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RESEARCH REPORT

Friedreich Ataxia in Classical Galactosaemia

Siobhán Neville • Siobhan O'Sullivan • Bronagh Sweeney • Bryan Lynch • Donncha Hanrahan • Ina Knerr • SallyAnn Lynch • Ellen Crushell

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Abstract Movement disorders such as ataxia are a recognized complication of classical galactosaemia, even in diet-compliant patients. Here, we report the coexistence of classical galactosaemia and Friedreich ataxia (FRDA) in nine children from seven Irish Traveller families. These two autosomal recessive disorders, the loci for which are located on either side of the centromere of chromosome 9, appear to be in linkage disequilibrium in this subgroup. Both conditions are known to occur with increased frequency amongst the Irish Traveller population.

Each member of our cohort had been diagnosed with galactosaemia in the neonatal period, and all are homozygous for the common Q188R mutation in the *GALT* gene. Eight of the nine patients later presented with progressive ataxia, between the ages of 5–13 years. Another child presented in cardiac failure secondary to dilated cardiomyopathy at 7 years of age. He was not ataxic at presentation and, one year from diagnosis, his neurological examination remains normal. The diagnosis of FRDA was confirmed by detecting the common pathogenic GAA expansion in both alleles of the frataxin gene (*FXN*) in each patient.

Neurological symptoms are easily attributed to an underlying diagnosis of galactosaemia. It is important to consider a diagnosis of Friedreich ataxia in a child from the Irish Traveller population with galactosaemia who presents with ataxia or cardiomyopathy.

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Competing interests: None declared

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Introduction

Classical galactosaemia is an autosomal recessively inherited disorder of galactose metabolism. Mutations in the *GALT* gene result in reduced activity of the galactose-1-phosphate uridyltransferase (GALT) enzyme leading to toxic accumulation of galactose and its metabolites (Murphy et al. 1999). Presentation is typically in the neonatal period, with feeding problems, hepatic failure and coagulopathy soon after the introduction of galactose-containing feeds (Rubio-Agusti et al. 2013). Without urgent treatment by dietary restriction of galactose, these infants are at risk of *E. coli* sepsis, multi-organ failure and death.

Classical galactosaemia is relatively common in the Irish population, with an overall birth incidence of 1 in 16,476 (Coss et al. 2013). This is in part due to a high incidence in the endogamous Irish Traveller population, with approximately 1 in 430 live births being affected in this group.

While dietary restriction of lactose and galactose is life saving in the neonatal period, long-term complications of galactosaemia occur even in diet-compliant patients (Coss et al. 2013). These include neurological complications such as ataxia and tremor. Although movement disorders are well described in galactosaemia, with reported rates varying from 6 to 45% (Coss et al. 2013; Ridel et al. 2005; Rubio-Agusti et al. 2013; Waggoner et al. 1990), their pathogenesis is, as yet, poorly understood.

Friedreich ataxia (FRDA) is a neurodegenerative disorder, which also demonstrates autosomal recessive inheritance. It is the commonest inherited ataxia, with a prevalence of up to 1 in 30,000 in parts of Western Europe, including Ireland (Collins 2013; Schulz et al. 2009). The prevalence in the Irish Traveller population has not been ascertained, although anecdotal evidence suggests it is common in this population (D. Barton and S.A. Lynch, personal communication). FRDA occurs due to mutations in the frataxin (*FXN*) gene, resulting in a marked reduction in production of this mitochondrial protein. Over 95% of cases are due to homozygous GAA triplet repeat expansions in the first intron of the *FXN* gene (Schulz et al. 2009). This is the mutational mechanism found amongst Irish Travellers.

FRDA usually presents in the first two decades of life with gait instability, ataxia, dysarthria and impaired sensation. Non-neurological manifestations include scoliosis, hypertrophic cardiomyopathy and diabetes mellitus. The disease is progressive, with an average of 10 years passing between onset of ataxia and the patient becoming wheelchair dependent (Collins 2013; Delatycki 2012). Current treatment of FRDA is supportive only, although novel agents are under investigation.

Here, we report the coexistence of genetically proven FRDA and classical galactosaemia in a cohort of paediatric patients from the Irish Traveller population.

Methods

Two centres were involved in this study: the National Centre for Inherited Metabolic Disorders at Children's University Hospital, Temple Street in Dublin, Ireland, and the Department of Metabolic Paediatrics at the Royal Hospital for Sick Children in Belfast, Northern Ireland. Between these two centres, care is provided for all paediatric galactosaemia patients on the island of Ireland.

A review of the 114 Irish and Northern Irish paediatric galactosaemia patients identified nine children who have also been diagnosed with FRDA. Following approval from the local Research and Ethics Committee, a detailed, retrospective chart review was performed for each of these nine cases. This included collecting basic demographic data, patient history and physical examination findings, as well as accessing imaging, test results and educational psychology reports.

An extensive literature review failed to reveal any previously reported incidences of coexistent galactosaemia and FRDA in this, or any other, population.

Results

Nine of a total 114 Irish and Northern Irish paediatric galactosaemia patients have been diagnosed with FRDA. These nine patients (four male, five female) include two sets of siblings. While the seven families are not known to be related, all are members of the Irish Traveller population. There is known parental consanguinity in seven of the nine cases.

All were diagnosed with galactosaemia in the newborn period (range: day of life 2–8) and all are homozygous for the common Q188R mutation (*GALT*).

The onset of ataxia ranged from 5 to 13 years of age, with the diagnosis of FRDA being made between 6 and 15 years (see Table 1). This diagnosis was confirmed by detecting the common pathogenic GAA expansion (>66 repeats) in both alleles of the *FXN* gene in each patient. The index case presented with gradually progressive ataxia and tremor from the age of five. Following a normal MRI brain, he was seen by a Consultant Paediatric Neurologist, (BL) who suspected FRDA based on clinical history and detailed neurological examination. This diagnosis was confirmed by frataxin gene analysis, by which time our patient was 10 years of age.

Subsequent to Case 1's diagnosis, his older sister was reviewed in clinic and found to have similar, albeit milder, ataxia, tremor and areflexia. Her MRI brain was also normal, and diagnosis of FRDA was confirmed on *FXN* gene analysis shortly after. Since that time, seven further patients have been diagnosed in Dublin and Belfast. The majority of these children had significantly shorter lag periods between symptom onset and diagnosis than our initial case, owing to a raised index of suspicion for the disease in this population. Two of these patients had brain imaging prior to diagnosis, which were again normal.

Case 8 had failed to attend his galactosaemia outpatient clinics for some years prior to presenting to another centre, aged 7 years, with decompensated cardiac failure secondary

Table 1 Diagnosis of FRDA

Case no.	Current age (years)	Age at symptom onset (years)	Age at diagnosis (years)	Duration pre-diagnosis (years)	Karyotype	MRI brain
1[a]	16	5	10	5	N	Y
2	17	10	11.5	1.5	N	Y
3	17.5	13	15	2	Y	N
4	16	13	14	1	N	Y
5	11	5	9	4	N	N
6	8.5	6	7.5	1.5	N	N
7	8	5	6	1	N	N
8	8	7.25	7.5	0.25	N	N
9	8.5	7.5	8.5	1	N	Y
Median (range)	11 (8–17.5)	7.25 (5–13)	9 (6–15)	1.5 (0.25–5)	[1/9]	[4/9]

[a] Index case
Y test conducted, *N* test not conducted

Table 2 Features of FRDA

Case no.	Ataxia	Areflexia	Tremor	Dysarthria	Pes cavus	Scoliosis	Cardiomyopathy	Hearing loss
1[a]	+++	+	+	+	+	+++	+	–
2	++	+	+	+	+	+	+	–
3	+	+	+	+	+	+	+	+
4	+	+	+	–	–	+	+	–
5	+	+	+	+	+	+	U	–
6	+	+	+	–	+	–	U	–
7	+	+	+	+	+	+	+	–
8	–	–	–	–	–	–	+++	–
9	+	+	–	–	U	–	U	–
Total ($n = 9$)	8	8	7	5	6	6	6	1

[a] Index case
+ feature present, +++ feature present and severe, – feature not present, *U* unknown as yet

to dilated cardiomyopathy. A full cardiomyopathy screen was performed, which yielded the same frataxin mutation seen in our other cases. One year on from diagnosis, his neurological exam remains normal.

The characteristics of FRDA seen in each of our cases are outlined in Table 2. At present, seven of our patients remain independently mobile; Case 1 is fully wheelchair dependent and Case 2 requires a manual wheelchair when mobilizing outside the home. Five patients have a mild degree of left ventricular hypertrophy on echocardiogram, while three of our most recently diagnosed patients are still awaiting cardiology review. To date, no patients have developed diabetes mellitus.

Discussion

The *GALT* and *FXN* genes are located on either side of the centromere of chromosome 9, at positions 9p13.3 and 9q21.11, respectively. Karyotyping was performed in one patient in order to rule out a common pericentric inversion of chromosome 9, which may have accounted for the co-segregation of these disorders. This, however, was normal. In the absence of such a structural chromosomal abnormality, the co-segregation of the two disorders in these families suggests that crossover during meiosis is occurring telomeric to both genes and that the two genes are in linkage disequilibrium (see Fig. 1).

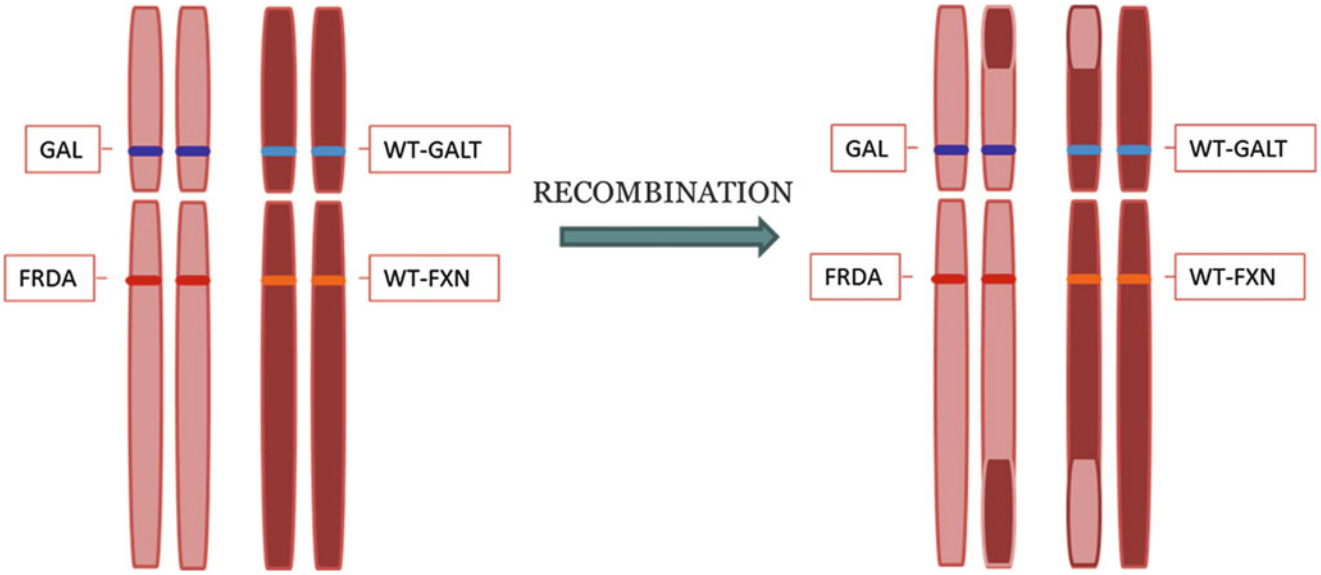

Fig. 1 Crossover of genetic material between homologous chromosomes during meiosis I. *WT-GALT* wild-type galactose-1-phosphate uridyltransferase enzyme gene (normal function), *WT-FXN* wild type frataxin gene (normal function), *GAL* classical galactosaemia-causing mutation in GALT, *FRDA* Friedreich ataxia-causing mutation in FXN

The coexistence of these conditions occurs only in a subset of the Traveller population, with other families known to be affected by either classical galactosaemia or FRDA alone. However, genetic recombination in our cohort of families may have resulted in some patients being homozygous for only one of the two diseases. To date, three siblings (from three different families) of our nine index cases have been diagnosed with galactosaemia alone. At four and seven years old, two of these children could be in the pre-symptomatic phase of FRDA; however, the third sibling is in his mid-teens and as yet is showing no evidence of ataxia, dysarthria or tremor. Pre-symptomatic testing for FRDA has not been performed in these three patients. In two cases (Republic of Ireland), testing has not been offered, as pre-symptomatic testing of a minor for a condition which has no treatment is considered unethical. Another case (Northern Ireland) has been offered pre-symptomatic testing; however, this has been declined by the parents. No family members are known to be affected with FRDA alone.

The diagnosis of galactosaemia can have a significant impact on the quality of life of both affected children and their carers (Bosch et al. 2009; Lambert and Boneh 2004). No literature describes the impact of a second chronic, life-limiting diagnosis in such children. The Traveller population in Ireland is known to have poorer quality engagement with health services (McGorrian et al. 2012). One patient in our cohort had been lost to galactosaemia follow-up for 3 years prior to presenting with severe decompensated cardiac failure, secondary to dilated cardiomyopathy. It was at this point that a diagnosis of FRDA was suspected and confirmed. He has no neurological symptoms of FRDA to date. This particular case highlights the possible need to reconsider the need for pre-symptomatic testing for FRDA in siblings of patients affected by both conditions, in particular those siblings with a diagnosis of galactosaemia.

Conclusion

Movement disorders are well described in classical galactosaemia, irrespective of dietary compliance (Rubio-Agusti et al. 2013; Coss et al. 2013). No previous reports of the coexistence of FRDA and galactosaemia exist. As a result, neurological symptoms may be easily attributed to an underlying diagnosis of galactosaemia, and a coexistent diagnosis could be missed. Clinicians should therefore have a high index of suspicion for a diagnosis of Friedreich ataxia in Irish Traveller patients with galactosaemia, who present with unusual symptoms.

Acknowledgments Thank you to Prof. David Barton at the National Centre for Medical Genetics for helpful discussion regarding genetic mechanisms and for insight into the prevalence of FRDA in the Traveller population.

Take-Home Message

Clinicians should have a high index of suspicion for Friedreich ataxia in Irish Traveller patients with classical galactosaemia who present with ataxia or cardiomyopathy.

Compliance with Ethics Guidelines

Conflicts of Interest

Siobhán Neville declares that she has no conflict of interest.

Siobhan O'Sullivan declares that she has no conflict of interest.

Bronagh Sweeney declares that she has no conflict of interest.

Bryan Lynch declares that he has no conflict of interest.

Donncha Hanrahan declares that he has no conflict of interest.

Ina Knerr declares that she has no conflict of interest.

Sally Ann Lynch declares that she has no conflict of interest.

Ellen Crushell declares that she has no conflict of interest.

Informed Consent

This is a descriptive report, and all patient information has been anonymized to ensure patients are not identifiable. All patients have provided informed consent to genetic testing.

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

Contributions of Individual Authors

Siobhán O'Sullivan, Ina Knerr and Ellen Crushell identified the patients relevant to this study.

Siobhán Neville, Siobhán O'Sullivan and Bronagh Sweeney collected the patient information.

Bryan Lynch and Donncha Hanrahan performed the neurological examinations.

Sally Ann Lynch advised on the inheritance pattern of these two conditions.

Siobhán Neville and Ellen Crushell drafted the original manuscript.

Siobhán Neville and Ellen Crushell designed the included figure and tables.

Ina Knerr, Siobhán O'Sullivan and Sally Ann Lynch amended the original manuscript.

All authors have read and approved the submitted manuscript.

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CASE REPORT

Phenotypic Expansion of Congenital Disorder of Glycosylation Due to *SRD5A3* Null Mutation

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Abstract We present a boy, admitted at 4 months, with facial dysmorphism, hypertrichosis, loose skin, bilateral inguinal hernia, severe hypotonia, psychomotor disability, seizures with hypsarrhythmia (West syndrome), hepatosplenomegaly, increased serum transaminases, iris coloboma, glaucoma, corneal clouding and bilateral dilated lateral ventricles, and extra-axial post-cerebellar space. Serum transferrin isoelectrofocusing (IEF) showed a type 1 pattern. Whole-exome genotyping showed a previously reported homozygous nonsense mutation c.320G>A; p. Trp107X in *SRD5A3*. Epilepsy and glaucoma have been reported only once in the 19 described *SRD5A3*-congenital glycosylation defect patients, and corneal clouding not at all.

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Introduction

SRD5A3-CDG is one of about 80 known congenital disorders of glycosylation (CDG). It is a defect in the penultimate step of the synthesis of dolicholphosphate, the carrier of the glycan intermediates in the assembly of $GlcNAc_2Man_9Glc_3$ in the endoplasmic reticulum. It affects protein N-glycosylation, as well as the synthesis of mannose-linked glycans, C-mannosylation, and glycophospholipid anchor synthesis. SRD5A3-CDG is characterized by neurological and ophthalmological findings such as nystagmus, visual impairment, microphthalmia, cataract, coloboma (iris, chorioretinal), optic disk hypoplasia, and optic nerve hypoplasia/atrophy (Assmann et al. 2001; Prietsch et al. 2002; Al-Gazali et al. 2008; Kahrizi et al. 2009, 2011; Morava et al. 2009, 2010; Cantagrel et al. 2010; Gründahl et al. 2012; Kara et al. 2014; Kasapkara et al. 2012).

Corneal clouding has not been reported in this disorder and epilepsy as well as glaucoma only once. We report a patient with corneal clouding, epilepsy, and glaucoma.

Clinical Report

This 4-month-old boy is the fourth child of first-cousin parents. Following an uncomplicated pregnancy, the patient was born with normal birth weight, height, and head

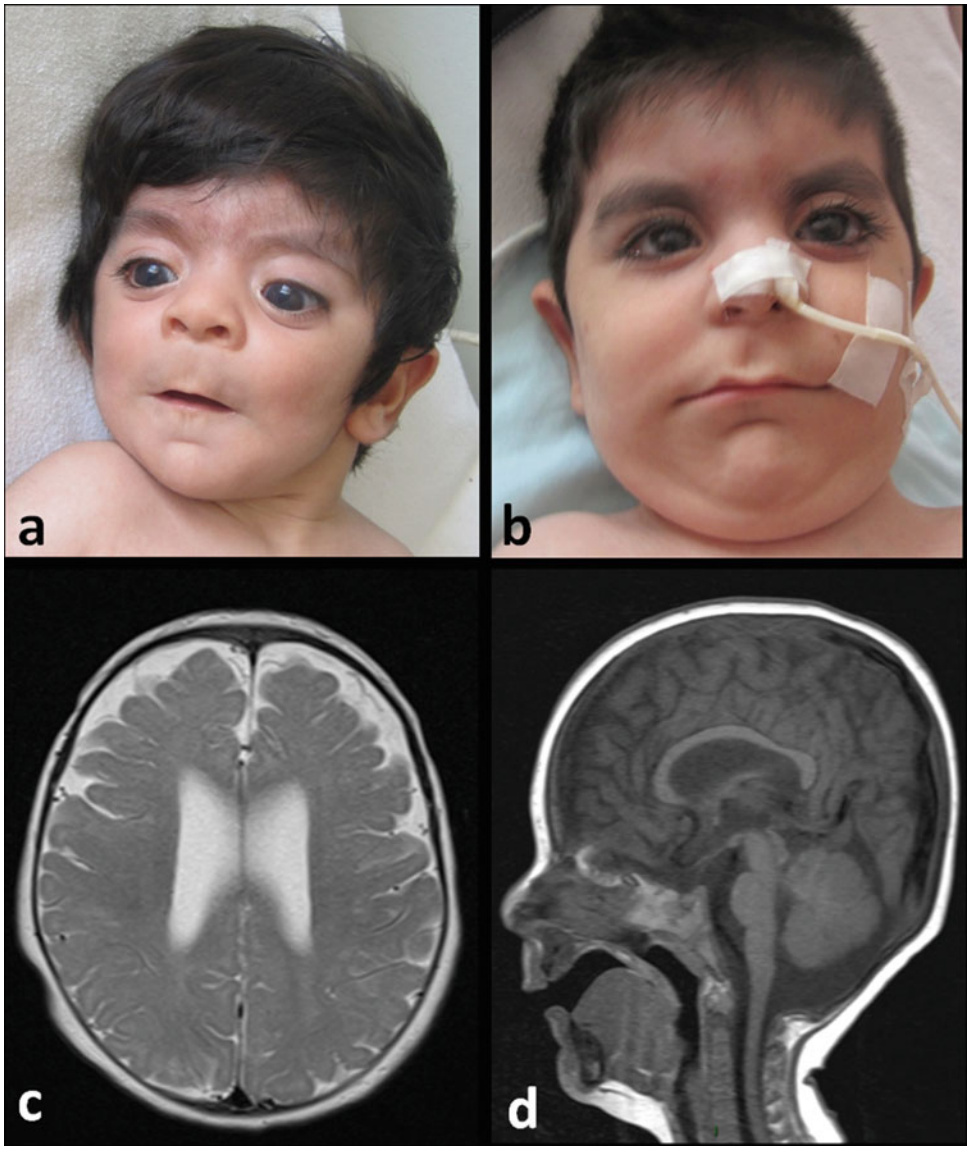

Fig. 1 The patient showed a coarse face with hairy forehead, depressed nasal bridge, proptotic eyes, low-set ears, smooth and long philtrum, and thin lips at 4 months (**a**) and at 31 months (**b**). Cranial MRI study at the age of 13 months showed bilateral dilated lateral ventricles and extra-axial post-cerebellar space in coronal T2 (**c**) and sagittal T1 (**d**)

circumference. His weight, height, and occipitofrontal circumference (OFC) were 5,600 g (−1.2 SD), 66 cm (+1.3 SD), and 41 cm (−0.2 SD), respectively, at 4 months of age. He was hypotonic and showed facial dysmorphism [hairy forehead, depressed nasal bridge, large fontanelle (6 × 8 cm), low-set ears, smooth philtrum, and thin lips], loose skin, hypermobility of elbows and fingers, hepatosplenomegaly, bilateral inguinal hernia, and hypertrichosis (Fig. 1a, b). Eye examination revealed iris coloboma, glaucoma, nystagmus, and corneal clouding. He had severe feeding difficulty. Serum transaminase (ALT 1,151 U/L, reference values 0–40 U/L; AST 144 U/L, reference values 0–40 U/L) and creatine kinase (291 U/L; reference value <170 U/L) levels were elevated. On abdominal ultrasonography there was a dilatation of the right pelvicalyceal system and ureter. Echocardiography revealed a patent foramen ovale. Osteoporosis was observed on skeletal X-ray. He underwent surgical repair for bilateral inguinal hernia at 8 months of age, and bilateral vitreo-trabeculectomy was performed at 9 months of age. Severe gastroesophageal reflux disease (GERD) was diagnosed at 2 years of age.

