lactic acidosis, she was found to be pregnant and in her second trimester. Despite compliance with monitoring and treatment of hypoglycemia, she developed sensorineural hearing loss and early-onset cognitive

impairment (Krishnamurthy et al. 2007). Another patient was hospitalized many times during her first 3 years of life, but she never felt that she was significantly affected by her illness after early childhood; in addition, she was the mother of two healthy children after two uneventful pregnancies (Asberg et al. 2010). Therefore, if a patient has suffered from recurrent hypoglycemic attacks during her adult life, it is suggested that pre-partum patient education and strict management during pregnancy are important for maternal neurological outcome (Table 1).

Glucose requirements are altered during pregnancy. Pregnant women must provide fuel not only for their own energy needs but also for the growth and metabolic requirements of the fetus. In the early pregnancy of a healthy woman, the increase in peripheral insulin sensitivity possibly causes diminished glucose availability in the circulation that can be compensated for by increased endogenous glucose production in times of need (Catalano et al. 1998). However, a patient with FBPase deficiency who cannot produce an endogenous glucose supply is prone to severe hypoglycemia and lactic acidosis when the exogenous glucose supply is interrupted due to emesis.

It is well known that an association exists between maternal hypoglycemia during pregnancy and fetal growth restriction as well as overall perinatal mortality. A higher incidence of small-for-gestational-age infants was found in the group with the low (<86 mg/dl) mean blood glucose level (Langer et al. 1994). Hypoglycemia might hinder fat-storage or growth-promoting factors in the fetal environment, which could result in low-birth-weight infants (Vadakekut et al. 2011). Moreover, Pugh et al. reported that patients with maternal hypoglycemia may be more likely to develop preeclampsia (Pugh et al. 2009). Increasing oral glucose requirements to prevent hypoglycemia can result in gestational diabetes.

Metabolic deterioration or fasting during delivery can be a serious risk for the child because lactic acidosis in the mother can cause serious problems in the neonate. Long-term anesthesia, a blood transfusion, and/or hypoglycemia can produce significant metabolic acidosis (Hashimoto et al. 1978). Because neonatal hypoglycemia has been demonstrated to be associated with long-term neurological dysfunction, including brain dysfunction, recurrent episodes of severe maternal hypoglycemia during pregnancy might affect the developing fetal brain.

In this case, via pre-pregnancy education, dietary counseling, and glucose infusion, we attained successful glycemic control during early pregnancy, which is the period when hypoglycemia is likely to occur. In the second and third trimesters, we instructed the patient regarding self-monitoring of blood glucose at home to prevent hypoglycemia and avoidance of overeating. By self-monitoring blood glucose and rapid medical intervention when oral intake was inadequate, she never experienced hypoglycemia with impaired consciousness. We screened for the risk of gestational diabetes mellitus due to over-eating by conducting a 75 g OGTT. At the cesarean section, we prevented hypoglycemia and lactic acidosis by a continuous glucose infusion during fasting both before and after the cesarean section. Moreover, we closely monitored blood glucose and arterial blood gases until adequate oral intake had been established. No neurological abnormalities in either the mother or the child manifested during the following 2 years.

In August 2013, we searched the electronic database of Medline for publications containing the terms “fructose-1,6-bisphosphatase deficiency” and “pregnancy” and “fructose-1,6-bisphosphatase deficiency” and “long-term prognosis.” We identified eight reports in the English literature, but found no case reports with detailed descriptions of the management of a pregnant woman with FDPase deficiency. This report suggests that for a patient with FBPase deficiency, the maternal and fetal complications can be reduced by pre-pregnancy education and self-monitoring of blood glucose to prevent hypoglycemia.

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Synopsis

For a patient with fructose-1,6-bisphosphatase deficiency, the maternal and fetal complications can be reduced by pre-pregnancy education and self-monitoring of blood glucose to prevent hypoglycemia.

Conflict of Interest

Genki Sugita, Hideaki Tsuyoshi, Koji Nishijima, and Yoshio Yoshida declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Animal Rights

This report does not contain any studies with animal subjects performed by any of the authors.

Details of the Contributions of Individual Authors

I acknowledge that all authors have contributed significantly to the planning, conduct, and reporting of the manuscript, and that all authors are in agreement with the content of the manuscript. The guarantor for this report is the corresponding author, Hideaki Tsuyoshi, M.D.

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ORIGINAL ARTICLE

Erratum to: AGC1 Deficiency Causes Infantile Epilepsy, Abnormal Myelination, and Reduced *N*-Acetylaspartate

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One of the author's names was inadvertently reversed in the published version of this chapter. The author's name is Yiran Guo, where his first name is Yiran and his last name is Guo. It should thus be abbreviated as Y. Guo.

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