Infantile spasms were noticed at the age of 7 months. Electroencephalography showed hypsarrhythmia. West syndrome was diagnosed and treated with IM ACTH. Levetiracetam was added to this treatment. His seizures were under control by levetiracetam for 1 year, and EEG showed some improvement. Brain MRI at 1 year old

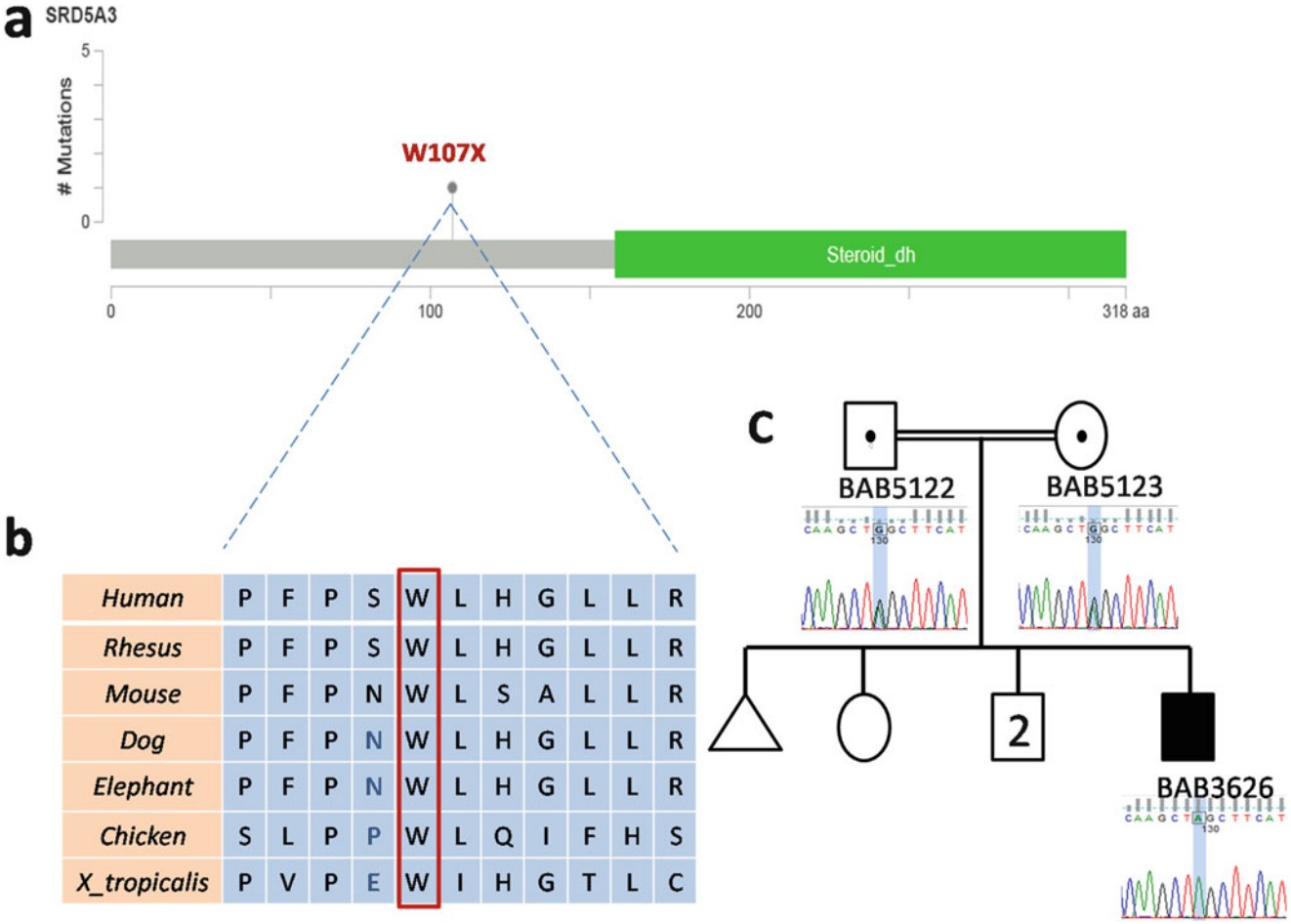

Fig. 2 Molecular and computational studies of the *SRD5A3* variant. (**a**) Protein structure of *SRD5A3* and location of the mutation in our patient. Our mutation affects the protein domain before steroid dehydrogenase domain. (**b**) Conservation alignment indicating that the tryptophan residue at position 107 is conserved across all vertebrates. (**c**) Pedigree of the family with Sanger sequencing results showing homozygosity of the c.320G>A; p.W107X variant in the proband and heterozygosity in both parents. Unaffected siblings were not available for segregation

revealed bilateral dilated lateral ventricles and extra-axial post-cerebellar space (Fig. 1c, d).

Weight, height, and head circumference were 10.5 kg (−3.5 SD), 92 cm (−1 SD), and 47 cm (−2.3 SD), respectively, at his last examination at 3 years and 4 months. A dark pigmentation on the dorsum of the feet was observed. Severe hypotonia persisted. He was unable to say single words and to walk. Head control was achieved at 18 months of age and sitting without support at 3 years of age and 4 months. Severe GERD and feeding problems persisted, and he had recurrent respiratory tract infections. His seizures were under control by levetiracetam, and EEG showed improvement in hypsarrhythmia. Serum IGF1 was normal (94.06 ng/ml; reference values 50–143), and antithrombin levels were decreased mildly, %73.5 (reference values %75–125).

Serum transferrin IEF showed which was consistent with type 1 pattern.

Mutation Analysis

Whole-exome sequencing (WES) was performed as described previously (Lupski et al. 2013). Whole-exome sequencing analysis showed a homozygous nonsense mutation c.320G>A; p.Trp107X (chr4:g.56,225,610 G>A [hg19]) in the *SRD5A3* gene that was predicted to terminate protein before the steroid reductase domain (Fig. 2a–c). The mutation was predicted to be deleterious by several bioinformatic tools including Polyphen2, LRT, MutationTaster, and SIFT. The mutation was not found in publicly available (i.e., ARIC, 1,000 genomes, NHLBI Exome Sequencing Project and dbSNP) or internal human genome databases (>4,000 exomes). The affected amino acid was evolutionarily well controlled in all vertebrates except jawless fish and zebra fish (no amino acid counterpart in these two vertebrates). Both parents were heterozygous consistent with Mendelian expectations for an

autosomal recessive trait. Furthermore, the mutation was previously reported in a CDG patient (Cantagrel et al. 2010).

Discussion

The serum transferrin IEF type 1 pattern pointed to a glycan assembly defect. Whole-exome sequencing revealed a homozygous mutation in *SRD5A3*, previously reported in one patient (Cantagrel et al. 2010). Nineteen patients have been reported with SRD5A3-CDG, belonging to 13 families. Among them are five adults belonging to two families. They showed various combinations of mainly facial dysmorphisms and neurological, ophthalmological, and cutaneous manifestations (Assmann et al. 2001; Prietsch et al. 2002; Al-Gazali et al. 2008; Kahrizi et al. 2009, 2011; Morava et al. 2009, 2010; Cantagrel et al. 2010; Gründahl et al. 2012; Kara et al. 2014; Kasapkara et al. 2012). Other clinical symptoms, present in a few patients, were feeding problems, failure to thrive, microcytic anemia, cardiac malformations/hypertrophy, hepatosplenomegaly, and cryptorchidism. Besides the type 1 serum transferrin IEF, laboratory investigations showed microcytic anemia, mildly increased serum transaminases, decreased antithrombin, protein C, protein S, and low IGF1 and IGFBP3. We compared the clinical features of the patient presented here with those reported 14 children with mutations in *SRD5A3* gene in Table 1.

The patient presented here showed the full clinical spectrum of this neuro-ophthalmo-cutaneous syndrome, caused by a known mutation. In addition, he had bilateral corneal clouding. Moreover, two of his symptoms have been reported only once in *SRD5A3*, namely, glaucoma and

Table 1 Comparison of the clinical features of our patient to those reported in patients with mutations in *SRD5A3*

	Present patient	Al-Gazali et al. (2008)	Morava et al. (2009, 2010)	Kasapkara et al. (2012)	Assmann et al. (2001)	Prietsch et al. (2002)	Gründahl et al. (2012)
			Polish (3)				
Ethnic background	Turkish	Baluchi (4)	Turkish (3)	Turkish	Turkish	Turkish	Pakistani
Facial dysmorphic features							
Brachycephaly	–	4/4	NA	NA	NA	NA	NA
Hairy forehead	+	4/4	NA	NA	NA	NA	NA
Arched eyebrows	+	4/4	NA	NA	NA	NA	NA
Hypertelorism	+	4/4	NA	NA	NA	NA	NA
Depressed nasal bridge	+	4/4	NA	NA	NA	NA	NA
Short upturned nose	+	4/4	NA	NA	NA	NA	NA
Smooth philtrum	+	4/4	NA	NA	NA	NA	NA
Large mouth	+	4/4	NA	NA	NA	NA	NA
Thin upper lip or thick lips	+	4/4	NA	+	NA	NA	NA
Abnormally formed, low-set ears	+	4/4	NA	+	NA	NA	NA
Neurological findings							
Developmental delay	+	4/4	5/6	+	+	+	+
Hypotonia	+	4/4	5/6	+	+	+	–
Ataxia/stereotypic movements	–	–	2/6	–	+	+	–
Spasticity	–	–	1/6	–	+	–	+
Midline brain malformation	–	3/4	–	–	–	–	–
Frontal polymicrogyria	–	2/4	–	–	–	–	–
Pituitary gland hypoplasia	–	3/4	–	–	–	–	–
Lateral ventricle dilatation	+	1/4	–	+	–	–	–
Global/cerebellar vermis hypoplasia	–	2/4	4/6	+	–	+	–
Extra-axial post-cerebellar space	+	1/4	–	+	–	–	–
Seizures	+	–	1/6	–	–	–	–

(continued)

Table 1 (continued)

	Present patient	Al-Gazali et al. (2008)	Morava et al. (2009, 2010)	Kasapkara et al. (2012)	Assmann et al. (2001)	Prietsch et al. (2002)	Gründahl et al. (2012)
			Polish (3)				
Ethnic background	Turkish	Baluchi (4)	Turkish (3)	Turkish	Turkish	Turkish	Pakistani
Ophthalmological abnormalities							
Microphthalmia	–	1/4	1/6	–	–	–	–
Nystagmus	+	–	5/6	–	+	+	+
Strabismus	+	–	–	+	+	+	NA
Cataract	–	–	2/6	–	–	–	NA
Coloboma	+	4/4	4/6	+	–	–	NA
Optic disk/nerve hypoplasia	–	2/4	3/6	+	+	+	NA
Glaucoma	+	–	1/6	–	–	–	NA
Visual loss	+	–	5/6	–	+	+	NA
Corneal clouding	+	–	–	–	–	–	NA
Cutaneous manifestations							
Erythroderma	–	1/4	–	+	NA	NA	–
Dry skin	–	4/4	3/6	+	NA	NA	–
Atopic dermatitis	+	2/4	–	–	NA	NA	–
Ichthyosis	–	3/4	–	+	NA	NA	–
Dark pigmentation	+	3/4	4/6	–	NA	NA	–
Thick palms and soles	–	3/4	–	–	NA	NA	–
Loose skin	+	3/4	–	–	NA	NA	–
Hypertrichosis	+	4/4	–	–	NA	NA	–
Other clinical symptoms							
Large fontanelle	+	–	–	–	–	–	–
Inguinal hernia	+	–	–	–	–	–	–
Joint hypermobility	+	–	–	–	–	–	–
Hepatosplenomegaly	+	–	–	–	–	–	–
Dilated cardiomyopathy	–	4/4	–	+	–	–	–
Feeding difficulty	+	1/4	2/6	+	–	–	–
Microcytic anemia	+	2/4	4/6	–	+	–	–
Increased transaminases	+	1/4	6/6	NA	+	–	–
Decreased antithrombin and protein C/S	+	4/4	6/6	NA	–	–	–
Low IGF1 and IGFBP3	+	3/4	NA	NA	–	–	–

NA not assessed

epilepsy. The reported epilepsy was absence seizures (Morava et al. 2010), while in the present patient it was West syndrome and localization-related epilepsy that responded well to levetiracetam. In conclusion, the present patient broadens the phenotype of SRD5A3-CDG.

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Take-Home Message

New features such as epilepsy, glaucoma, and corneal clouding were described in the patient with CDG1q.

Details of the Contributions of Individual Authors

BT was the leading physician who diagnosed the condition and to the writing of the manuscript.

DP: sequencing the sample and writing the manuscript.

AÖ, ÇZ, and CY were the physicians who followed up the patient during his admission to the pediatric ophthalmology, neurology, and metabolism departments.

JS: sequencing the sample.

RAG: sequencing the sample and organizing the project.

DMM: sequencing the sample.

JRL: sequencing the sample, organizing the project, and writing the manuscript.

JJ was revising the manuscript critically for important intellectual content and analysis of serum transferrin IEF level.

Conflict of Interest

BT, DP, ÇZ, CY, JS, RAG, DMM, JRL, and JJ declare that they have no conflict of interest.

Informed Consent

Informed consent was obtained from the parents of the patient.

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CASE REPORT

Periventricular Calcification, Abnormal Pterins and Dry Thickened Skin: Expanding the Clinical Spectrum of *RMND1*?

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Abstract *Background*: We report a consanguineous Sudanese family whose two affected sons presented with a lethal disorder characterised by severe neonatal lactic acidosis, hypertonia, microcephaly and intractable seizures. One child had additional unique features of periventricular calcification, abnormal pterins and dry thickened skin.

Methods: Exome enrichment was performed on pooled genomic libraries from the two affected children and sequenced on an Illumina HiSeq2000. After quality control and variant identification, rare homozygous variants were prioritised. Respiratory chain complex activities were measured and normalised to citrate synthase activity in cultured patient fibroblasts. RMND1 protein levels were analysed by standard Western blotting.

Results: Exome sequencing identified a previously reported homozygous missense variant in *RMND1* (c.1250G>A; p.Arg417Gln), the gene associated with combined oxidation phosphorylation deficiency 11 (COXPD11), as the most likely cause of this disorder. This finding suggests the presence of a mutation hotspot at cDNA position 1250. Patient fibroblasts showed a severe decrease in mitochondrial respiratory chain complex I, III and IV activities and protein expression, albeit with normal RMND1 levels, supporting a generalised disorder of mitochondrial translation caused by loss of function.

Conclusions: The current study implicates *RMND1* in the development of calcification and dermatological abnormalities, likely due to defective ATP-dependent processes in vascular smooth muscle cells and skin. Review of reported patients with *RMND1* mutations shows intra-familial variability and evidence of an evolving phenotype, which may account for the clinical variability. We suggest that COXPD11

should be considered in the differential for patients with calcification and evidence of a mitochondrial disorder.

Introduction

Mitochondrial disorders are clinically heterogeneous and typically affect multiple organ systems. These disorders tend to be severely debilitating, progressive and often fatal. Mitochondrial disorders can be caused by mutations in the maternally inherited mitochondrial genome (mtDNA), which encodes 13 essential proteins of the oxidative phosphorylation (OXPHOS) system, or by mutations in one of >1,300 nuclear genes that encode mitochondrially targeted proteins (Koopman et al. 2012). Mitochondrial diseases have poor genotype-phenotype correlation, and most patients' clinical features do not fall discretely into any one particular syndrome/category, making molecular diagnosis challenging. The introduction of targeted multigene panel testing and exome sequencing has greatly increased molecular diagnostic yield (Taylor et al. 2014).

We report a consanguineous family of Sudanese origin whose two affected sons presented with a lethal syndrome characterised by severe neonatal lactic acidosis, hypertonia and intractable seizures (Fig. 1a). One child also had periventricular calcification, abnormal pterins and dry thickened and pigmented skin. After negative single-gene testing, whole-exome sequencing was undertaken to identify the cause of the suspected nuclear-driven mitochondrial disorder in this family.

Methods

Sample Collection

Written informed consent was obtained from the parents for publication of this report, including patient photographs. Genomic DNA was extracted from peripheral blood lymphocytes of the two affected children and unaffected parents. The study protocol was approved by the ethics committee of Temple Street Children's University Hospital (Dublin, Ireland).

Whole-Exome Sequencing

Whole-exome sequencing was undertaken for both affected children. Libraries were prepared from DNA of the two children and pooled in equal amounts (IntegraGen, France). The exonic DNA was enriched with the SureSelect v5 Human All Exon Kit (Agilent Technologies, Santa Clara) and sequenced on an Illumina HiSeq2000 (Illumina, San Diego, California, USA) at IntegraGen (France). The 100 bp paired-end reads were aligned to the hg19 human reference genome. Quality control and variant identification were performed as previously described (Casey et al. 2015). Assuming an autosomal recessive model, we prioritised variants that were (i) autosomal; (2) absent or present with a frequency <1% in dbSNP130, NHLBI Exome Variant Server database and 1,000 Genomes; (3) homozygous; and (4) absent in our 60 control exomes (Supplementary Table S1). As the two patient samples were pooled pre-capture, only variants for which both affected children are homozygous are called as homozygous in the pooled sample.

Respiratory Chain Complex Analysis

Respiratory chain complex activities were measured and normalised to citrate synthase activity in cultured fibroblasts as previously described (Kirby et al. 2007). Cell lysates were prepared from patient fibroblasts (II:1) and analysed by Western blotting as previously described (Brito et al. 2015). Antibodies against RMND1 (Sigma HPA031399), NDUFB8 (Abcam ab110242), SDHA (Abcam ab14715), UQCRC2 (Abcam ab14745), COXI (Abcam ab14705), COXII (Abcam ab110258), ATP5A (ab14748) and VDAC1 (Abcam ab14734) were used, followed by HRP-conjugated secondary antibodies (DakoCytomation).

Case Reports

Patient II:1

Patient II:1 was born at term, birth weight 2.34 kg (0.4th centile) and occipitofrontal circumference 32 cm (0.4th centile). There was respiratory difficulty requiring ventilation. A pigmented skin rash was noted on the trunk. At 2 h of life, bilateral clonic seizures emerged. There was marked peripheral hypertonia with fisting of the hands. At 6 weeks there was microcephaly (34.5 cm <<0.4th centile), stiffness, excessive startle, rigid lower limbs, stiff flexed upper limbs, dry thickened skin and little spontaneous movement (Fig. 1b, c). There was no visual fixation or following. Thereafter, there was no neurodevelopment. The seizures were refractory to antiepileptic drug treatment with frequent bouts of status epilepticus, and the infant died at 11 months of age.

Investigations

Screen for congenital infections (toxoplasmosis, rubella, cytomegalovirus, herpes simplex and HIV) was negative. Metabolic acidosis was noted on initial blood gases. Blood count and film, blood glucose, serum calcium, serum magnesium, coagulation studies, immunoglobulins, abdominal and pelvic ultrasounds and skeletal survey were normal.

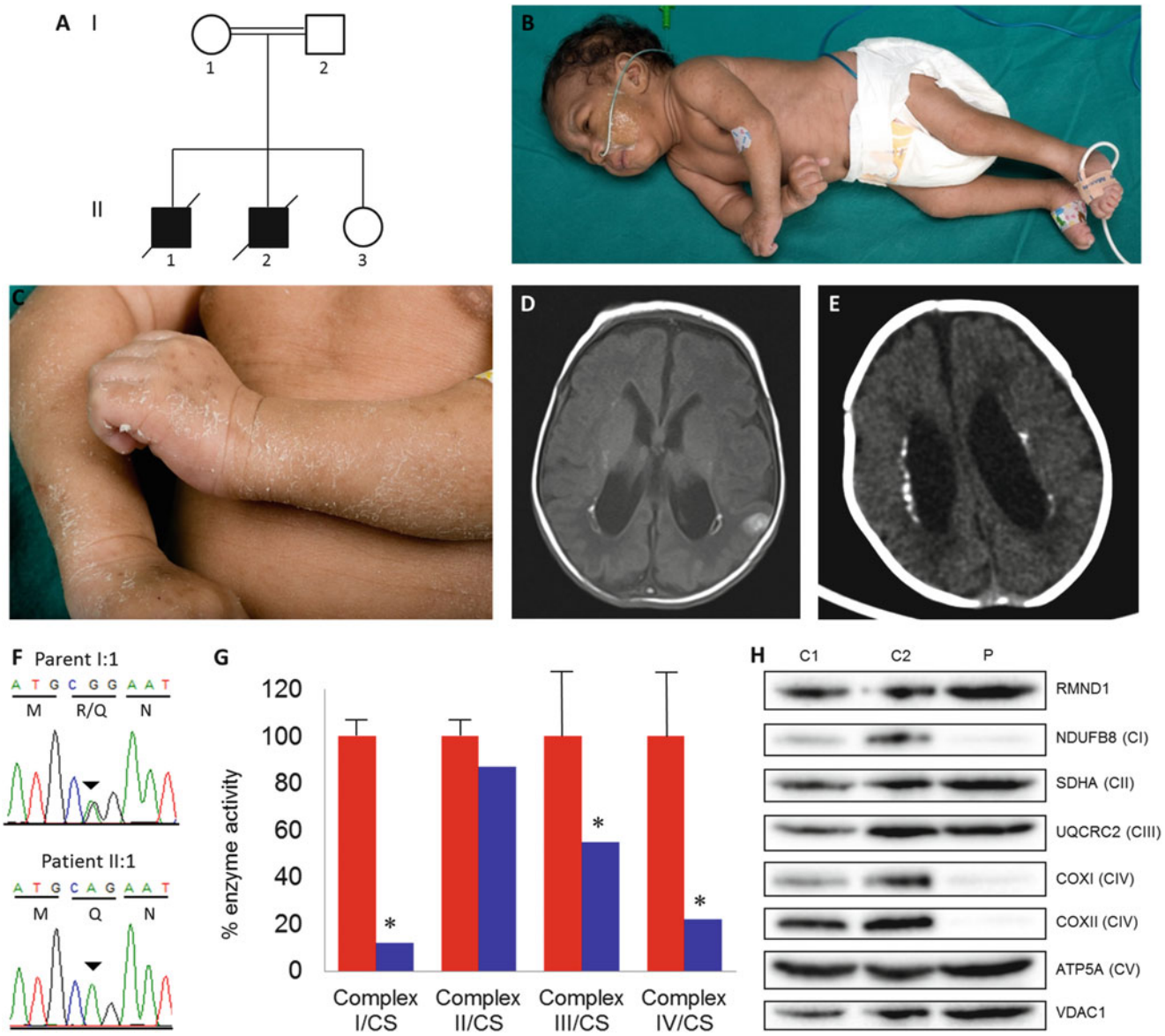

Fig. 1 Clinical, genetic and proteomic analyses. (**a**) Pedigree of a consanguineous Sudanese family who have had two affected sons and one healthy daughter. DNA was not available for II:3. (**b**) Photograph of patient II:1 showing stiffness in both arms, fisting of the hands and flexion of the toes. (**c**) Photograph of patient II:1 showing dry thickened skin with a pigmented skin rash. (**d**) Axial T1 MR image at day 7 of life shows posterior predominant ventricular dilation with bilateral symmetric white matter volume reduction. The periventricular calcifications detected by ultrasound are evident here as bilateral punctate hyperintensities. A focal area of hyperintensity in the left parietal parenchyma is in keeping with an acute haemorrhagic infarction. It was associated with restricted diffusion. (**e**) A CT scan at 4 weeks of age confirms the periventricular calcification bilaterally. Moderate ventricular dilation was unchanged. (**f**) The *RMND1* NM_017909.3 c.1250G>A variant was validated by Sanger sequence analysis. Traces are shown for parent I:1 and affected child II:1. The inverted triangle indicates the position of the mutated G base which changes Arg (R) to Gln (Q) at residue 417. (**g**) Assessment of individual respiratory chain enzyme activities in fibroblasts identified severe OXPHOS deficiencies involving complexes I, III and IV with sparing of complex II activity in patient II:1 (*blue bars*) compared to controls (*red bars*). Mean enzyme activities shown for fibroblast controls ($n = 10$) are set at 100%. (**h**) Western blot analysis of cell lysates from control (C1 and C2) and patient II:1 (P) fibroblasts. Antibodies against RMND1, NDUFB8 (complex I), SDHA (complex II), UQCRC2 (complex III), COXI (complex IV), COXII (complex IV), ATP5A (complex V) and VDAC1 (mitochondrial loading control) were used

Cranial ultrasound (Day 1) showed moderate ventricular dilation with periventricular calcification. Magnetic resonance imaging (MRI) brain (Day 7) showed reduced white matter volume posteriorly and a small acute haemorrhagic infarct (Fig. 1d). Computed tomography (CT) of the brain at 4 weeks showed moderately dilated lateral ventricles and confirmed the bilateral periventricular calcification (Fig. 1e). Electroencephalography (Day 10) showed slowing of the background with excessive sharp activity. This evolved over the following months to a severely encephalopathic pattern with periodic lateralised epileptiform discharges.

Analysis of urine organic acids identified increased excretion of lactate with marked ketonuria (3-hydroxybutyrate), mild dicarboxylic aciduria and hydroxydicarboxylic aciduria and increased excretion of tricarboxylic acid cycle intermediates fumarate and malate, suggestive of a mitochondrial disorder. Blood lactate was 2–6 mmol/L (normal range 0.6–2.4 mmol/L), CSF lactate was 5.7 mmol/L (normal range 1.0–2.2 mmol/L), and blood lactate-pyruvate ratio was increased at 28, pointing towards an oxidative phosphorylation defect. Assessment of fatty acid β-oxidation flux in cultured fibroblasts showed abnormalities consistent with a primary defect of the mitochondrial respiratory chain (Olpin et al. 1997).

Analysis of cerebrospinal fluid (CSF) neurotransmitters identified abnormal pterins, neopterin 375 (7–65 nmol/L), dihydroneoptrin 16.5 (0.4–13.9 nmol/L) and low vitamin B6 at 22 nmol/L (44–89 nmol/L). Serum and CSF alpha interferon levels were normal.

Chromosome analysis showed a normal 46XY karyotype. A number of diagnoses were considered including small-vessel brain disease (*COL4A1*-related disorder) and Aicardi-Goutieres syndromes because of the calcification and abnormal CSF pterins. However, clinical sequencing of *COL4A1*, *TREX*, *RNASEH2B*, *RNASEH2C*, *RNASEH2A* and *SAMHD1* did not identify any pathogenic variants.

Patient II:2

During the second pregnancy, the mother had pedal oedema, high blood pressure and fever at week 33. Regular fetal ultrasound scans did not detect intracranial calcifications. Patient II:2 was born at term by emergency caesarean section due to fetal bradycardia and required resuscitation. From birth, he showed almost identical clinical manifestations to his older brother (II:1): seizures on day 1, axial hypotonia and lactic acidosis. Dysmorphic features included a relatively large anterior fontanelle, small toes and small suboptimally curved pinna. Seizures, initially focal clonic and later myoclonic and tonic, were noted within 1 h of life. Cranial ultrasound on day 1 of life showed a speckled appearance raising the possibility of pre-calcification, though quality was suboptimal. CT brain scan did not identify any clear evidence of calcification. Despite supportive care, he died on day 4 of life.

Results

Exome Sequencing

Variant prioritisation identified 16 novel or rare homozygous variants shared by both affected children (Supplementary Table S3). Only one of the 16 genes is known to be mitochondrial, *RMND1* NM_017909.3 c.1250G>A, p.(Arg417Gln). Validation and segregation analysis of the *RMND1* c.1250G>A variant was undertaken by polymerase chain reaction and bidirectional Sanger sequencing (Supplementary Table S2). Sanger sequencing confirmed that both affected brothers are homozygous for the c.1250G>A *RMND1* variant and the parents as obligate carriers (Fig. 1f).

RMND1 encodes a member of the evolutionary conserved sif2 protein family which localises to the mitochondria and is involved in mitochondrial translation (Janer et al. 2012; Garcia-Diaz et al. 2012). Mutations in *RMND1* have been associated with a severe neonatal encephaloneuromyopathy termed combined oxidative phosphorylation deficiency 11 (COXPD11; *614917). COXPD11 is an autosomal recessive disorder characterised by deficiencies of multiple respiratory chain complexes leading to neonatal hypotonia and lactic acidosis.

Respiratory Chain Complex and RMND1 Protein Analyses

Analysis of patient fibroblasts showed a severe biochemical defect involving respiratory chain complexes I, III and IV with sparing of complex II (Fig. 1g), in accordance with a generalised disorder of mitochondrial translation. Steady-state levels of RMND1 protein were similar in patient fibroblasts compared to controls, despite a decrease in protein levels of complex I (NDUFB8) and complex IV (COXI and COXII) subunits (Fig. 1h). These data are consistent with previous work showing that the steady-state levels of RMND1 were unchanged, but formation of an RMND1 complex was impaired in a patient with the same p.(Arg417Gln) mutation (Janer et al. 2012), supporting a loss-of-function mechanism.

Discussion

We report a homozygous missense p.(Arg417Gln) variant in *RMND1* in two Sudanese brothers with a lethal mitochondrial disorder presenting as neonatal lactic acidosis, hypertonia and intractable seizures. The identified *RMND1* c.1250G>A, p.(Arg417Gln) variant has previously been reported by Janer and colleagues in a consanguineous sib-pair of Pakistani origin (Janer et al. 2012). Finding the same disease variant in a second population suggests the presence of a mutation hotspot at cDNA position 1250, given the absence of any known shared ancestry between the Pakistani and Sudanese populations.

To date, 12 patients (8 families) with 4 different *RMND1* mutations have been reported in the literature (Table 1) (Garcia-Diaz et al. 2012; Janer et al. 2012; Taylor et al. 2014). The core features of lactic acidosis, hypertonia and

Table 1 Clinical and biochemical features of patients with *RMND1* mutations

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Age of onset	2 months	6 days	18 days	n.r.	n.r.	n.r.	6 months	3 months	18 months	6 months	<1 month	18 months	1 day	1 day
Encephalopathy	+	+	+	+	+	+	–	+	+	–	–	–	+	+
Severe lactic acidosis	+	+	+	+	+	+	+	+	+	–	+	–	+	+
Intractable seizures	+	+	+	–	–	–	n.r.	n.r.	n.r.	n.r.	n.r.	+	+	+
Microcephaly	+	+	–	–	–	–	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	+
Hypotonia	n.r.	+	+	+	+	+	+	+	+	+	+	+	+	+
Respiratory failure at birth	–	–	+	+	+	+	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	+
Deafness	–	–	–	–	–	–	+	+	+	+	+	+	–	–
Tongue fasciculations	n.r.	n.r	+	–	+	–	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	–
Renal tubular acidosis	n.r.	n.r.	n.r.	n.r	n.r.	n.r.	–	–	–	+	–	+	–	–
Renal dysplasia	n.r.	n.r.	n.r.	n.r	n.r.	n.r.	+	–	+	–	–	–	–	–
Cardiac anomaly	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	–	–	+	+	–	–	–	–
Bilateral equinus foot deformity	n.r.	n.r.	+	+	n.r.	+	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	–	–
Other	–	–	abs ten ref	–	abs Moro ref, musc skel def	–	–	↓ Hb	–	–	–	–	pv calc, abn pterins, dry thick skin	Large ant font, small toes, small pinna
Blood lactate (0.63–2.44 mM)	n.r.	↑ 3.56	↑ 3.2	↑ 3.5	↑ 2.5–8.4	↑ 5.7	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	↑ 2–6	↑ 3.9
OXPHOS	n.r.	↓ C I, III–V	n.r.	↓ C I–IV	n.r.	n.r.	↓ CI, III, IV	↓ CI, IV	↓ CI, III, IV	↓ CI, III, IV	↓ CI, III, IV	↓ CI, CIV	↓ CI, III, IV	n.p.
Age at time of report/ death (†)	13 months†	5 months †	18 months†	12 days †	8 months	4 months	4 years	1 year†	5 years	10 years†	18 months	5 years†	11 months†	4 days†

Comparison of the clinical and biochemical profiles of patients with *RMND1* mutations. Information on previously reported patients was obtained from the studies of Janer et al., Garcia-Diaz et al. and Taylor et al. Note that blood ammonia, urine amino acids and lysosomal enzymes were normal where reported

Abbreviations: ↑, increased compared to normal; ↓, decreased compared to normal; n.p., test not performed; n.r., information not reported; abs ten ref, absent tendon reflexes; abs Moro ref, absent Moro reflex; ant font, anterior fontanelle; ↓ Hb, iron deficient anaemia; musc skel def, muscular skeletal deformities; pv calc, periventricular calcification. Patient identifiers: A, Janer P3; B, Janer P4; C, Garcia-Diez VI-1; D, Garcia-Diez VI-3; E, Garcia-Diez VI-7; F, Garcia-Diez VI-8; G, Taylor P1; H, Taylor P2; I, Taylor P3; J, Taylor P4; K, Taylor P5; L, Taylor P6; M, Current study II:1; N, Current study II:2. Note that the stillborn baby (VI-9) with *RMND1* mutation reported in the study by Garcia-Diaz is not included in this table; † indicates the age of the child when they died (deceased)

seizures are common to all patients. In addition, some patients may have other features including deafness, renal dysplasia and cardiac anomalies. As most of the patients with reported deafness are the oldest of the *RMND1* cohort, it is likely that *RMND1* mutation causes an evolving phenotype where clinical signs manifest with age.

While the patients in this study have the core clinical features of COXPD11, one child (II:1) has a number of additional features not previously associated with COXPD11, namely, periventricular calcification, abnormal pterins and dry thickened skin. Are these features a direct result of the *RMND1* mutation, or could they be due to a second genetic disorder in the eldest patient? To investigate the possibility of a second disorder, we analysed the exome data to look for dominant or recessive variants in genes associated with one or more of the following clinical features; brain calcification, abnormal pterins and dry thickened skin (Supplementary Table S4). We did not identify any rare potentially pathogenic variants in these genes, including Aicardi-Goutieres syndrome genes, which could account for the extra clinical features in the eldest patient. However, we cannot fully exclude the possibility of a mutation in an as yet uncharacterised calcification/dermatological gene or a mutation outside of the exome.

There is evidence in the literature to support a causal role for the *RMND1* mutation in both calcification and dermatological abnormalities. Firstly, there are three previous reports of patients with combined OXPHOS defects and brain calcifications (thalamic, cerebral and basal ganglia) (Kaiming et al. 2014; Robinson et al. 1992; Van Straaten et al. 2005). The relationship between impaired COX activity and excessive vascular calcification has been demonstrated in mutant *Lmna* mice (Villa-Bellosta et al. 2013). The current study suggests that *RMND1* could also be involved in the development of calcification due to defects in oxidative phosphorylation in vascular smooth muscle cells, though it is likely to be a rare feature. Given the speckling observed on cranial ultrasound in the second baby (patient II:2), we postulate that calcification might have developed had he lived longer. It is not unusual for there to be differences in the timing of emergence of clinical features in autosomal recessive disorders.

Secondly, patient II:1 was noted to have pigmentation and dry thickened stiff skin. Histological examination of a skin biopsy was normal. However, the underlying fascia was not biopsied. There are known interactions between mitochondria and cytokeratins, and 10% of patients with mitochondrial disorders present with dermatological features including hair abnormalities, rashes, pigmentation abnormalities and acrocyanosis (Feichtinger et al. 2014). Given the numerous ATP-dependent processes occurring in the skin, it is not surprising that mutations affecting mitochondrial function cause dermatological features. However, it is not clear why only a subset of patients with OXPHOS deficiencies develop skin changes. Indeed, in this study there was intra-familial variability (not uncommon in mitochondrial disease) with intracranial calcification and dermatological abnormalities present in only one of the two siblings.

In conclusion, our study implicates *RMND1* in the development of calcification and dermatological features and highlights the variable and progressive nature of COXPD11.

Acknowledgements We sincerely thank the participating family for their involvement in this study and the use of genetic samples and clinical information.

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Synopsis

RMND1 mutation can cause calcification, abnormal pterins and dermatological features and should be considered in the differential for patients with mitochondrial disorders that include calcification.

Compliance with Ethics Guidelines

Conflict of Interest

Jillian P. Casey, Ellen Crushell, Kyle Thompson, Eilish Twomey, Langping He, Sean Ennis, Roy K. Philip, Robert W. Taylor, Mary D. King and Sally Ann Lynch 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's parents for inclusion in the

study. Additional informed consent was obtained from the parents to include identifying information in this article.

Author Contributions

JPC, SAL and EC were responsible for the study concept and design and obtained funding. EC, RKP, MDK and SAL performed clinical assessment and diagnostic investigations and were responsible for obtaining clinical samples and patient management. EC, ET and MDK reviewed and interpreted the biochemical, radiological and neurological findings, respectively. JPC analysed and interpreted the exome data and did a literature review on previous patients. EC, JPC, MDK, SE and SAL assessed the genetic findings in relation to the patient phenotype. KT, LH and RT analysed patient fibroblasts for respiratory chain defects and altered mitochondrial protein levels and interpreted their findings. JPC drafted the manuscript, and all authors were involved in critical revisions.

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RESEARCH REPORT

TMEM165 Deficiency: Postnatal Changes in Glycosylation

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Abstract Congenital disorders of glycosylation form a rapidly growing group of inherited metabolic diseases. As glycosylation affects proteins all over the organism, a mutation in a single gene leads to a multisystemic disorder. We describe a patient with TMEM165-CDG with facial dysmorphism, nephrotic syndrome, cardiac defects, enlarged cerebral ventricles, feeding problems, and neurological involvement. Having confirmed the diagnosis via prenatal diagnostics, we were able to observe the glycosylation right from birth, finding a pathological pattern already on the first day of life. Within the next few weeks, hypoglycosylation progressed to less sialylated and then also to hypogalactosylated isoforms. On the whole, there has not been much published evidence concerning postnatal glycosylation and its adaptational process. This is the first paper reporting changes in glycosylation patterns over the first postnatal weeks in TMEM165-CDG.

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Introduction

Glycosylation is a complex and highly conserved way of co- and posttranslational modification of proteins and lipids. More than 50% of eukaryotic proteins are predicted to be glycoproteins, of which more than 90% carry N-linked oligosaccharides (Apweiler et al. 1999). *N*-Glycosylation is a process taking place in the endoplasmic reticulum (ER) and in the Golgi apparatus. In the ER, an oligosaccharide precursor is synthesized on a lipid anchor, dolichol, and is then transferred to the asparagine residue of a nascent protein. This protein-linked oligosaccharide is further processed in the endoplasmic reticulum and in the Golgi apparatus. The glycans in glycoproteins serve various functions: prevention of protein aggregation during folding, stabilization of the protein, regulation of interaction and recognition processes, regulation of protein degradation, and many more (Helenius and Aebi 2004).

Defects in glycosylation can lead to congenital disorders of glycosylation (CDG), a rapidly growing group of metabolic diseases. In terms of understanding the underlying defects, a distinction can be made between defects in dolichol-linked oligosaccharide synthesis and its transfer to the polypeptide (CDG-I) and in defects in the further processing of the protein-linked oligosaccharide (CDG-II). Since many new variants of glycosylation have been discovered in the last few years – and yet more are expected to be discovered – that do not all fit into this classification, a new nomenclature was proposed (Jaeken et al. 2009). Different forms of CDG should be named after the affected gene with the suffix "-CDG".

While there are many publications on different kinds of glycosylation disorders, there is only little (and inconsistent) evidence about the results of biochemical examinations in the first days and weeks of life. In this article, we

will give evidence for distinct changes in postnatal glycosylation in TMEM165-CDG (OMIM:614726).

Material and Methods

Mutation Analysis

Primers for sequencing of TMEM165 genomic DNA (NG_032881.1), designed with Primer3 software (Rozen and Skaletsky 1998) and purchased from Invitrogen (Carlsbad, CA, USA), are listed in the online supplementary material (see Table 1S). For PCR amplification 1 μL cDNA (fibroblasts 0.2 μg/μL; blood 0.04–0.05 μg/μL) was supplemented with 2 μL PCR buffer 10× (Qiagen), 4 μL Q-solution 5× (Qiagen), 2 mM dNTP mix (GE Healthcare, Buckinghamshire, UK), 9 μL water, 0.1 μL Taq DNA polymerase (Qiagen), 1 μL primer forward, and 1 μL primer reverse (20 pmol/μL). Samples were incubated for 5 min at 94°C. After 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, a final incubation was performed at 72°C for 10 min. PCR products were purified using the PCR Product Pre-Sequencing Kit (USB Products/Affymetrix, Ohio, USA), and sequencing was performed with the BigDye Terminator Kit 3.1 (Applied Biosystems/Life Technologies, Darmstadt, Germany). 1.5 μL of purified PCR product was supplemented with 1.5 μL sequencing primer (10 pmol/L), 1 μL buffer, 0.5 μL BigDye, and 5.5 μL water. This was heated at 96°C for 2 min and then 25 cycles of 94°C for 10 s, 50°C for 5 s, and 60°C for 2 min followed. For final purification, the Sephadex/Millipore System (GE Healthcare, Buckinghamshire, UK/Merck Millipore, Schwalbach, Germany) was utilized. The sequence was analyzed on the ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA, USA).

Isoelectric Focusing (IEF)

Isoelectric focusing of transferrin was carried out as described in Niehues et al. (1998).

Immunoprecipitation and SDS-PAGE

Immunoprecipitation and sodium dodecyl sulfate–polyacrylamidegel electrophoresis (SDS-PAGE) for investigation of truncated carbohydrate side chains of transferrin was carried out as described in Niehues et al. (1998).

Nano-electrospray Ionization Time-of-Flight Mass Spectrometry (nanoESI-TOF MS)

NanoESI-TOF MS was performed as described previously by Park et al. (2014).

MALDI Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS was carried out as described by Wada et al. (2012).

2D Gel Electrophoresis

Two plasma samples of the patient were collected at different times of life after parental informed consent was obtained. The sample preparation was performed as follows: 1 μL of plasma was mixed with 1 μL of a sodium dodecyl sulfate/dithioerythritol solution (SDS 10% w/v, DTE 2.3% w/v) and incubated at 95°C for 10 min. Afterward it was mixed with 250 μL of a sample buffer containing 8 M urea, 2% chaps, 50 μM DTT, 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue (Bio-Rad Laboratories, Hercules, CA, USA) and frozen at −20°C until further use.

The first dimension run (isoelectric focusing) was performed on the Protean i12 IEF System (Bio-Rad). 7 cm linear IPG strips (pH3-10) were rehydrated with 125 μL of the prepared sample solution at 10°C for 12 h at the temperature of 10°C. After rehydration, IEF was performed according to the following protocol.

In a first step, 250 V was applied for 15 min before the voltage was gradually increased to 4,000 V over the course of 1 h. This voltage was maintained until 15,000 volt-hours were reached. Afterward 500 V was applied until the gel strips were removed from the apparatus. The IPG strips were either frozen at −20°C until further use or directly transferred onto Mini-PROTEAN precast gels (Bio-Rad) for the second dimension run. This run was performed on the Mini-PROTEAN system (Bio-Rad). In brief, 50 V was applied until a running front formed and the voltage was consequently increased to 150 V until the front had reached to the bottom of the gels.

Silver staining of the 2D gels was performed as described by Bjellqvist et al. (1993).

Results

Clinical Data

The patient was the second child born to consanguineous parents after their first child had died from TMEM165-CDG at the age of 5 months. At 6 months of gestation, amniotic fluid was drained due to polyhydramnios. In this context, material for genetic analysis was obtained and sequencing of TMEM165 showed homozygosity for the same mutation as previously found in the sibling.

Birth was at term. Directly thereafter, the patient suffered from respiratory distress and CPAP support was necessary. Facial dysmorphic features and a weak abdominal wall were noticed. Echocardiography revealed a small apical VSD, a PFO, and a small PDA with mild signs of right ventricular hypertrophy. Brain ultrasound showed enlarged lateral and third ventricles. In the first days of life, there was suspicion of neonatal seizures, but EEG recording showed no abnormalities. Neurological examination revealed a large, temporarily tensed fontanel, muscular hypertonia with opisthotonic posture, and a sundown position of the eyes.

At the age of 1 week, mild proteinuria was observed, the extent increasing over the next weeks to nephrotic syndrome with slowly progressive renal failure. Edema as well as pericardial and pleural effusions developed, causing an intermittent need for oxygen supply. Infusion of human albumin had only very limited effect. Repeated hospitalizations were necessary due to recurrent edema and feeding problems requiring tube feeding.

The patient died at the age of 5 months due to complications of nephrotic syndrome and renal failure.

Mutation Analysis of TMEM165

Sequence analysis of TMEM165 showed homozygosity for the missense mutation c.323 A>G (p.E108G) in exon 2. The same mutation had been found in a homozygous state in the older sister, both parents have been shown to be heterozygous carriers of this mutation. In exome variation databases (Exome Variant Server), this mutation was not found in more than 5,000 sequenced probands.

IEF and SDS-PAGE

Isoelectric focusing (IEF) of transferrin showed a pathological pattern with increased amounts of tri-, di-, and monosialotransferrin already in the first blood sample taken 3 min after birth. In the follow-up controls, the pattern changed with increasing amounts of di-, mono-, and asialotransferrin and decreasing tetra- and trisialotransferrin until there was hardly any tetrasialotransferrin left (Fig. 1).

In SDS-PAGE, changes in apparent molecular mass led to a slight shift of the patient's transferrin toward faster migrating isoforms. In comparison to a patient with PMM2-CDG where the bands represent normally glycosylated transferrin and transferrin missing one or two carbohydrate side chains, respectively, the loss in our patient was less than a whole carbohydrate side chain (2 kDa) (Fig. 2).

Mass Spectrometry

Mass spectrometry (nanoESI-TOF MS) of transferrin from the day of birth detected normally glycosylated transferrin and another isoform missing one sialic acid. In the follow-up controls, the proportion of truncated forms steadily increased with different forms missing one or more sialic acids and then also galactose residues (Fig. 3). This was also observed in samples of the affected sister (not shown).

Mass spectrometry of apolipoprotein C3 (MALDI-TOF MS) showed an increasing proportion of truncated and unglycosylated apoprotein C3 as a marker for a defect in *O*-glycosylation. These findings also increased over time (not shown).

2D Gel Electrophoresis

In general, the second sample showed a fainter pattern compared to sample 1 while being incubated for the same time in silver staining solution.

Changes in charge and molecular mass were present in various proteins. Most notably, α_1-antitrypsin was shown to present with three more negatively charged subspecies in the later sample (Fig. 4).

Discussion

Up to date, there is no CDG screening test for newborns, due to the fact that (1) there is no test available for a reliable screening on the third day of life and (2) no treatment for most types of CDG syndromes is available. Only children with conspicuous clinical features are selectively screened for CDG. Thus, CDG syndromes are often diagnosed in the first year of life, but normally not within the neonatal period or the first 3 months of life (Funke et al. 2013). In the present case, a sibling already diagnosed with TMEM165-CDG allowed prenatal diagnosis. This gave us the opportunity to monitor the changes in glycosylation patterns directly from birth.

The TMEM165 gene (HGNC:30760) is located on chromosome 4q12. The gene consists of six exons, the corresponding protein has 324 amino acid residues. It belongs to the highly conserved UPF0016 family of membrane proteins. Members of this family are found among many organisms, bacteria as well as eukaryotes (Fig. 5). TMEM165 is supposed to be a transporter for calcium ions, involved in Golgi pH homeostasis by a Ca2+/H+ antiport (Demaegd et al. 2013). It is located within the Golgi apparatus, plasma membrane, and late endosomes/lysosomes (Rosnoblet et al. 2013). Up to now, five patients affected with TMEM165-CDG have been described with skeletal dysplasia, growth retardation, and psychomotor retardation as key features (Foulquier et al. 2012). Early fatal outcome due to nephrotic syndrome has not been described before.

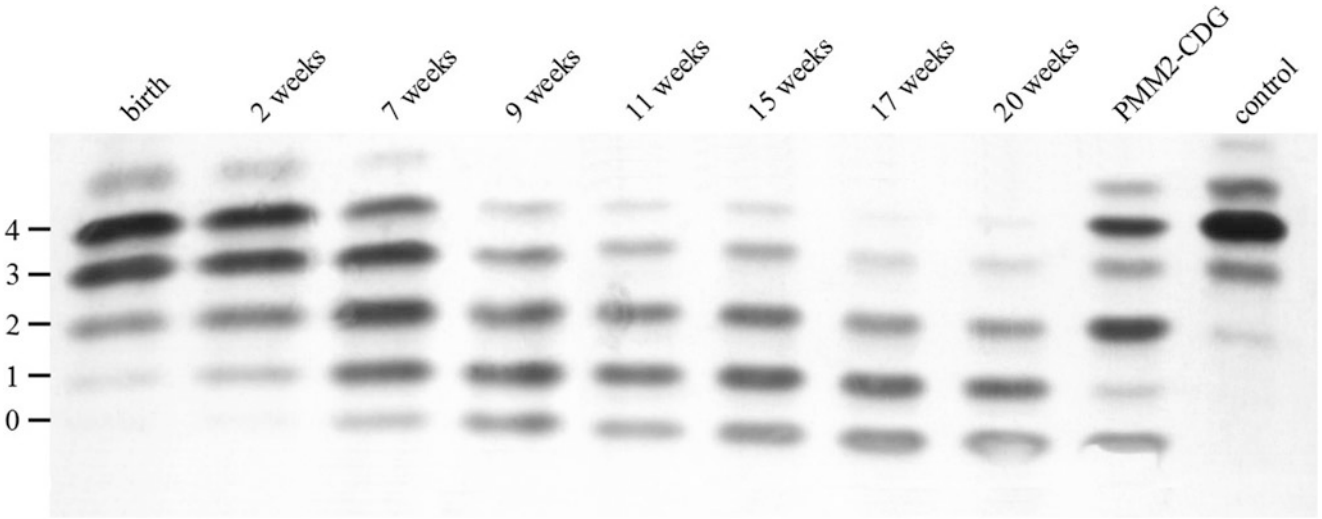

Fig. 1 IEF of transferrin (Coomassie staining). Numbers at the *left* represent the number of negatively charged sialic acid residues. A distinct change to less sialylated specimens is visible over time

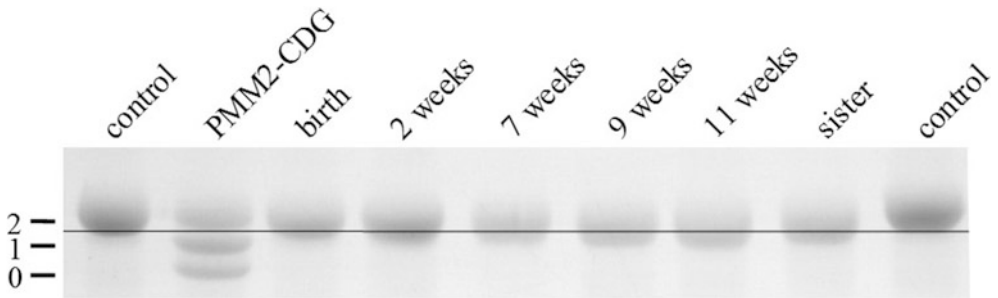

Fig. 2 SDS-PAGE of transferrin (Coomassie staining). Numbers at the *left* indicate the amount of glycans. The band shifts toward the anode, indicating a loss of molecular mass of less than 2 kDa

Directly after birth, glycosylation was abnormal in this case with increased amounts of tri-, di-, and monosialotransferrin, with tetra- and trisialotransferrin still forming the largest fractions. Over the next few weeks, the spectrum of transferrin changed to less sialylated and then also to hypogalactosylated forms, resulting in the nearly complete loss of tetrasialotransferrin. From week 9 forward, galactose and sialic acid residues are missing in equal proportions, indicating that the critical point was galactosylation rather than sialylation. We do not know if this pattern would have further changed since the patient died a few days after the last blood sample was collected. *O*-Glycosylation was also impaired right from birth with patterns deteriorating over time.

It is remarkable that an abnormal glycosylation pattern was already observed only 3 min after birth. It has previously been reported that abnormal glycosylation in PMM2-CDG develops not until the second or third week of life (Clayton et al. 1993). Fetal blood obtained in the 19th week of gestation showed normal transferrin glycosylation. Only in the second to third postnatal week the pattern changed in the pathological way normally observed in PMM2-CDG. Denecke et al. (2005) confirmed that IEF of transferrin in the 19th gestational week was normal in a fetus affected with ALG3-CDG. Other proteins like α1-antitrypsin were also shown to be normally glycosylated in an affected individual in utero. Other examples for negative IEF screening for different forms of CDG syndrome (PMM2-CDG, ATP6V0A2-CDG, SRDA3-CDG) in the first weeks of life have been reported (Funke et al. 2013). Why transferrin IEF is a poor marker neonatally for various CDGs is not entirely clear. Possible explanations are maternal proteins crossing of the placental barrier or a maternal factor influencing fetal glycosylation.

Synthesis of transferrin in the fetus has been observed as early as the ninth week of gestation but is quantitatively different compared to adults (Melartin et al. 1966). Relatively stable concentrations of transferrin postnatally without a significant decrease indicate sufficient synthesis by the fetus and neonate (Hitzig 1961). Transferrin has been

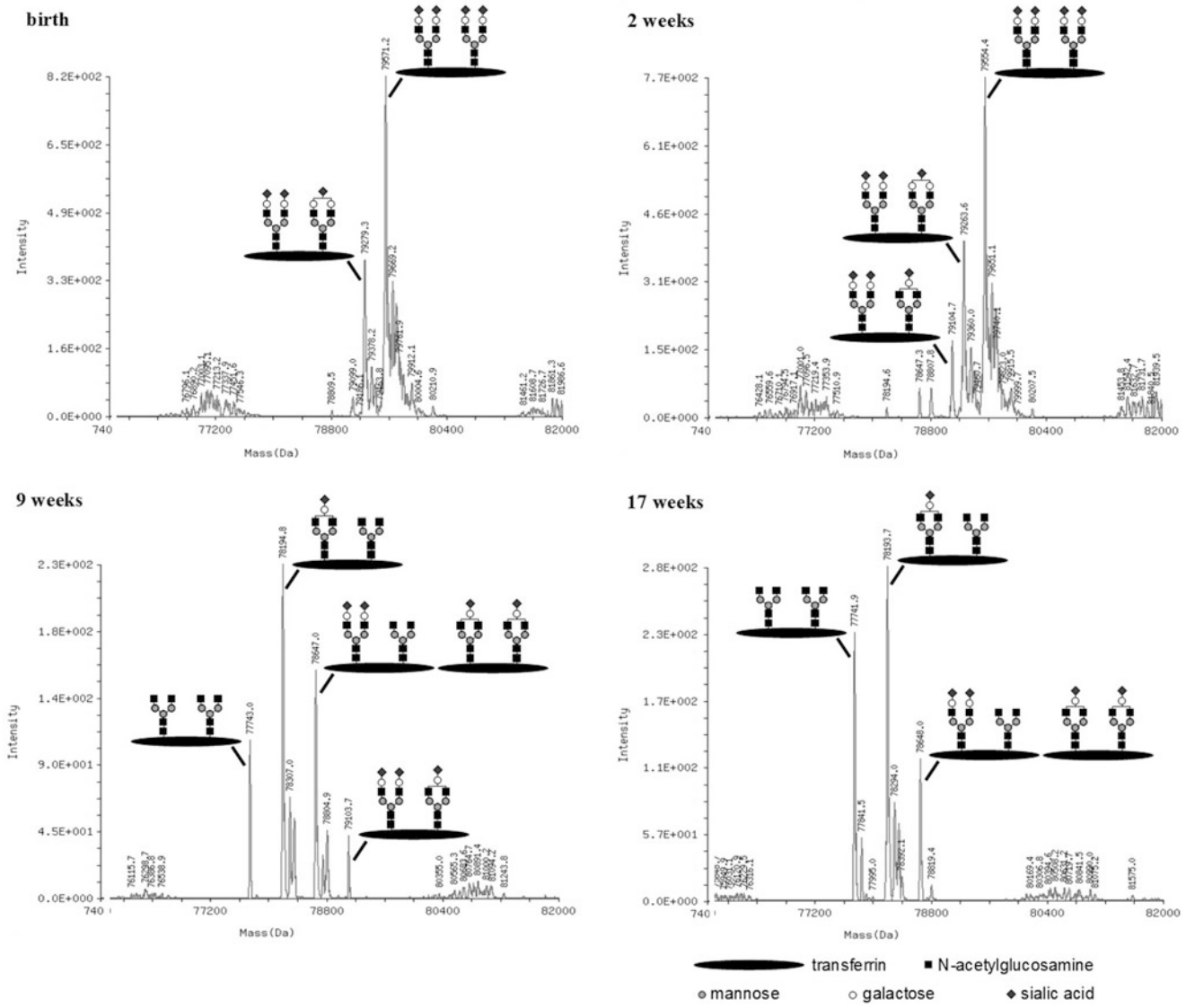

Fig. 3 nanoESI-TOF MS of transferrin: a shift to less sialylated (and galactosylated) specimen is obvious in this analysis

shown to be able to cross the placenta, but only to a very limited extent (Gitlin et al. 1964). The concentration of radioiodinated transferrin in cord plasma was below 20% of the concentration in the mother at delivery. In some cases, little or no labeled transferrin was found in fetal plasma at all. Due to missing data concerning rates of degradation in the fetus and rates of transfer from fetus to mother, the plasma concentrations could not be exactly translated into placental transfer rates.

Placental transfer of transferrin alone cannot explain why the IEF of transferrin is entirely normal in the reported cases, as the predominant amount of transferrin is from fetal origin. With a half-life of 8–10 days (Chung 1984), even if transferrin in the newborn is in part of maternal origin, the proportion of maternal transferrin is negligible after 3–4 weeks. This total loss of maternal transferrin can explain a postnatal change to a more pathological glycosylation pattern, but not the initially normal results observed in different types of CDG.

de Jong and van Eijk (1988) demonstrated that during pregnancy, highly sialylated transferrin is increased in relative as well as in absolute amounts. The increase was also detectable in women using oral contraceptives, indicating that it may be caused by hormonal changes. It was also shown that this change could be observed in guinea pig fetuses as well, with the transferrin spectrum normalizing within 20 days after birth. These results point to a hormonal factor favoring higher degrees of sialylation in the maternal as well as in the fetal organism. Yet there was a difference between women in pregnancy and those taking oral contraceptives – while the absolute concentration of transferrin grew in the same extent, the relative gain

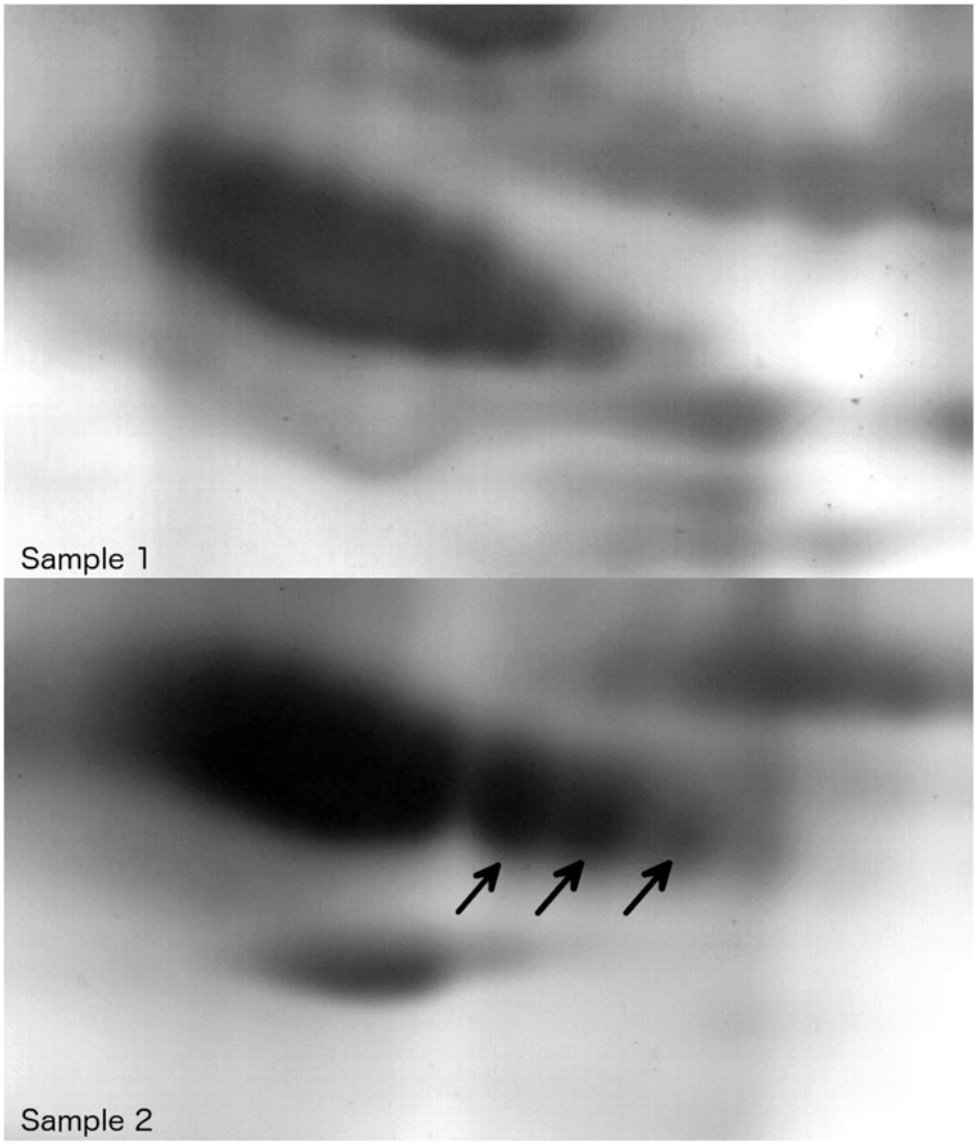

Fig. 4 2D gel electrophoresis: comparison of α_1-antitrypsin in plasma samples 1 (*above*) and 2 (*below*) of the patient. Note the different migration patterns in the sample collected later during the course of the disease

of highly sialylated transferrin was less pronounced than in pregnancy, maybe due to a different estrogen/progesteron ratio or other hormones (de Jong et al. 1992). The existence of hormone-responsive elements within the promotor regions that might be the mediators for this hormonal influence on glycosylation has been proven for some glycosyltransferases (Medvedova et al. 2003), and that applies also to different expression patterns of glycosyltransferases in embryonic tissues (Uehara and Thelu 2001; Zhou et al. 1998; Granovsky et al. 1995) that might arise from this hormonal influence.

Stibler and Skovby (1994) reported another case of prenatal diagnostics in whom chorionic villus biopsy and amniotic fluid at gestational weeks 11 and 17 in twins with PMM2-CDG showed normal results for transferrin. In contrast to what was mentioned above, already on the first day of life, a CDG type I pattern was observed in transferrin IEF of blood samples. During the following 2 months, an increase in the concentration of carbohydrate-deficient transferrin was observed from 3.5/4.9 mg/L to 189/197 mg/L (data for twin 1/twin 2).

A pathological pattern in IEF of transferrin directly after birth, followed by an aggravation in the following weeks, was also observed by van de Kamp et al. (2007) with regard to two siblings affected with PMM2-CDG. In both cases, a pathological transferrin IEF pattern was observed

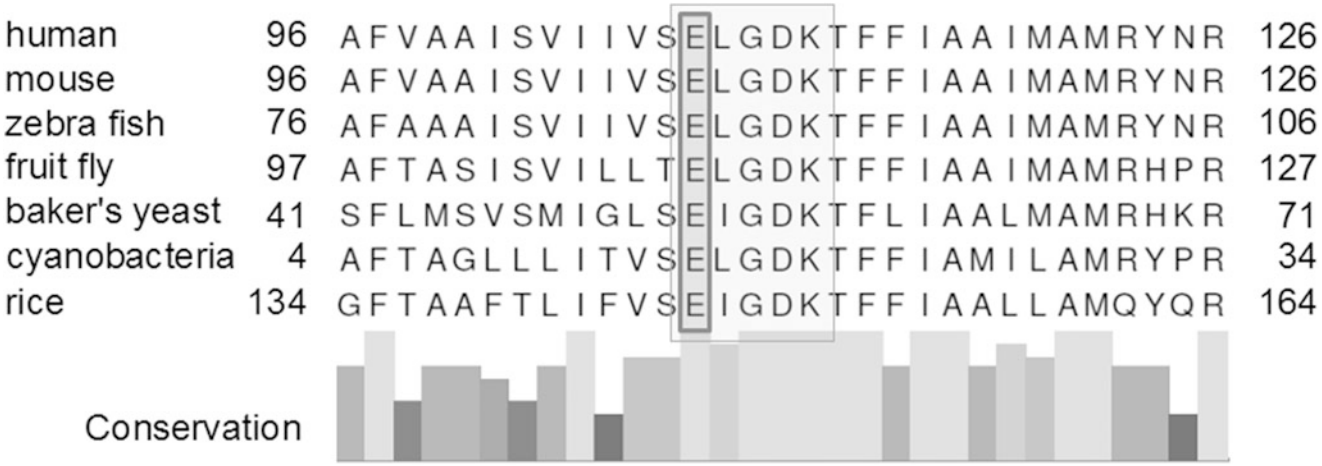

Fig. 5 Cross-species alignment of TMEM165 and other members of the UPF0016 family. Alignment was prepared using the alignment tool from UniProt homepage (The UniProt Consortium 2014). Reference sequences for human (NP_060945.2), mouse (NP_035756.2), zebra fish (NP_997848.1), fruit fly (NP_650426.1), baker's yeast (*Saccharomyces cerevisiae*, NP_009746.1), *c*yanobacteria (*Synechocystis* sp., NP_442278.1), and rice (NP_001068053.1). *Columns below* indicate the degree of conversation. The motif ELGDK (*light gray box*) is highly conserved and characteristic for proteins of the cation transporter UPF0016 family, indicating its functional importance. Most proteins of this family have two regions containing this motif, each with three predicted transmembrane regions and a central hydrophilic loop (Demaegd et al. 2013). Rosnoblet et al. (2013) suggested that the E108LGDK motif might be crucial for putative cation recognition signals. Since the patient's mutation affects the glutamic acid on position 108 ("E", *dark gray box*) in human TMEM165, one of the two negatively charged amino acids in this motif, this mutation might critically interact with the normal protein function as a calcium transporter. This could explain the severe phenotype in the patient

on the second day of life. In the second child, there were follow-ups on days 22 and 39, respectively, when an increase in di- and asialotransferrin was noticed.

Other authors found abnormally glycosylated transferrin already in utero in the 27th and 30th gestational week, respectively (Edwards et al. 2006; Léticée et al. 2010), in patients affected with PMM2-CDG.

2D electrophoresis revealed a change in glycosylation not only for the common CDG biomarker transferrin but also for a variety of other glycoproteins such as the example shown in Fig. 4 (α_1-antitrypsin). This indicates that TMEM165 deficiency affects the glycosylation of various proteins and that the changes are not restricted to transferrin. The faint pattern of the second sample can be explained by the patient's nephrotic syndrome since it has been observed that the consequent protein loss can account for faint patterns in IEF (Kranz et al. 2004).

Results in our case show that glycosylation defects can be diagnosed by IEF already directly after birth (and probably already in the late intrauterine period) at least in some cases. The current opinion is that prenatal diagnosis for CDGs should only be made by molecular analysis (Matthijs et al. 2004). Of course this is the best way, giving a definite and reliable answer. In cases, however, in whom the underlying molecular defect is not known, IEF of transferrin might also give some information in the late prenatal and in the early postnatal period. With regard to the false-negative results described earlier, a negative result cannot definitely exclude a CDG syndrome. In case of ongoing suspicion, diagnostics should be repeated up to the age of at least 1 month for a definite exclusion of CDG. But in case of a positive result, transferrin IEF might shorten the time of uncertainty for parents.

It can only be speculated why there are CDG patients with normal IEF in the first days and weeks of life and others with a pathological pattern already in utero. This phenomenon is not restricted to specific types of CDG, but witnessed over a broad spectrum of different forms. A possible answer is that the more severe the impact of the underlying molecular defect is, the earlier a pathological pattern can be detected in transferrin analysis. Yet it seems that there is indeed a protecting factor during intrauterine life. Since it is hard to imagine that a fundamental pathway like glycosylation has major differences in the fetus compared to the neonate circumventing different metabolic blocks, it is likely that there is a protecting factor coming from the mother.

It would be very interesting to find out the nature of this protecting factor of neonatal glycosylation. From what we know, it should be a factor coming from the mother that is withdrawn by the time of birth, maybe a hormonal change. Cell culture work with CDG fibroblasts and maternal serum might be a way to identification of this factor which could render new therapeutic approaches to different types of CDG.

Acknowledgement We dedicate this paper to our friend and colleague Christian Körner. He was one of the founders of CDG research and will be deeply missed by colleagues and patients.

Take-Home Message

Glycosylation patterns undergo an adaptational process over the first postnatal weeks that can be shown by transferrin analysis. Postnatal screening for CDG has pitfalls due to a correcting factor likely derived from the mother.

Compliance with Ethic Guidelines

Conflict of Interest

All authors declare no conflict of interests.

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 consent was obtained from the parents.

Details of the Contributions of Individual Authors

S. Schulte Althoff: acquisition and analysis of data, drafting and revision of the manuscript

M. Grüneberg, J. Reunert, J. H. Park, S. Rust, Y. Wada: acquisition and analysis of data, revision of the manuscript

C. Mühlhausen, R. Santer: medical treatment of the patient, acquisition and analysis of data, revision of the manuscript

T. Marquardt: supervision, data acquisition and interpretation, revision of the manuscript

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CASE REPORT

Transaldolase Deficiency: A New Case Expands the Phenotypic Spectrum

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Abstract Transaldolase (TALDO) deficiency has various clinical manifestations including liver dysfunction, hepatosplenomegaly, anemia, thrombocytopenia, and dysmorphic features. We report a case presenting prenatally with hyperechogenic bowel and intrauterine growth restriction. The infant was born small for gestational age, with cutis laxa and hypertrichosis. Postnatally, meconium plug was identified, complicated with intestinal obstruction necessitating laparotomy, partial resection of the intestine, and ileostomy. Liver biopsy revealed cholangiolar proliferation and portal fibrosis. He also suffered from persistent congenital thrombocytopenia requiring platelet transfusions and severe hypothyroidism with normal anatomical and structural gland responding only to the combination of T3 and T4 treatment. Neurologically, severe hypotonia and anisocoria were noted at the age of 2 months. Brain MRI was normal. Shortly after the abdominal surgery, a rapid liver failure ensued, which eventually led to his death. Specific metabolic tests ruled out glycosylation disorders, yet urine analysis using 1H NMR showed accumulation of sedoheptulose which was previously described in patients with transaldolase deficiency. Sequencing of the gene-encoding transaldolase (*TALDO1*) revealed a homozygous stop mutation c.669C>G; p.Tyr223*. In conclusion, we present an infant with a novel homozygous mutation in *TALDO1*, causing TALDO deficiency, and extend the clinical characteristics of this rare syndrome.

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Introduction

Transaldolase (TALDO, EC 2.2.1.2) is a key enzyme in the pentose phosphate pathway (PPP). It is crucial in the nonoxidative part, immediately after transketolase (EC 2.2.1.1). These two enzymes enable an important connection between the PPP and glycolysis (Perl 2007). TALDO interconverts a three-carbon moiety between different sugars; thus, it enables the production of fructose-6-phosphate and erythrose-4-phosphate from glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. In the absence of transaldolase intermediate products such as ribitol, D-arabitol and erythritol may accumulate, and their

abnormal elevated concentrations can be measured directly (Wamelink et al. 2007). Although the diagnosis is rare, the description of more cases is needed for further delineation of the enzyme's deficiency. TALDO deficiency was previously described in several patients, causing a severe disease of the liver, skin, and blood (Eyaid et al. 2013; Verhoeven et al. 2001, 2005). Fetal abnormal findings and maternal disease during pregnancy are infrequent, but were documented previously, presented by IUGR (Verhoeven et al. 2001), maternal HELLP syndrome (Verhoeven et al. 2005), oligohydramnios, fetal splenomegaly, and fetal distress (Wamelink et al. 2008a). Here, we present a case of fetal hyperechogenic bowel, which after birth developed a multisystemic disease, eventually leading to his death. He was found to harbor a novel homozygous mutation in *TALDO1*.

Clinical Report

A 35-year-old pregnant woman was referred to the genetic clinic at 21 weeks and 6 days of pregnancy, due to grade 2 hyperechogenic bowel detected in the fetus. The estimated hyperechogenic bowel size was 39 × 21 mm. Fetal echocardiogram was interpreted as normal. Maternal history included delivery of two normal children born at term and five miscarriages. The mother was heterozygous for Factor V Leiden and was treated with enoxaparin (Clexane) up to 35 weeks' gestation. Parental karyotyping was normal. The parents are first-degree cousins, of Arabic Muslim origin. Follow-up during pregnancy revealed fetal dilated bowel loops and intrauterine growth retardation. Genetic evaluation of both parents for 24 common known mutations of cystic fibrosis was negative as was the infectious workup – for cytomegalovirus (CMV) and toxoplasmosis. First-trimester screening was normal, without added risk for any aneuploidy.

The couple refused amniocentesis, and further fetal ultrasonographic scan demonstrated bowel echogenicity without dilated bowel loops. A male infant was born at 37 weeks of gestation by vaginal delivery. Birth weight was 2,050 g, the length was 40 cm, and head circumference was 31 cm. He had cutis laxa and hypertrichosis. At 2 days of age, meconium plug was diagnosed, necessitating laparotomy, resection of 15 cm of occluded small intestine, and ileostomy. Surgical closure of the ileostomy at 2 months of age was complicated by *Pseudomonas aeruginosa* peritonitis. During this operation, a liver biopsy was performed due to the impression of an abnormal liver color. Histopathology demonstrated cholangiolar proliferation and portal fibrosis. Following this operation, the infant deteriorated clinically with rapid liver failure which eventually led to his death. The liver malfunction presented mainly with increased direct bilirubin levels (up to 35 mg/dl), increased ammonia levels, low clotting factor levels (Factor V-12%, Factor VII-8%), however, without considerable elevation in hepatocellular or cholestatic liver enzymes. Congenital thrombocytopenia was observed requiring perioperative platelet transfusions, yet neither anemia nor leucopenia was found. Blood smear showed a low number of small platelets. The thyroid axis hormones were examined routinely and revealed severe hypothyroidism with minimal change under levothyroxine sodium (T4) treatment. Euthyroidism was achieved only by using a combination of triiodothyronine (T3) and T4 treatment. A thyroid scan demonstrated a normal anatomical and structural gland, raising the possibility of enzymatic or receptor-related endocrine disorder. Neurologically, the infant had severe hypotonia since birth. At 3 days of age, after recovering from the first abdominal surgery, he had a comatose event lasting 3 days, from which he recovered spontaneously. Infectious, metabolic, and toxic evaluations were normal. Brain magnetic resonance imaging (MRI) was normal. At the age of 2 months, he developed acute anisocoria. Brain computerized tomography (CT) demonstrated dilated brain ventricles with no focal finding, edema, or midline deviation and normal structures of gray and white matter. Assays of transferrin electrophoresis for glycosylation disorders were normal.

Urine test for metabolites of the pentose cycle using proton nuclear magnetic resonance (1H-NMR) demonstrated a high level of polyols, including sedoheptulose, which characterizes transaldolase deficiency. Erythronic acid level was below detection (normal), as shown in Fig. 1b. The concentration of sedoheptulose found here was 1,350-µmol/mmol creatinine, similar to a patient described by Engelke et al. (2010) which showed a sedoheptulose level of 1,700-µmol/mmol creatinine at the same age (1 month). The normal range for sedoheptulose at ages 0–3 months was previously reported to be lower than 40-µmol/mmol creatinine (Engelke et al. 2010). The concentration of polyols cannot be provided quantitatively since the specific molecules underlying this heavily overlapping signal region are not known. Nevertheless, the integrated intensity ratio of the polyol region (3.60–4.15 ppm) to that of creatinine (signal at 3.13 ppm) was 6–13-fold higher in the spectrum obtained from the patient's urine compared to spectra obtained from four healthy children aged 3 to 7 years. The limit of detection of erythronic acid under the conditions used here is 60-µmol/mmol creatinine or 130 µM absolute concentration. Therefore, the level of erythronic acid in our case, if any, is lower than this limit. This detection limit is based on (1) the identification and quantification of erythronic acid using the doublet signal of the proton in position 2, at 4.326 ppm (Engelke et al. 2010),

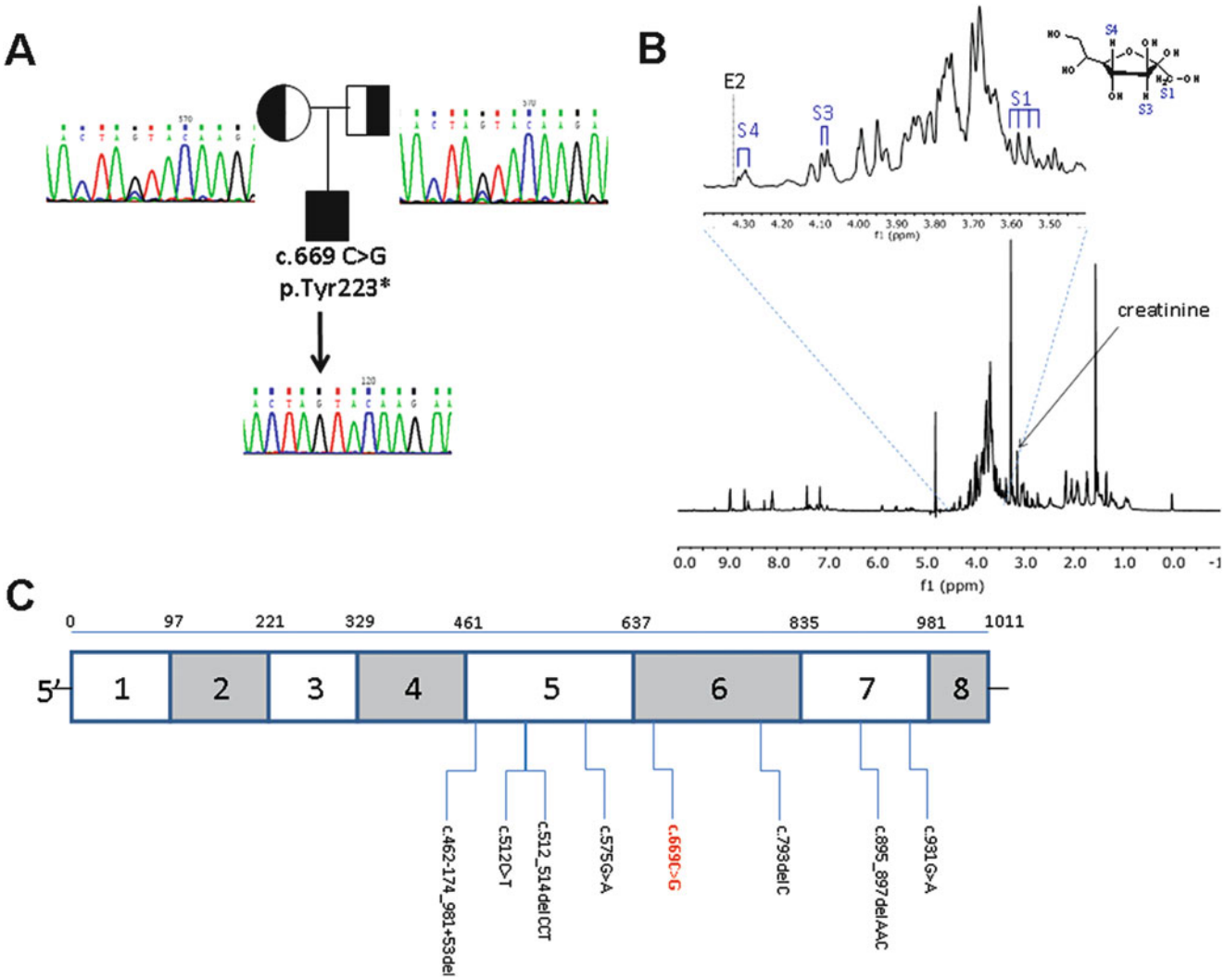

Fig. 1 TALDO deficiency in the patient. (**a**) *TALDO1* homozygous pathogenic mutation c.669C>G; p.Tyr223* in the patient and his parents. Sanger sequencing of the patient and his parents: both parents are heterozygous for the mutation and a wild-type allele. *TALDO1* mutation is not found in controls of the same background (not included). (**b**) A 1H-NMR spectrum of the patient urine sample showing abnormally high levels of sugars at about 3.3–4.5 ppm. Sedoheptulose (a ketoheptose) was identified using the S1, S3, and S4 signals of sedoheptulose which were previously characterized in a urine sample from a patient with TALDO deficiency (Engelke et al. 2010). Erythronic acid was not detected in the patient's urine, as depicted by the lack of the characteristic doublet signal at 4.325 (position marked E2) (Engelke et al 2010). (**c**) TALDO1 gene cDNA schematic structure. Mutations are presented on the diagram. The following are the mutations previously described: c.512_514delCCT, p.Ser171del; c.512C>T, p.Ser171Phe; c.574C>T, p.Arg192Cys; c.575G>A, p.Arg192His; c.793delC, p.Gln265ArgfsX56; c.895_897delAAC, p.Asn299del; c.931G>A, p.Gly311Arg; c.462-174_981+53del. The mutation in the patient described in this article is colored in *red*

and (2) the specific concentration of creatinine in the current sample which was independently measured. Array comparative genomic hybridization (CGH) analysis showed male karyotype with no copy-number changes. Based on the known parental consanguinity, an identity by descent approach utilized CGH for assessment of runs of homozygosity (ROH), a combined homozygous region of 234 Mbp was identified. By focusing on metabolic recessive genetic causes for cutis laxa (Valayannopoulos et al. 2006; Mohamed et al. 2011), within these areas of homozygosity, we could exclude known congenital disorders of glycosylation, Menkes disease, and other previously published forms of autosomal recessive cutis laxa syndromes, and we were left with transaldolase deficiency encoded by *TALDO1*, localized at chromosomal region 11p15.5, amidst a homozygous region of 7.4 Mbp, out of a total of 234-Mbp homozygous regions.

Sequencing *TALDO1* identified a homozygous nonsense point mutations c.669C>G; p.Tyr223* [NM_006755.1] (Fig. 1a). The presence of the above mutation is predicted to target the *TALDO1* mRNA for nonsense-mediated decay (NMD).

Materials and Methods

Array CGH

Ten milliliter of peripheral blood was centrifuged at 2,500 *g* for 10 min and 200-µl buffy coat was taken for isolation of genomic DNA using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA (0.1 µg) was labeled using the Affymetrix Cytogenetics Reagent Kit, and labeled DNA was applied to an Affymetrix Cytogenetics Array (2.7 million probes, Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instructions. The array was scanned, and the data were analyzed using the Affymetrix Chromosome Analysis Suite.

Urine Sample Preparation

The urine samples were centrifuged before analysis. A volume of 70 µl of a 20-mmol/l trimethylsilyl-2,2,3,3-tetradeuteriumpropionic acid (TSP, sodium salt; Sigma-Aldrich) D_2O solution was added to 700 µl of urine as a chemical shift reference ($\delta = 0.00$) and as a lock signal. The pH of the urine was adjusted to 2.51 ± 0.05 with HCl. The sample of 770 µl was then transferred to a 5-mm NMR tube.

1H-NMR Spectroscopy

An 11.8 T spectrometer (Varian, Palo Alto, CA) equipped with a TRX probe was used for direct ^1H detection. Water pre-saturation was followed by 45° pulses with a repetition time of 10 s. One hundred and twenty-eight transients were recorded at 25°C. The spectra were collected with 16 K data points and zero filled to 32 K. A line broadening of 0.2 Hz was applied, and phase and baseline were corrected manually.

The chemical shift was referenced to TSP at 0 ppm. Metabolite signal areas were compared with the area of creatinine singlet (3.13 ppm; the N–CH3 protons) to determine metabolite concentrations expressed as µmol/mmol creatinine. Spectral analysis was carried out using MNova (Mestrelab Research, Santiago de Compostela, Spain).

TALDO1 Sequencing

The seven coding exons of the *TALDO1* gene were PCR amplified using blood-extracted DNA (primer sequences are available upon request). The PCR products were purified and sequenced on a 3130 Genetic Analyzer using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Table 1 Clinical features of previously described patients with TALDO deficiency and comparison to the present patient

Feature	Reported cases (percent of cases)	Present patient
Consanguinity	25/31 (80.6)	+
Dysmorphism	18/31 (58)	+
Liver dysfunction	26/31 (83.8)	+
Hepatosplenomegaly	30/31 (96)	+/− (only splenomegaly)
Anemia	27/31 (87)	−
Thrombocytopenia	27/31 (87)	+
Cardiac	23/31 (74)	+
Neonatal edema	8/31 (25.8)	−
Renal	11/31 (35.5)	+
Respiratory	5/31 (16)	−
Developmental delay	2/31 (6)	NA
Additional features found	Hypertrichosis Hypotonia	Hypothyroidism Hyperechogenic bowel Hypertrichosis Neonatal hypotonia

Data collected from previous reports (Leduc et al. 2014; Verhoeven et al. 2001; Verhoeven et al. 2005; Valayannopoulos et al. 2006; Wamelink et al. 2008a; Al-Shamsi et al. 2015; Tylki-Szymanska et al. 2009; Tylki-Szymanska et al. 2014; Eyaid et al. 2013; Balasubramaniam et al. 2011)

Discussion

Patients with transaldolase (TALDO) deficiency have a variable phenotype yet with common features. Table 1 depicts the various clinical manifestations previously reported in TALDO deficiency.

It is evident that most reported patients are offsprings of consanguineous marriages as expected in such a rare disorder. Most patients have liver dysfunction, hepatosplenomegaly, and hepatic fibrosis (83.8%, Table 1). TALDO deficiency is associated with dysmorphic features including cutis laxa, anti-mongoloid slant and low-set ears, dolichocephaly, exophthalmia, and broad nasal bridge. Also reported are hirsutism/hypertrichosis, heart defects, renal problems, and intermittent hypoglycemia. Mental and motor development is usually normal in surviving patients. In rare cases, clitoromegaly, micropenis, sensorineural and conductive deafness, and rickets were noted (Eyaid et al. 2013; Wamelink et al. 2008a). Brain MRI and magnetic resonance spectroscopy (MRS) did not reveal abnormal findings. TALDO protein and function have been reviewed thoroughly by Wamelink et al. (2008b). As known, TALDO is a key enzyme in the pentose phosphate pathway, responsible of exchanging a

three-carbon moiety between different carbohydrates, enabling either the production of more nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate, or by being inhibited, enabling production of more nucleic acids. Therefore, TALDO function is essential for cell survival (Wamelink et al. 2008b; Perl 2007; Perl et al. 2011).

The novel *TALDO1* mutation found in the present described family is a homozygous STOP mutation. This mutation creates an mRNA predicted to be degraded through nonsense-mediated mRNA decay (NMD) process. This case displays perinatal findings not previously reported in TALDO deficiency, namely, fetal echogenic bowel, meconium ileus, neonatal involvement of CNS, and severe hypothyroidism. Generally, hyperechogenic bowel is encountered in 1% of all pregnancies. Associated conditions are aneuploidy, infections (CMV, toxoplasmosis), and cystic fibrosis (CF) (Al-Kouatly et al. 2001). There are different conditions associated with failure to pass meconium – including Hirschsprung's disease, meconium plug syndrome, meconium ileus, and anorectal malformations (Loening-Baucke and Kimura 1999). Meconium ileus is known to be associated with cystic fibrosis (van der Doef et al. 2011). In the case presented, fetal hyperechogenic bowel, along with meconium ileus, was associated with TALDO deficiency. Shortly after birth, the bowel obstruction caused severe deterioration. We can now postulate that the liver malfunction may explain the meconium plug – as malsecretion of bile acids could lead to the creation of sticky and viscous stool (Harries 1978). In addition, the postnatal comatose event lasting 3 days postoperatively may be related to the impaired liver function and its failure to effectively eliminate anesthetic drugs. As described, no other cause for this event was found. The severe hypothyroidism described in this case is unusual. Triiodothyronine in treating infantile hypothyroidism is rarely needed (Wartofsky 2013) and can probably be explained by disrupted pentose phosphate pathway, known to be important for thyroid function (Wartofsky 2013). It is possible that this neonate harbors a variation in a modifier gene, which may cause a severe form of clinical presentation. Also, this could be the result of a second mutated gene. These possibilities can be explored in whole-genome sequencing or whole-exome sequencing, which were not part of this report.

In the paper by Engelke et al. (2010), six patients with TALDO deficiency were described, all reported with high levels of erythronic acid (EA) compared with age-matched controls, including one patient at the age of 1 month. The mechanism responsible for the elevation of EA is not clear. EA is a product of the pentose phosphate pathway, produced from D-erythrose-4-phosphate, which, in turn, is a product of active transaldolase. However, more research is needed to evaluate the role of EA as a biomarker in TALDO deficiency.

Figure 1c outlines the distribution qof the published TALDO1 mutations showing that as of now all mutations are clustered in exons 5 through 7. There is no clear genotype-phenotype correlation. The severe presentation of TALDO deficiency may of course be ascribed to variations in additional genes serving as phenotype modifiers. There is also the possibility of coexistence of a second disease. This can be further explored using whole-exome or whole-genome sequencing which was not performed in this case. It is also possible that environmental factors, such as anesthetic drugs used in the perinatal operation, had a role in the severe deterioration of the disease described.

In conclusion, the severe disease caused by this specific nonsense TALDO1 mutation further delineates the variable presentation of this important-to-recognize disease.

Synopsis

This article presents a new case of transaldolase deficiency with symptoms previously undescribed, diagnosed with nmr spectrometry and cgh array technology.

Compliance with Ethical Guidelines

Conflict of Interest

Ehud Banne, Vardiella Meiner, Avraham Shaag, Rachel Katz-Brull, Ayelet Gamliel, Stanley Korman, Horowitz Cederboim, Morasha Plesser Duvdevani, Ayala Frumkin, Amir Zilkha, Vadim Kapuller, Dan Arbell, Elite Cohen, and Smadar Eventov-Friedman declare that they have no conflict of interest.

Informed Consent

All procedures described in this manuscript were performed in accordance with the ethical standards of the responsible Institutional Review 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's family for blood and urine tests.

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RESEARCH REPORT

Pearson Syndrome: A Retrospective Cohort Study from the Marrow Failure Study Group of A.I.E.O.P. (Associazione Italiana Emato-Oncologia Pediatrica)

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Abstract Pearson syndrome (PS) is a very rare and often fatal multisystemic mitochondrial disorder involving the liver, kidney, pancreas, and hematopoietic and central nervous system. It is characterized principally by a transfusion-dependent anemia that usually improves over time, a tendency to develop severe infections, and a high mortality rate. We describe a group of 11 PS patients diagnosed in Italy in the period 1993–2014. The analysis of this reasonably sized cohort of patients contributes to the clinical profile of the disease and highlights a rough incidence of 1 case/million newborns. Furthermore, it seems that some biochemical parameters like increased serum alanine and urinary fumaric acid can help to address an early diagnosis.

Abbreviations

ADA	Adenosine deaminase
A.I.E.O.P.	Associazione Italiana di Ematologia ed Oncologia Pediatrica
ANC	Absolute neutrophil count
BFU	Burst-forming units
BM	Bone marrow
BMF	Bone marrow failure
CFU-E	Colony forming unit-erythroid
CFU-GM	Colony forming unit-granulocyte macrophage

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CRF	Case report form
CT	Computed tomography
DBA	Diamond–Blackfan anemia
EPO	Erythropoietin
FUP	Follow-up
GCSF	Granulocyte colony stimulating factor
Hgb	Hemoglobin
IDDM	Insulin-dependent diabetes mellitus
KSS	Kearns–Sayre syndrome
LS	Leigh syndrome
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial DNA
PRBC	Packed red blood cells
PS	Pearson syndrome
VWT	Ventricular wall thickness

Introduction

PS is a multisystem disorder caused by large-scale rearrangements of mitochondrial DNA (mtDNA), with consequent defects in the mitochondrial respiratory chain (Rötig et al. 1995). PS typically consists of refractory, hypoplastic macrocytic anemia with vacuolated marrow precursors, lactic acidosis, and exocrine pancreatic dysfunction; anemia is frequently associated with a variable degree of thrombocytopenia and neutropenia (Rötig et al. 1995). Additional reported manifestations include proximal myopathy, neurologic symptoms (seizures, ataxia, movement disorders), skin lesions, and proximal renal tubular acidosis (Rötig et al. 1995; Atale et al. 2009). Most infants reportedly die before 3 years of age (Rötig et al. 1995). In some cases, a phenotypic transformation to Leigh syndrome (LS) is observed (Santorelli et al. 1996), characterized by dysphagia, hypotonia, ataxia, peripheral neuropathy, and ophthalmoparesis, or Kearns–Sayre syndrome (KSS) (Mcshane et al. 1991; Lee et al. 2007), characterized by progressive external ophthalmoplegia, retinopathy, ataxia, cardiac conduction abnormalities, and deafness.

Patients and Methods

This retrospective study was designed by the Bone Marrow Failure (BMF) Study Group of A.I.E.O.P. (Associazione Italiana Emato-Oncologia Pediatrica) and approved by the Ethics Committee of the Civico Hospital, Palermo. All procedures followed were in accordance with the ethical standards of the committee in charge of human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2000. Informed consent for inclusion in the study and genetic studies was obtained from the parents or the legal guardians of all patients.

A case report form (CRF) with 167 questions was sent to all 55 A.I.E.O.P. centers. In some cases, a single patient was treated by more than one center in different periods, so we received 14 CRFs regarding 11 PS patients, all diagnosed on the basis of clinical phenotype and genetic testing; 4 patients had been more succinctly presented in another report (Tumino et al. 2011).

Overall survival (OS) was calculated from the date of birth to death from any cause, or date of last follow-up (FUP). Survival analysis was performed using the open source statistical software R (R Development Core Team 2011) and the R package survival (Therneau 2012).

Results

Eleven PS patients (M/F: 5/6) from healthy unrelated parents were studied: their principal characteristics are summarized in Tables 1 and 2. The median age at diagnosis was 299 days.

Clinical Phenotype

All mothers were Caucasian except one who was of South American origin. The median age of the mothers of Italian origin was similar to the Italian population: 32.0 vs. 32.2 years. Only one mother had a previous miscarriage. All pregnancies were uneventful. No patient was born preterm, and only 3 out of 11 (27%) had a neonatal weight less than 2,500 g: the smallest was 2,230 g. In 6 pregnancies (54%), a cesarean section was performed, and in 3 of them, the cause was acute fetal illness. In these 3 newborns, metabolic acidosis and an extremely severe anemia were present: hemoglobin (Hgb) of 1.8, 2.9, and 5.5 g/dL, respectively, and in 2/3 hypoglycemia was associated.

All patients but one had a heart evaluation in the course of disease: ventricular wall thickness (VWT), depolarization abnormalities, and prolonged QT were observed in 4, 2, and 1 patient, respectively.

Only one of the patients suffered from skin abnormalities (diffuse hyperpigmentation and a large *café* au lait *spot*). Exocrine pancreatic impairment was found in 3 patients (27%), and 1 of them also presented type 1 insulin-dependent diabetes mellitus (IDDM). IDDM was also diagnosed in another patient without involvement of exocrine pancreas. Complete adrenal insufficiency was found in 2 children (18%) and hypoparathyroidism in another.

In 8/11 (72%) patients, hepatomegaly was observed, and in 5/8 it appeared at less than 6 months of age. Splenomegaly was seen in only 3/11 patients (27%): in one it was present at birth and in the other two appeared at 4 and 15 months of age, respectively. Abdominal ultrasound

Table 1 Clinics

Pt	HM	SM	Ins. Ex. Pancreas	IDDM	Growth Impair.	VWT	Neurol. Sympt.	KSS	Eye Problems	Transf. Indep.	Sev. Infect.	D/A (years)	Cause of death
1	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	D (6.4)	Severe acidosis
2	No	No	No	No	Yes	No	No	No	No	Yes	No	A (2.9)	/
3	Yes	No	No	No	No	NK	No	No	Yes	Yes	No	Lost at FUP (3.7)	/
4	Yes	No	Yes	No	Yes	Yes	Yes	No	No	No	Yes	D (5.7)	Renal failure
5	No	No	No	No	Yes	No	Yes	No	Yes	Yes	Yes	A (6.6)	/
6	No	No	No	No	Yes	Yes	Yes	No	NK	NP	No	D (0.33)	NK
7	Yes	Yes	No	No	Yes	No	No	No	Yes	Yes	Yes	D (8.0)	Sepsis
8	Yes	No	No	No	No	Yes	Yes	No	Yes	NP	Yes	D (0.53)	Sepsis
9	Yes	No	No	Yes	No	No	Yes	Yes	No	Yes	Yes	D (10.42)	AML
10	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	D (10.44)	Renal failure
11	Yes	Yes	Yes	No	No	Yes	No	No	NK	Yes	Yes	D (3.9)	Sepsis

Pt patient, *HM* hepatomegaly, *SM* splenomegaly, *IDDM* type 1 insulin-dependent diabetes mellitus, *VWT* ventricular wall thickness, *KSS* Kearns–Sayre syndrome, *Transf. Indep* transfusion independency, *Sev. Infect* severe infections, *D/A* dead/alive, *FUP* follow-up, *AML* acute myeloid leukemia, *NK* not known, *NP* not pertinent

Table 2 Laboratory

Pt	mtDNA del.	↑ Serum lactate	↑ Serum alanine	↑ Urine lactate	↑ Urine fumarate	↑ Urine malic acid	Fetal Hgb	EPO	Reticulocytes >60,000 μL	BM
1	5,000 N.S.	Yes	Yes	Yes	Yes	No	ND	ND	Yes	Vacuoles and ↓ cellularity
2	8,648–15,368	Yes	Yes	Yes	Yes	Yes	ND	↑	Yes	Vacuoles and ↓ cellularity
3	4,000 N.S	Yes	ND	No	No	No	ND	↑	Yes	Dyserythropoiesis
4	5,000 N.S.	Yes	Yes	Yes	Yes	Yes	↑	↑	Yes	Vacuoles
5	8,843–13,459	No	No	ND	ND	ND	↑	↑	Yes	Vacuoles and ↓ cellularity
6	8,400–13,500	Yes	Yes	Yes	Yes	Yes	ND	ND	Yes	Vacuoles and ↓ cellularity
7	6,634–9,935	Yes	Yes	ND	ND	ND	ND	↑	Yes	Vacuoles
8	10,049–15,088	Yes	Yes	Yes	Yes	No	ND	↑	No	↓ Cellularity
9	19,447–15,395	Yes	Yes	No	No	No	↑	ND	Yes	Vacuoles and ↓ cellularity
10	7,000 N.S.	Yes	Yes	Yes	Yes	Yes	↑	ND	Yes	Vacuoles and ↓ cellularity
11	5,000 N.S.	Yes	Yes	Yes	Yes	No	↑	ND	Yes	↓ Cellularity

Pt patient, *mtDNA delet* mtDNA deletion, *ND* not determined, *EPO* erythropoietin, *BM* bone marrow

was performed during the course of disease in 10 patients, and an increase in kidney echogenicity was observed in 5 patients, two of whom developed tubular acidosis and later severe renal failure. Triglycerides, cholesterol, and transaminases were normal in all patients. Two children presented slightly elevated values of gamma-glutamyltransferase. Growth impairment was observed in 7 (63%) of our patients. Furthermore, 2 patients (18%) presented severe malabsorption with a need for total parental nutrition, and 1 child had a severe duodenal ulcer.

Neurological examination was normal at birth in all children, but an impairment, above all characterized by retarded speech development, hypotonia, and muscle hypotrophy, developed in 7 patients (63%), 3 of whom later evolved towards a full KKS phenotype. A neonatal cerebral ultrasound was performed on 6 patients, and it was normal in all of them. Magnetic resonance imaging (MRI) of the brain was performed on 6 patients. In 2 cases (one with KSS), an abnormally high signal at brainstem level was observed; a similar anomaly was noted in a brain computed tomography (CT) scan of another patient affected by KSS. In another two cases (one with KSS), subcortical white matter hyperintensities were observed. Visual evoked potentials, performed on four patients, were not informative; in contrast, the auditory evoked potentials, performed on five patients, were abnormal only in the two patients affected by KSS, showing a compromised brainstem. Electroencephalography, performed on 8 patients, was found altered only in the three patients with KSS. Eye examination was performed on 9/11 patients, and in 6 (66%) a variety of problems (principally of the cornea and retina) were present. Two of the three patients later developing KSS presented congenital ptosis. No case of hearing loss was reported.

From the hematological point of view at the first blood count, pancytopenia was present in five children, anemia associated with leucopenia and/or neutropenia in five patients, and isolated anemia in 1 child. The median value of Hgb was 5.7 g/dL, the lowest value of platelets was 72,000 μL, and neutropenia (present in 10 patients) was severe (absolute neutrophil count (ANC) $<0.5 \times 10^9$/L) in 1, moderate (ANC $0.5–1 \times 10^9$/L) in 8, and mild (ANC of 1.3×10^9/L) in another. Interestingly, in the course of disease in 10/11, at least one count of reticulocytes higher than 60,000 μL and of ANC higher than 0.5×10^9/L were encountered. Anemia associated with other cytopenias or not was the most relevant onset symptom in all patients, even though in the three children in whom the transfusion dependency started at 12–25 months of life (see later), a growth impairment at that time had already been addressed.

Fetal Hgb was assessed in five patients after the neonatal period, and it was found elevated (>2%) in all of them. Erythropoietin (EPO), dosed in 6/11 patients, was always high. Immunoglobulin levels, lymphocyte count, and vitamin B12/folate were evaluated in 10, 5, and 9, respectively, and were normal in all cases. BM examination performed on all patients showed a precursor vacuolization associated with reduced cellularity in 6 children (54%), whereas vacuoles without decreased cellularity were observed in only two patients; in one patient, there was only a mild dyserythropoiesis, and in two other patients, only a reduction of cellularity. Perls staining revealed the presence of sideroblasts in six out of seven tested patients (85%).

BM colonies were investigated in seven patients, and in all tests burst-forming units (BFU), colony-forming unit-erythroid (CFU-E), and colony-forming unit-granulocyte macrophage (CFU-GM) were moderately to extremely reduced. In our population, there was one patient (n. 9) who evolved into acute myeloid leukemia (after allogenic bone marrow transplantation). No solid tumor was observed.

Biochemical Profile

Serum lactate was dosed in every patient and found elevated in all but one with a 1.34–4.77-fold increase: in three children the levels of lactate were intermittently high. Urine organic acids were analyzed in nine patients, and in 7/9 (77%) an increased excretion of lactate was noted: in these seven children, an elevated excretion of fumaric acid was found too. An increase of malic acid was found in 4/9 patients (44%), whereas a methylglutaconic aciduria mentioned in two papers (Gibson et al. 1992; Lichter-Konecki et al. 1993) could be found only in two children. A plasmatic amino acid analysis was performed on ten children: in 9/10 alanine was high. Constitutional karyotype was found normal in all patients; diepoxybutane test, performed on 7 patients, was negative in all, and adenosine deaminase (ADA), tested in four patients, showed increased values in two of them.

Treatments

All patients were transfused with packed red blood cells (PRBC), six with platelets, and two with plasma (during the course of severe sepsis). The PRBC transfusion dependency started in four patients at birth, in four patients before 4 months of life, in two patients at 12 months of age, and at 25 months of life in the remaining one. A spontaneous improvement of the Hgb values was noted in eight of the nine patients with a sufficiently long FUP (88%): the last PRBC transfusion was done at a median age of 2.01 years. Therapy with erythropoietin (EPO) was attempted in three children, and in no case did it prove to be beneficial. Granulocyte colony stimulating factor (GCSF) was administered in three patients and proved to be beneficial in one. Pancreatic extracts were effective in the three children with exocrine pancreatic deficiency.

Survival

After a median FUP of 5.7 years, 8/11 patients died (two at less than 6 months of life), 1 was lost at FUP at 45 months of age, and only 2/11 patients are alive (at the age of 2.9 and 6.6 years, respectively), accounting for crude survival rate of 20% (Fig. 1).

Causes of death were sepsis (exclusive cause) in three cases, acute myeloid leukemia in one case, intractable

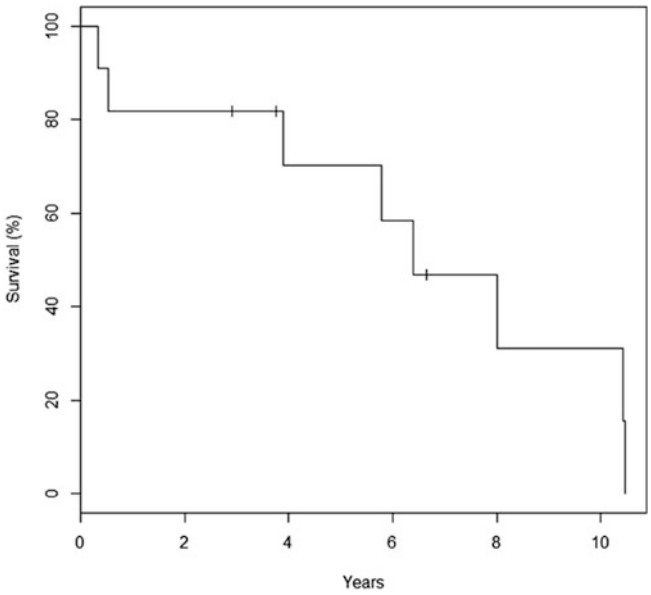

Fig. 1 Kaplan Meier survival curve

metabolic acidosis in one case, and severe renal failure in two cases; in one patient (deceased at 4 months of age), it was impossible to clearly establish the cause of death. All three patients with KSS died.

Discussion

Mitochondrial abnormalities are causes of some severe human diseases. The full-blown picture of PS is typically characterized by refractory sideroblastic anemia with vacuolization of marrow precursors, lactic acidosis, neurological, renal, hepatic, and exocrine pancreatic dysfunction (Rötig et al. 1995; Atale et al. 2009). The incidence is unknown. Since the 55 units of the A.I.E.O.P. network are spread over the entire national territory, it seems unlikely that any child affected by BMF has never been observed in an A.I.E.O.P. center, and so this series is likely to represent the total or near total of PS children in Italy in the period from 1993 to 2014. Based on the average annual birthrate, we can deduce that the likely incidence of PS in Italy is approximately 1/1,000,000 newborns.

The complete mitochondrial genome is small (16.6 kb), and some copies (normal and mutated mtDNA) passed on to progeny via the cytoplasm and accounted for by maternal inheritance are present in the same cell (heteroplasmy). It is probable that a mitochondrial disease can appear only when the proportion of mutated mtDNA exceeds a given threshold. In PS the mtDNA mutations are rather homogeneous since the same 5.0 kb deletion constitutes the most common lesion (Rötig et al. 1995) and is typically sporadic. In our cohort, similar to other series (Rötig et al. 1995; Topaloğlu et al. 2008), no apparent correlation was found between the size and site of mtDNA deletion, clinical presentation, and final outcome.

Affected children are frequently reported born preterm, but all the patients in our series were born later than 38 weeks, and only three presented a birth weight under 2,500 g. In our analysis, apart from one case of bicameral right ventricle, and two of ptosis, no evident malformation or dysmorphism was observed. Splenic atrophy was reported in two patients of the original paper of Pearson et al. (1979), but no other case, including the present series, has yet been published.

The absence of exocrine pancreatic deficiency is reported in 23–63% of cases (Rötig et al. 1995; Atale et al. 2009; Broomfield et al. 2015): in our series the high proportion of 73% was free of this complication. Likewise and in contrast to the high incidence of hepatic failure (33%) reported by Rötig et al. (1995), none of our patients presented signs of serious liver involvement.

Two patients (18%) developed a severe tubulopathy progressing to severe renal failure, which is consistent with the 23–24% reported in another 2 series (Atale et al. 2009; Rötig et al. 1995). Both patients presented increased echogenicity of kidneys. The growth deficiency, described as typical of PS (Atale et al. 2009), was also observed in our series as it was seen in 63% of patients.

The neurological features of PS are quite variable, ranging from normal to highly severe. The neurological examination can be abnormal very precociously, and in some cases, it is possible to document progression in the neurological disability. Sometimes a final evolution to KSS (Mcshane et al. 1991; Lee et al. 2007) and LS (Santorelli et al. 1996) can be documented. Neuroimaging is quite variable: from completely normal to severely abnormal findings of white matter, deep gray nuclei, cerebellum, and brainstem (Lee et al. 2007; Morel et al. 2009). Neurologic problems are quite frequent in our series (8/11), above all seizures and hypotonia, and three patients (27%) developed full KSS. An abnormally high signal at brainstem level on MRI, or CT, was the most frequent finding in neuroimaging (three out of eight patients evaluated).

Echocardiography was performed on ten patients, and in four of them, a ventricular hypertrophy was observed. Occasionally, an impaired cardiac function has been described (Krauch et al. 2002; Broomfield et al. 2015), but to our knowledge, the frequent association between PS and VWT has never been reported previously even though hypertrophic cardiomyopathy is a well-known feature of many mitochondriopathies (Kopajtich et al. 2014; Wang et al. 2008; Kupari 1984). Elevated fetal Hgb, occasionally described in PS patients (Superti-Furga et al. 1993), and elevated EPO were observed in all patients evaluated.

All but one of the patients had elevated lactate, and interestingly, serum alanine was elevated in eight out of nine patients evaluated. This probably reflects, more than the altered NADH:NAD flux, the disruption of the Krebs

cycle which causes the accumulation of pyruvate that can be reversibly converted to lactate or to alanine (Zschocke et al. 2004). Similarly, the increase of fumarate (7/9) and malic acid (4/9) can be explained by the fact that they are intermediate products generated by pyruvate carboxylase during pyruvate metabolism. Actually, although an increased urinary excretion of organic acids (independent of tubulopathy) has already been reported (Atale et al. 2009), this was not specifically focused on fumaric acid. Based on the above findings, it is important to highlight that both increased alanine serum levels and fumaric acid urinary excretion, even though not specific markers (since they can also be present in other mitochondriopathies (Broomfield et al. 2015), can be helpful above all for the differential diagnosis from other non-mitochondrial bone marrow failures.

Vacuoles of myeloid or erythroblastic progenitors in the BM, as well as the presence of ringed sideroblasts after Perls staining, are highly suggestive of PS (Pearson et al. 1979): in our series vacuolization was observed in 8/11 patients and Perls staining was positive in 6/7 patients.

For the 11 medical doctors taking care of the patients during the diagnostic phase, Diamond–Blackfan anemia (DBA) was the most frequent differential diagnosis (9/11 physicians); 2/11 initially suspected a "metabolic disease." In a recent series of DBA patients (Gagne et al. 2014), a small percentage was found to be affected by PS: adenosine deaminase (ADA), typically high in DBA patients, can be of no help for differential diagnosis according to our data, since its elevation, already described in at least one report (Superti-Furga et al. 1993), was present in two out of four patients evaluated. Notwithstanding, we think that, also based on the findings of the present analysis, clinical and biochemical elements other than ADA can be helpful: above all, neurological symptoms and vacuoles in BM, together with increased levels of serum lactate, serum alanine, and urine fumarate, can be decisive in the differential diagnosis from DBA.

PRBC transfusion independency, occasionally reported in other papers (Muraki et al. 1997), was achieved by eight out of nine evaluable patients, and consequently it seems to be highly probable in the case of survival after the first 2–3 years of life.

PS is usually reported to lead to premature death even though survival up to young adulthood has occasionally been reported (Muraki et al. 1997). Our findings, with eight deaths/ten patients, confirm that the prognosis is dismal, with mortality more common at 5–11 years of age (five out of eight evaluable patients), whereas in other older series, more than 50% of patients died before 3.5 years of life (Rötig et al. 1995). Treatments other than supportive therapy and transfusions appear to be of limited utility: administration of bicarbonate can control metabolic acidosis, and episodic treatments with GCSF can reverse infections in the course of severe neutropenia. In our cohort, similarly to other recent series (Broomfield et al. 2015), the general improvement of supportive therapy probably explains the improvement in survival.

Conclusion

We report a reasonably sized cohort of PS, compared to those described previously. Our report is based on an accurate survey among physicians of the national A.I.E.O.P. network and enables us to estimate the likely incidence of this disease in our country as being about 1/million newborns. Moreover, our study highlights some clinical insights and a biochemical profile characterized by increased serum alanine and urinary fumaric acid that can be helpful in the initial diagnostic work-up.

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Take-Home Message

PS is a severe mitochondrial disorder characterized by increased alanine and lactate serum levels and fumaric acid urinary excretion and a frequent recovery of bone marrow (BM) in the case of survival after the first 2–3 years of life.

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Compliance with Ethical Guidelines

Conflict of Interest

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Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Contributor's Statements

Drs. Farruggia and Di Cataldo conceptualized and designed the study and the data collection instruments. Drs. Farruggia, Pillon, and Dufour carried out the initial analyses and drafted the initial manuscript. Drs. Farruggia, Puccio, and Macaluso coordinated data collection and performed the statistical analysis. Drs. Macaluso, Palmisani, Pinto, Lo Valvo, Cantarini, Tornesello, Corti, Fioredda, Varotto, Martire, Russo, and Moroni contributed to the enrollment of patients, diagnosis, and sample collection.

All authors critically reviewed the paper, approved the final manuscript as submitted, and agreed to be accountable for all aspects of the work.

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RESEARCH REPORT

Bioimpedance Analysis as a Method to Evaluate the Proportion of Fatty and Muscle Tissues in Progressive Myopathy in Pompe Disease

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Abstract During progressive myopathy, the space of atrophic muscle tissue is gradually filled by fatty tissue. The proportion of these two tissue types relative to body mass provides an indication of the extent of muscle tissue destruction, i.e., the progression and severity of the disease.

In this study we use Pompe disease as an example to report the new possibility of using bioimpedance analysis (BIA) to assess the relative proportion of fatty and muscle tissue in diseases associated with muscle atrophy, thus enabling the assessment of disease progression and the effectiveness of treatment. Results from BIA analysis were compared with magnetic resonance images.

The results of muscle magnetic resonance images and BIA analysis were similar, which suggests that BIA may provide valuable diagnostic guidance for the assessment of the progression of the disorder.

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Introduction

Bioimpedance analysis (BIA) is a noninvasive testing method allowing for accurate analysis of body composition using electrical resistance of various tissues of the body: so-called impedance (Lewitt et al. 2010). It uses the ability of the muscle tissue to conduct electrical current. The test measures impedance, i.e., electrical resistance and reactance of the tissues through which low-intensity electrical current is passed ($\leq$1 mA). The phenomenon of resistance is associated with resistivity of specific tissues, while reactance is mainly due to the electrical capacitance of cell membranes, which, due to their structure, act as capacitors (Lewitt et al. 2010). The body composition assessment method based on electrical bioimpedance analysis is used in medicine, mainly to assess the risk and degree of overweight and obesity, where the fatty tissue to body weight ratio is estimated. Researches which based on the noninvasive measurements of the electrical impedance characteristics in medicine are more frequent: BIA was used in comparison of segmental with whole-body impedance measurements in peritoneal dialysis (Nescolarde et al. 2008). Electrical impedance myography (EIM) is a noninvasive technique for the assessment of neuromuscular health of individual muscles or groups of muscles and was used in the assessment of boys with Duchenne muscular dystrophy (Rutkove et al. 2014).

In diseases such as myopathies, muscle cells die with the progress of the disease, and the atrophic muscle tissue is replaced by the fatty tissue (Mercuri et al. 2002a). Muscle atrophy gradually leads to loss of function of the motor system and the respiratory muscles (Pellegrini et al. 2005; Gaeta et al. 2013). The dynamics of the atrophic process in myopathies is usually assessed on the basis of functional tests. In some cases, magnetic resonance imaging allows an

approximate evaluation of the relative proportions of the muscle and fatty tissues in the selected segments of the whole body (Pellegrini et al. 2005; Gaeta et al. 2013; Pichiecchio et al. 2004).

In this study, BIA was obtained in patients with Pompe disease (glycogen storage disease type II, GSD II). Pompe disease is an autosomal-recessive metabolic myopathy, caused by deficiency of the lysosomal acid α-glucosidase. The deficiency of this enzyme leads to an accumulation of glycogen taking place mainly in muscles and leading to their progressive destruction. The spectrum of clinical phenotypes includes infantile-onset form, juvenile form, and late-onset form. In the classic form (infantile onset), the first symptoms appear in the first months of life with hypotonia, muscle weakness, and cardiomegaly. In juvenile forms, the first symptoms, including progressive proximal and axial muscle weakness, appear between 2 and 5 years of age.

The late-onset form has a slower progression. Skeletal muscle involvement is more prominent with a predilection for the lower limbs. Late-onset features include hypotonia and progressive muscle weakness – mainly in respiratory muscles, leading in advanced stages to ventilator-assisted breathing.

In Pompe disease, the main affected tissue is muscle tissue – as the disease progresses, this tissue becomes atrophic and is replaced by fatty tissue (Del Gaizo et al. 2009; Angelini et al. 2013; Alejaldre et al. 2012).

This study is innovative because BIA was used for the first time to assess the relative proportions of the fatty and muscle tissues in diseases associated with muscle atrophy, thus enabling the assessment of disease progression and the effectiveness of treatment.

Material and Methods

Study Subjects

The study was conducted in The Children's Memorial Health Institute in a group of 20 patients with Pompe disease (10 women and 10 men) aged from 6.7 years to 52 years (mean age: 27.7 years). All the patients had a diagnosis of Pompe disease confirmed by biochemical and molecular analysis (1 infantile-onset form; 8 juvenile form; 11 late-onset form) (Table 1). In 20 patients, body composition analysis by electrical bioimpedance method (BIA) using an InBody J10 (Biospace) device was conducted. Impedance measurements were performed by using 3 different frequencies 5, 50, and 250 kHz at each 5 segments of the body. In 17 patients, MRI images of the thigh muscles were obtained using a 1.5 T scanner (Siemens Sonata Vision) using Axial T1-weighted spin echo sequences with TR = 300 ms, TE = 10 ms. Slice thickness was equal 10 mm, gap 1 mm and FOV 400 mm.

Study Design

As the disease progresses, the space of atrophic muscle tissue is gradually filled by the fatty tissue. The relative to body mass proportions of these two tissue types are allowed to determine the extent of muscle tissue destruction, i.e., the progression and severity of the disease.

The study objective was to use the BIA analysis method to determine the proportion of the amount of the fatty and muscle tissues in patients with progressive myopathies. This is allowed to evaluate the severity of the disease.

BIA Analysis

The bioimpedance method assesses the tissue components of the body, involving a measurement of electrical resistance in the human body. Resistance varies depending on the composition of the subject's body. The assessment of body composition and the proportion between fatty and muscle tissues is obtained after entering data on age and gender; the device measures body height and weight and performs measurements of resistance and reactance. The test provides information on body composition: body water (l); the amount of protein (kg); the amount of mineral substances (kg); fatty tissue mass (kg); and % of body fat of the whole body weight, skeletal muscle mass (kg), and the distribution of fatty and muscle tissues in various segments of the body. The measurement was performed in a patient in their underwear, according to the instructions of measurement. Criteria of patient inclusion in the study: Pompe disease confirmed by an enzymatic assay, age: min. 3 years, body weight 10–250 kg, height: 95–220 cm. Study exclusion criteria are inability of standing without assistance, implanted pacemaker, pregnancy, or breastfeeding. To observe the changes in the composition of the human body, the analyses should be performed in comparable measurement conditions as far as possible; therefore, the subject should not exercise before the measurement, should not be measured immediately after a meal (min. 2 h), should not take a bath immediately before the measurement, and should be measured after a bowel movement, and the measurements should be performed before noon. The patient assumed a standing position, with the head remaining in the Frankfurt plane. Both hands held the hand electrode rods in such a manner that four fingers were placed on the surface of the electrode, and the thumb was placed on the electrode button. Bare feet were placed on the electrodes in such a way that the heels touched the peripheral electrode, and the sole of each foot remained on the elliptical surface. The results were shown as a graph,

Table 1 Patients characteristics

Patient	Gender	Curent age	Age of first symptoms (years)	Age of diagnosis (years)	Mutation	Form
1	M	6.7	No symptoms, family screening	6/12	G377S/c.2495_2496 delCA	Juvenile
2	F	6.8	6/12	2	L291F, 871C>T/R600C, 1798C>T	Invant
3	F	10	No symptoms, family screening	2	IV1-13T>G/c.2662G>T	Juvenile
4	F	13.2	3	4	C1129G>A/c.2495_2496 delCA	Juvenile
5	M	13.7	No symptoms, family screening	6	IVS1-13T>G/c.2662G>T	Juvenile
6	M	14.5	3.5	4	IVS1-13T>G/c.925G>A	Juvenile
7	F	15.9	>1	2.5	IVS1-13T>G/c.307T>G	Juvenile
8	F	16	8	11	IVS1-13T>G/C103G	Juvenile
9	M	23	6	15	IVS1-13T>G/c.2662G>T	Juvenile
10	F	29	7	25	IVS1-13T>/C103G	Late onset
11	F	32	16	30	IVS1-13T>G/c.307T>g	Late onset
12	M	33	15	25	IVS1-13T>G/c.307T>G	Late onset
13	M	36	27	29	c.364A>G/c.1796C>T	Late onset
14	F	36	25	34	IVS1-13T>G/C103G, 307T>G	Late onset
15	M	36	18	34	IVS1-13T>G/c.307T>g	Late onset
16	F	38	6	31	c.364A>G/c.1796C>T	Late onset
17	M	45	35	40	IVS1-13T>G/ -	Late onset
18	M	47	35	46	IVS1-13T>G/c.307T>G	Late onset
19	M	51	33	48	IVS1-13T>G/C103G, 307T>G	Late onset
20	F	52	>30	46	IVS1-13T>G/525delT	Late onset

with values given in kilograms and percentages. References used in software are standard range of PBFs which are 10-20% of standard body weight for men and 18–28% of standard body weight for women. For patients under 18 years of age InBody J10 uses standard PBF for appropriate age classes (Heyward and Stolarczyk 1996; Lohman 1992; Tahara et al. 2002; Fomon et al. 1982). In Body J10 software, standard skeletal muscle mass (kg) is estimated with the assumption: 40–47% for boys and men and 40–42% for girls and women of standard body weight (Heymsfield et al. 1990; Ito et al. 2001).

According to the Cole classification of BMI (Cole et al. 2007), two patients had severe thinness, 2 patients were underweight, 13 patients had a normal range of BMI, and 3 patients were overweight. *Z*-score values were calculated for each patient for the body weight, muscle tissue, and body fat.

For BIA the following scale was used. This scale was invented by the authors for the purposes of this study. “Mild” indicates when body weight (kg) is normal with muscle tissue (kg) and percentage of body fat (PBF %) within normal limits or if body weight is below normal and both muscle and PBF are below standard charts. “Moderate” indicates when body weight is normal but there is a disproportion between muscle tissue and adipose tissue, for example, muscle tissue is normal and PBF is above the norm or when muscle tissue is below normal and PBF is normal. “Severe” indicates when weight is normal or above normal, muscle tissue is below normal, and fat tissue is above normal.

MRI Analysis

MRI images of the thigh were obtained using a 1.5 T scanner (Siemens Sonata Vision). In Pompe disease, the imbalance between muscle tissue and fatty tissue is mainly visible in the thighs (Alejaldre et al. 2012) because the thigh has a simple geometry and tissue composition. Each muscle group was graded according to the degree of degeneration using the scale proposed by Mercuri (Mercuri et al. 2002b): “mild” if only traces of increased signal intensity could be observed in an otherwise well-preserved muscle; “moderate” if less than 50% of the muscle showed an increased signal intensity; and “severe” if at least 50% showed increased signal intensity.

Table 2 BIA and MRI results

				BIA			
Patient	Age (years)	Gender	Curent age	Body mass (kg) [references]	Muscle tissue (kg) [references]	Percent body fat [references]	BIA (scale)	MRI (scale)
1	6.7	M	6.7	24.1 [21.2–28.6]	9.4 [9.4–11.6]	16.1 [10–20]	Mild	Mild
2	6.8	F	6.8	24.9 [22.2–30.0]	8.7 [9.6–11.8]	25.8 [12–22]	Severe	–
3	10	F	10	36.3 [39.6–53.6]	15.5 [17.9–21.9]	17.7 [16–26]	Mild	Mild
4	13.2	F	13.2	40.5 [45.5–61.5]	15.4 [20.3–24.8]	25.1 [18–28]	Moderate	Mild
5	13.7	M	13.7	58.1 [60.2–81.4]	29 [30.4–37.2]	9.5 [10–20]	Mild	Mild
6	14.5	M	14.5	60.9 [54.6–73.8]	30.4 [27.5–33.5]	11.9 [10–20]	Mild	Mild
7	15.9	F	15.9	51 [48.1–65.1]	19.1 [21.5–26.3]	28 [18–28]	Severe	Severe
8	16	F	16	55.7 [47–63.6]	21.9 [21–25.6]	27.8 [18–28]	Mild	Mild
9	23	M	23	39.4 [54–73]	20.2 [27.1–33.1]	3 [10–20]	Not classified	Moderate
10	29	F	29	56.6 [48.1–65.1]	19.8 [21.5–26.3]	34.4 [18–28]	Severe	–
11	32	F	32	55.4 [53.7–72.7]	20.7 [24.2–29.6]	29.8 [18–28]	Severe	Moderate
12	33	M	33	68.9 [61.3–82.9]	30 [31–37.8]	20.9 [10–20]	Moderate	Severe
13	36	M	36	59.7 [50.6–68.4]	25.9 [22.7–27.7]	20.9 [18–28]	Mild	Mild
14	36	F	36	38.1 [45.9–62.1]	15.3 [20.4–25]	22.3 [18–28]	Not classified	Moderate
15	36	M	36	85.4 [63–85.2]	28.3 [31.9–38.9]	39.1 [10–20]	Severe	Severe
16	38	F	38	73.5 [57–72.2]	28 [25.8–31.6]	29.6 [18–28]	Moderate	Moderate
17	45	M	45	81 [57.2–77.4]	30.8 [28.8–35.2]	30.7 [10–20]	Moderate	Severe
18	47	M	47	71.6 [56.9–76.9]	24.8 [28.6–35]	35.8 [10–20]	Severe	–
19	51	M	51	76.4 [57.7–78.1]	31.5 [29.1–35.5]	27 [10–20]	Moderate	Moderate
20	52	F	52	72.4 [50.4–68.2]	22.8 [22.6–27.6]	42 [18–28]	Severe	Severe

Ethical Consideration

The protocol was approved by the Human Subjects Institutional Review Board at the Children's Memorial Health Institute. Written informed consent had to be provided by the parents or legal guardians. The study was designed and conducted in compliance with the principles of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guidelines for Good Clinical Practice.

Results

BIA

BIA results including the body mass, the muscle tissue mass, and percentage of adipose tissue together with the reference range (WHO reference charts) for each parameter are shown in Table 2. Six of the 20 patients were classified as "mild" form, five as "moderate" form, and seven as "severe" form. Two patients were not classified in any of the groups due to low body weight and inconclusive test results (Fig. 1). For example, patient 14 should have put on 16 kg but 12 kg of this was muscle tissue and only 4 kg was adipose tissue. Thus, even at this stage, there was a visible disproportion between adipose tissue and muscle tissue.

MRI

Muscle groups were graded according to the degree of degeneration using the scale proposed by Mercuri (Mercuri et al. 2002b). Figure 2 shows examples of MRI. Seven of 17 patients were classified as "mild" form, five as "moderate" form, and five as "severe" form. Because of equipment malfunction, MRI images were not obtained for three patients.

BIA vs. MRI

In 12 cases the results of BIA analysis and MRI image were accordance and were classified to the same grade; only in 3 cases the results were not accordance and were graded into different classes of severity of the disease. In 2 cases analysis for BIA was not unequivocal, the

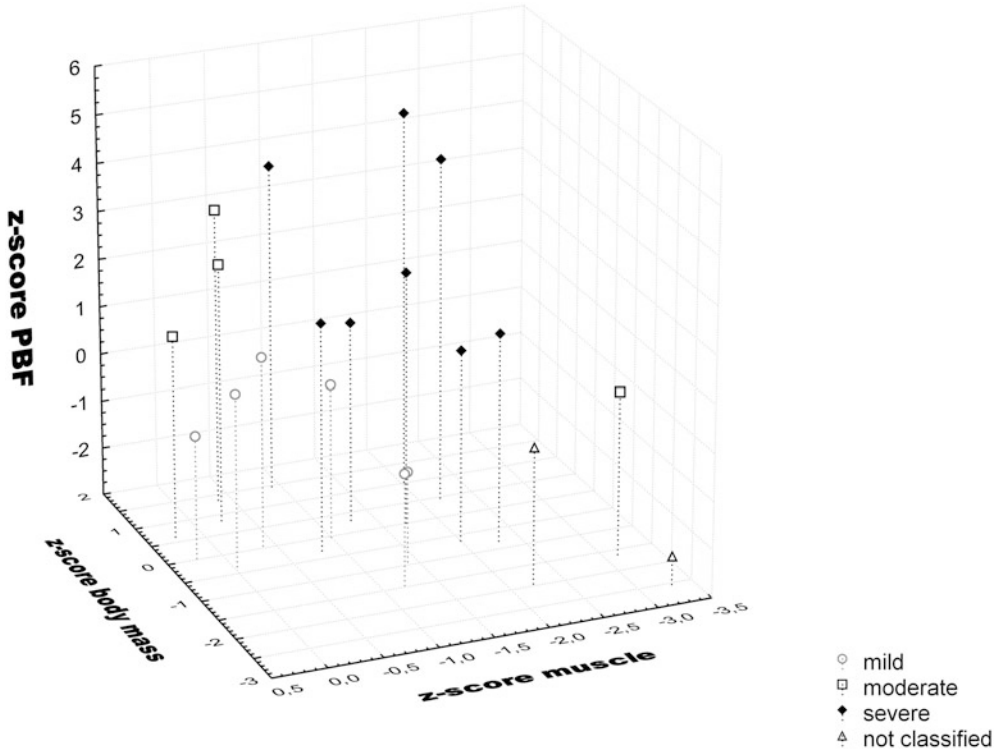

Fig. 1 Relation between muscle tissue, body mass and body fat in *z*-score values

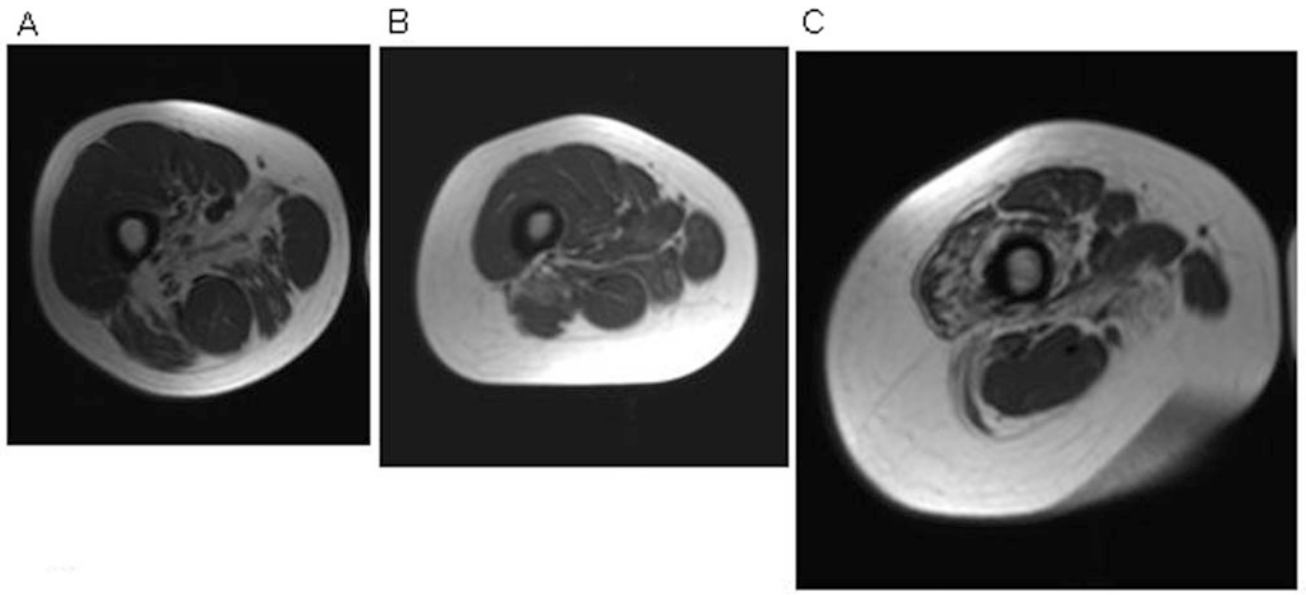

Fig. 2 Examples of MRI. (**a**) Mild; (**b**) moderate; (**c**) severe

body weight was considerably below the standard charts, analysis showed both the scarcity of fat tissue and muscle tissue, and therefore, it was difficult to classify these patients to one of the groups. MRI was not performed in 3 patients.

Discussion

In recent years, several studies have reported the use of muscle MRI as a useful noninvasive technique for the assessment of neuromuscular disorders (Mercuri et al. 2002a;

De Jager et al. 1998; Paradas et al. 2010; Fisher et al. 2005; Wattjes et al. 2010). In this study, the authors introduce a completely new, simple to perform, and reliable diagnostic method to assess the progress of the muscle atrophy process in myopathies. The accuracy of BIA is comparable with magnetic resonance (Salinari et al. 2002). BIA is much cheaper, faster, and easier to perform. The use of BIA to assess the relative proportions of fatty and muscle tissues in diseases associated with muscle atrophy is an innovative method not previously used in myopathies. The results of our study show that there is a correlation between the percentage of body fat by segment and the degree of replacement of the muscle tissue with fatty tissue, as shown in MRI. As the disease progresses, the percentage of body fat increases and reduces the percentage of muscle tissue. Furthermore, it correlates with the age of the patients and the severity of the disease evaluated clinically. The results prove the high sensitivity of the BIA method and suggest great potential for using electric bioimpedance analysis as a cognitive tool enabling the assessment of the proportion of fatty and muscle tissues in progressive myopathies as well as the potential for using the device in diagnostics. Notwithstanding this, a detailed study is required involving a larger group of subjects with progressive myopathies. The device for bioimpedance measurement used in this study allowed for measurements only in the standing position, but there are devices available that can perform measurements in the supine position. This could be useful in patients with severe muscle atrophy preventing them from moving without assistance. BIA devices allow for accurate measurement of bioimpedance for five body segments, four limbs and the trunk; they are based on the technology of the 8-point tactile electrode system, ensuring accuracy and reproducibility of measurements using eight different points of measurement: the thumb and fingers of both hands and the front and back parts of the soles of both feet (Kaido et al. 2013).

The scale developed by the authors needs to be verified with more tests. An interpretation of the results of analysis for patients with body weight significantly below the reference charts appears to be problematic. In cases where a body weight was considerably below the standard charts, analysis showed both the scarcity of fat tissue and muscle tissue, and therefore, it was difficult to classify patients into one of the groups.

Conclusions

Loss of muscle tissue in progressive myopathies is associated with an increased fat amount in the muscles. Measurement of the proportion of these two tissues allows to specify the degree of atrophy of skeletal muscles and thus the degree of severity of the disease. Sensitive and reliable tool for this purpose is the BIA as it was demonstrated on the example of testing patients with varying degrees of severity of Pompe diseases. BIA because of their sensitivity, simplicity, and cheapness can be used to assess muscle atrophy in other myopathies.

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Compliance with Ethics Guidelines

Conflict of Interest

Agnieszka Różdżyńska-Świątkowska, Elżbieta Jurkiewicz, and Anna Tylki-Szymańska 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 (5). Informed consent was obtained from all patients for being included in the study.

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CASE REPORT

Infantile Refsum Disease: Influence of Dietary Treatment on Plasma Phytanic Acid Levels

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Abstract Infantile Refsum disease (IRD) is one of the less severe of Zellweger spectrum disorders (ZSDs), a group of peroxisomal biogenesis disorders resulting from a generalized peroxisomal function impairment. Increased plasma levels of very long chain fatty acids (VLCFA) and phytanic acid are biomarkers used in IRD diagnosis. Furthermore, an increased plasma level of phytanic acid is known to be associated with neurologic damage. Treatment of IRD is symptomatic and multidisciplinary.

The authors report a 3-year-old child, born from consanguineous parents, who presented with developmental delay, *retinitis pigmentosa*, sensorineural deafness and craniofacial dysmorphisms. While the relative level of plasma C26:0 was slightly increased, other VLCFA were normal. Thus, a detailed characterization of the phenotype was essential to point to a ZSD. Repeatedly increased levels of plasma VLCFA, along with phytanic acid and pristanic acid, deficient dihydroxyacetone phosphate acyltransferase activity in fibroblasts and identification of the homozygous pathogenic mutation c.2528G>A (p.Gly843Asp) in the *PEX1* gene, confirmed this diagnosis. Nutritional advice and follow-up was proposed aiming phytanic acid dietary intake reduction. During dietary treatment, plasma levels of phytanic acid decreased to normal, and the patient's development evaluation showed slow progressive acquisition of new competences.

This case report highlights the relevance of considering a ZSD in any child with developmental delay who manifests hearing and visual impairment and of performing a systematic biochemical investigation, when plasma VLCFA are mildly increased. During dietary intervention, a biochemical

improvement was observed, and the long-term clinical effect of this approach needs to be evaluated.

Introduction

Infantile Refsum disease (IRD; OMIM#601539) is the less severe form of Zellweger spectrum disorders (ZSDs), which are a continuum of phenotypes with clinical, biochemical and genetic overlap – Zellweger syndrome, neonatal adrenoleukodystrophy and IRD. Presenting neonatally with hypotonia, large fontanels, failure to thrive and cholestatic jaundice, IRD is later recognizable by developmental delay, *retinitis pigmentosa*, sensorineural deafness, hepatomegaly, growth retardation and milder dysmorphic features (Baumgartner et al. 1998; Poll-The et al. 2004). The disease progresses slowly and some patients may survive into adulthood, depending on the level of clinical care (Poll-The et al. 2004; Crane et al. 2005).

The IRD diagnosis is made by a combination of clinical manifestations with biochemical and molecular testing, as well as macro- and microscopic examinations and immunocytochemistry analysis of peroxisomes (Aubourg and Wanders 2013). Diagnostic tools rely on the fact that IRD is an autosomal recessive peroxisomal biogenesis disorder (PBD) caused by pathogenic variants in the *PEX* genes, which encode peroxins that are essential to peroxisomal assembly and the protein import system, resulting in none or a few abnormally formed peroxisomes, whose functions are generally impaired (Aubourg and Wanders 2013). Since very long chain fatty acids (VLCFA) beta-oxidation is a unique metabolic function of peroxisomes, the identification of an increased level of VLCFA in plasma, fibroblasts and amniotic fluid cells is a biomarker of ZSD (Wanders 2014). Elevated plasma levels of phytanic and pristanic acids and bile acid precursors (BAP), as well as reduced plasmalogen levels in erythrocytes, are additional biochemical abnormalities that point to a ZSD (Wanders 2014). A reduced dihydroxyacetone phosphate acyltransferase (DHAP-AT) activity in fibroblasts and amniocytes confirms the postnatal and prenatal diagnosis of ZSD (Wanders et al. 1995). Finally, identification of pathogenic mutations in a *PEX* gene (http://www.dbpex.org) is useful for diagnosis, prognosis, and management of a ZSD, but it also enables carrier testing of at-risk relatives and prenatal or preimplantation diagnosis (Ebberink et al. 2011).

Management of IRD is multidisciplinary and treatment remains symptomatic (Braverman et al. 2013). Reports of treatment impact in disease progression are limited. However, sporadically reports have showed that changes in diet lead to specific biochemical effects (Robertson et al. 1988; Moser et al. 1999).

Herein, we describe an IRD patient with mild biochemical phenotype who had a reduction of plasma phytanic acid levels after onset of dietary management. Slight developmental progress was observed and retinopathy did not evolve significantly during follow-up.

Methods

The following peroxisomal biochemical analyses were performed, as previously reported, with minor modifications: plasma and fibroblast VLCFA quantification (Moser et al. 1999), plasma phytanic and pristanic acid level quantification (Dacremont et al. 1995), fibroblast DHAP-AT enzymatic activity measurement (Wanders et al. 1995), erythrocyte plasmalogen level quantification (Bjorkhem et al. 1986), plasma BAP measurement (Shimada et al. 2001), plasma polyunsaturated fatty acid level measurement (Bailey-Hall et al. 2008) and antibody anti-catalase immunofluorescence labelling (Wanders et al. 1989). Genomic DNA was extracted from peripheral blood, using Qiagen BioRobot EZ1 apparatus with EZ1 DNA blood 350 µl kit (according to the manufacturer's instructions). PCR products of all *PEX1* exons and flanking regions (reference number, NM_000466.2) were analysed by Sanger sequencing using BigDye Terminator v1.1 Cycle Sequencing Kit and 3130xl Genetic Analyzer (Applied Biosystems) (PCR primers and conditions are available upon request).

The nutritional management to reduce phytanic acid intake from the diet consisted in decreasing global fat intake, mainly from ruminant meats, dairy products and high fat content fish. Griffiths Mental Development Scales were used to evaluate psychomotor development.

Results

A 3-year- and 6-month-old girl was referred to the Medical Genetics outpatient clinic due to *retinitis pigmentosa*, sensorineural hearing loss (SNHL), developmental delay and dysmorphic craniofacial features (Fig. 1a). She was born from consanguineous healthy parents, who were first cousins, and she had two older twin brothers, who manifested hypotonia and psychomotor developmental delay associated with extreme prematurity. During gestation, nuchal translucency above the 95th percentile for gestational age prompted a fetal karyotype and a fetal echocardiography, which were unremarkable. She was born at 38 weeks, and, in the neonatal period, hypotonia and jaundice were diagnosed. Low-amplitude and high-frequency bilateral vertical nystagmus was first noticed at 3 months of age. At 6 months, bilateral pigment deposits became apparent in the retinal mid-periphery of both ocular fundi. The following months, these pigment deposits assumed round circular shapes instead of the more typical bone spicule form and approached the posterior pole, while the maculas acquired

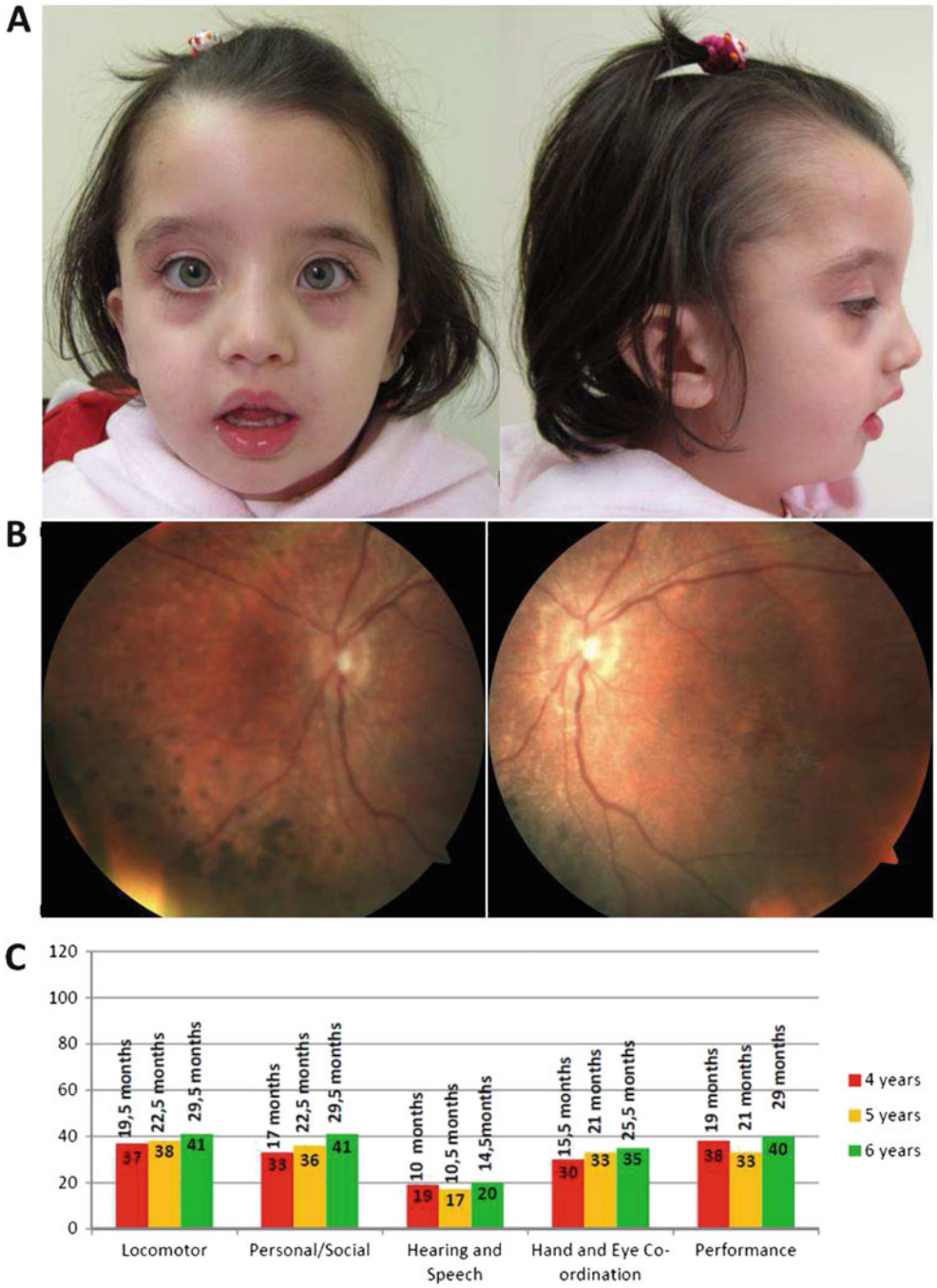

Fig. 1 (**a**) Facial dysmorphic features observed in the proband, at 4 years old, included high forehead, absent orbital ridges, and micrognathia. (**b**) Bilateral retinographies, obtained at 4 years old, showing diffuse pigment epithelium changes in the retinal mid-periphery, as well as mottled appearance in the macular area, compatible with *retinitis pigmentosa*. (**c**) Performance level (age of development in months) in five subscales of the Griffiths Mental Development Scales at 4, 5 and 6 years of age

a salt and pepper appearance and eventually pigment scattering (Fig. 1b). Electroretinography was performed according to ISCEV standards at 4 years of age and demonstrated an extinguished rod response with a diminished maximum combined rod-cone response, compatible with *retinitis pigmentosa*. Bilateral and symmetric hyperopic astigmatism was also diagnosed and is currently corrected with glasses. Due to intellectual disability, visual acuity could not be reliably tested. Despite the severe and generalized photoreceptor dysfunction, both parents and other caretakers report good functional vision for indoor and outdoor activities, without tripping or collisions that

suggested a markedly reduced visual field, and do not report significant limitations in scotopic conditions (nyctalopia), typical of severe *retinitis pigmentosa*. Both fundoscopic lesions and hyperopia remained stable during the last 3 years of follow-up. Cerebral MRI did not show brain abnormalities at 10 months old. Seizures were not reported. Profound bilateral SNHL at frequencies 2,000–4,000 Hz was confirmed at 2 years and 11 months old, by auditory evoked potentials. However, conditioned play audiometry showed a 40 dB loss at 250–2,000 Hz bilaterally with the use of hearing aids, placed at approximately 3 years old, and an increased social interaction was concomitantly observed. She seated alone at 8 months and started walking at 24 months. At 4 years, developmental evaluation showed a severe developmental delay across all areas (GQ = 31) (Fig. 1c). Height and weight progressed along the 5th percentile curve, while head circumference was growing along the 25th percentile curve. At 3 years old, electrocardiography was normal and echocardiography showed patent *ductus arteriosus* with small left-right shunt but good biventricular function.

Diagnosis of ZSD was considered, due to hearing and visual impairment associated with developmental delay and mild dysmorphic features. At 16 months, quantification of plasma levels of VLCFA did not clearly support this diagnosis, since C26:0 and C24:0/C22:0 ratios were within the normal range and the C26:0/C22:0 ratio was slightly increased (Table 1). However, at 33 years and 11 months old, increased plasma levels of VLCFA in combination with increased phytanic acid and pristanic acid levels supported the hypothesis of a peroxisomal disorder, either a ZSD or a D-bifunctional protein deficiency. The decreased activity of DHAP-AT in fibroblasts was in accordance with the diagnosis of a ZSD. A pathogenic homozygous missense mutation c.2528G>A, p.(Gly843Asp), was detected in the *PEX1* gene, establishing a ZSD diagnosis for this patient. Parents are carriers of the same pathogenic variant. Catalase immunofluorescence analysis showed a punctuate fluorescence pattern in control cells, due to the catalase presence within the peroxisome compartment. In this patient, however, the catalase is predominately scattered in the cytosol, and this leads to a diffuse rather than a punctuate fluorescence pattern (Fig. 2). From this spectrum, considering the clinical phenotype, this patient can be classified as an IRD.

After diagnosis, nutritional advice and follow-up was proposed. In the first nutritional appointment, at four years and two months old, the fat intake recorded was 29% of the total energy intake (1800 kcal/day). The nutritional prescription reduced the total of fat into 15% of the total energy intake. Diet was also supplemented with maltodextrins, in order to prevent catabolism, resulting in a higher total energy intake (1900 kcal/day). At 7, 12, 18 and 25 months after starting the dietary intervention, plasma levels of phytanic acid lowered to normal range. The subsequent nutritional appointments confirmed the excellent compliance with the proposed diet. The successive developmental evaluations revealed slight progress in all evaluated areas (at 6 years, GQ = 34), most significantly in locomotor and autonomy/sociability (Fig. 1c). However, her performance was significantly conditioned by a short attention span in more structured tasks.

Discussion

We report a 3-year-old child with IRD, in whom a thorough characterization of the clinical phenotype was critical to support the hypothesis of a peroxisomal disorder, in face of first-tier mild biochemical abnormalities. IRD was considered due to developmental delay associated with hearing loss and progressive retinopathy, since facial dysmorphic features were misleading. Although neurological dysfunction observed in the neonatal period resembled a neuromuscular disorder, later in life, our patient's phenotype also resembled Usher type II, congenital defects of glycosylation or mitochondrial respiratory chain defects. Diagnosing IRD may be complicated due to its phenotypic as well as genotypic heterogeneity, leading eventually to an underestimation of its true prevalence. Since the onset and severity of manifestations is variable and numerous clinical differential diagnoses exist, a clinical suspicion of IRD – based on neurologic, developmental and sensory deficits – should prompt a systematic biochemical investigation of PBD (Baumgartner et al. 1998), including measurements in plasma, erythrocytes and skin fibroblasts, to show defects in the α-oxidation, β-oxidation and synthesis of ether phospholipids.

The repeated measurement of plasma VLCFA, along with phytanic acid and pristanic acid, proved valuable in the identification of a ZSD in this specific patient, which was subsequently confirmed by analysis of the DHAP-AT activity in fibroblasts. Biochemical diagnosis of IRD may be challenging in patients with normal or mildly increased plasma VLCFA levels (Gootjes et al. 2004; Zeharia et al. 2007). Indeed, older individuals were shown to have lower plasma ratios of C24:0/C22:0 and C26:0/C22:0 fatty acids when compared with children under one year (Hall et al. 1988). Since normal VLCFA plasma levels do not exclude the diagnosis of IRD, this clinical case highlights the need of repeated plasma VLCFA measurements or analysis of additional biochemical parameters, including plasma phytanic acid or erythrocyte plasmalogens, to increase the diagnostic rate of IRD patients (Krause et al. 2009).

The mild clinical and biochemical phenotype in this patient is likely to be explained by the type of mutation

Table 1 Biochemical characterization of the proband with the pathogenic variant c.2528G>A (p.Gly843Asp), in homozygosity, in the *PEX1* gene

Age at metabolite measurement[a]		Measurements before diagnosis	Measurements at diagnosis		Measurements performed after onset of dietary management			
		1 Y + 4 m	3 Y + 11 m	4 Y + 1 m	4 Y + 10 m	5 Y + 3 m	5 Y + 9 m	6 Y + 4 m
Plasma	Reference values							
VLCFA								
C26:0 (μg/ml)	0.16–0.57	0.57 (N)	0.64 (↑)		0.61 (↑)	0.61 (↑)	0.61 (↑)	0.61 (↑)
C24:0/C22:0	0.63–1.10	1.04 (N)	1.14 (↑)		1.22 (↑)	1.07 (N)	1.05 (N)	1.10 (N)
C26:0/C22:0	0.004–0.022	0.036 (↑)	0.052 (↑)		0.074 (↑)	0.044 (↑)	0.034 (↑)	0.056 (↑)
Phytanic acid (μg/ml)	0–3.1		12 (↑)		1.5 (N)	1.4 (N)	0.6 (N)	0.7 (N)
Pristanic acid (μg/ml)	0–0.9		2.4 (↑)		0.6 (N)	0.5 (N)	0.1 (N)	
Aspartate transaminase (U/ml)	10–30		54 (↑)			42 (↑)		64 (↑)
Alanine transaminase (U/ml)	10–36		24 (N)			27 (N)		32 (N)
Bile acid precursors								
DHCA (μg/ml)	0–0.01		0.24 (↑)		0.12 (↑)	0.04 (↑)		
THCA (μg/ml)	0–0.03		0.93 (↑)		0.68 (↑)	0.62 (↑)		
DHCA/CDCA	0–0.02		0.77 (↑)		0.20 (↑)	0.27 (↑)		
THCA/CA	0–0.04		5.6 (↑)		2.35 (↑)	0.82 (↑)		
PUFA								
Arachidonic acid (C20:4n-6) (μg/ml)	105–244		131 (N)		206 (N)		170 (N)	178 (N)
EPA (C20:5n-3) (μg/ml)	4.4–30		13 (N)		10 (N)		12 (N)	8.6 (N)
DHA (C22:6n-3) (μg/ml)	25.9–84.2		22.9 (↓)		49.5 (N)		15.9 (↓)	23.1 (↓)
Erythrocytes								
Plasmalogens[b]								
C16:0DMA/C16:0	0.042–0.103				0.073 (N)			
C18:0DMA/C18:0	0.115–0.193				0.116 (N)			
Fibroblasts								
VLCFA								
C26:0 (μg/mg protein)	0.03–0.18			0.36 (↑)				
C24:0/C22:0	0.95–2.75			2.36 (N)				
C26:0/C22:0	0.03–0.17			0.77 (↑)				
DHAP-AT (nmol/2 h/mg protein)	3.4–16			1.6 (↓)				

VLCFA very long chain fatty acids, *DHCA* dihydroxy cholestenoic acid, *THCA* trihydroxy cholestenoic acid, *CDCA* chenodeoxycholic acid, *CA* cholic acid, *PUFA* polyunsaturated fatty acids, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *DMA* dimethyl acetal, *DHAP-AT* dihydroxyacetone phosphate acyltransferase

[a] Age is reported in years (Y) and months (m)

[b] Plasmalogen values in erythrocytes may normalize with age. ↑, above maximum reference value. ↓, below minimum reference value. N, between minimum and maximum reference values

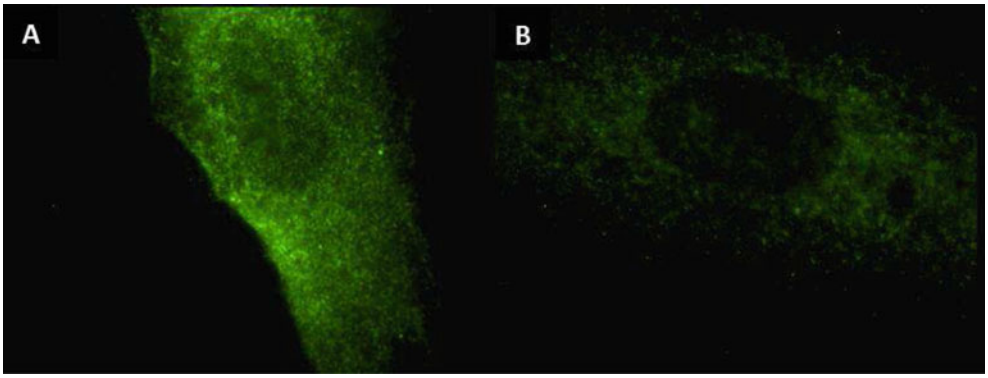

Fig. 2 Distribution of peroxisomes in cultured skin fibroblasts from a healthy control (**a**) and from the patient (**b**), visualized by immunofluorescence microscopy using an anti-catalase antibody

detected in the *PEX1* gene, as well as by peroxisomal mosaicism, which was observed in the patient's fibroblasts. This child is homozygous for the pathogenic missense variant c.2528G>A (p.Gly843Asp) in *PEX1*, affecting the second ATP-binding domain of the protein and which may enable transcription into mRNA and translation into protein to a certain extent (Crane et al. 2005). As a consequence, the binding between PEX1 and PEX6 is reduced but not abolished. This interaction is essential for the peroxisomal protein import system (Tamura et al. 2001). In addition, this pathogenic variant influences the PEX1 activity in a temperature-sensitive manner, i.e. while at 37°C import of matrix proteins into "ghost" peroxisomes is observed in some cells (peroxisomal mosaicism), at 30°C, peroxisomal import is almost completely recovered, as well as peroxisomal metabolic functions, and at 40°C, no peroxisomal import is observed (Imamura et al. 1998). Overall, this pathogenic variant retains a residual peroxisomal function which, along with peroxisomal mosaicism, results in less severe biochemical deficiencies, a milder clinical phenotype and prolonged survival (Osumi et al. 2000; Poll-The et al. 2004; Crane et al. 2005).

This IRD patient was treated with a phytanic acid-restricted diet, since there is increasing evidence on its toxicity, namely, disturbing normal lipid homeostasis (van den Brink and Wanders 2006). Additionally, carbohydrate supplementation was crucial to maintain an adequate energy intake. This led to persistent plasma phytanic acid level normalization after the onset of the dietary regimen. Similar biochemical results were previously reported in few patients with IRD treated with a low phytanic acid diet (Robertson et al. 1988; Pakzad-Vaezi and Maberley 2014). However, the long-term clinical benefit of this approach remains to be elucidated. Slow developmental progression was concomitantly observed in this patient. Nonetheless, since the prognosis of IRD is variable, the slow disease progression or halting of the disease progression may be due to the natural history of the disease. Since phytanic acid and pristanic acid originate exclusively from exogenous sources, its accumulation in ZSD patients is dependent on diet, as well as age (Wanders et al. 2011). Theoretically, the measurement of plasma phytanic acid levels may be regarded as a response marker to the low phytanic acid intake diet. Furthermore, accumulation of phytanic acid over time, through dietary intake, is a diagnostic marker of PBD (Aubourg and Wanders 2013). Nonetheless, since intermittent normalization of plasma phytanic acid levels may also be observed in IRD patients, possibly depending on their diet (Poll-The et al. 2004), its value as a biochemical diagnostic parameter may be less reliable, prompting the need to combine it with other biochemical diagnostic parameters.

The missense variant c.2528G>A (p.Gly843Asp) in *PEX1* is the most commonly found pathogenic mutation in different patient cohorts (25–40% of the ZSD patients) (Ebberink et al. 2011) and is associated with a mild phenotype, meaning that IRD patients may live several decades (Crane et al. 2005). Accordingly, an earlier diagnosis will enable a more effective intervention in these patients. Systematic evaluation of long-term biochemical, clinical and developmental effects of a low phytanic acid intake diet in IRD patients may prove whether this could be a useful supplement to the recommended management of this disorder.

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Synopsis

In spite of being a peroxisomal biogenesis disorder, infantile Refsum disease may have residual peroxisomal activity once we obtained a decrease of phytanic acid plasma levels, along with a low phytanic acid diet, in a 3-year-old child with the common pathogenic p.(Gly843Asp) mutation in the *PEX1* gene.

Compliance with Ethics Guidelines

Conflict of Interest

Maria João Nabais Sá, Júlio C. Rocha, Manuela F. Almeida, Carla Carmona, Esmeralda Martins, Vasco Miranda, Miguel Coutinho, Rita Ferreira, Sara Pacheco, Francisco Laranjeira, Isaura Ribeiro, Ana Maria Fortuna and Lúcia Lacerda 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. Informed consent was obtained from the patient's parents for being included in the study. Proof that informed consent was obtained is available upon request.

Details of the Contributions of Individual Authors

Maria João Nabais Sá, Júlio C. Rocha and Lúcia Lacerda contributed pertinent aspects of the planning, conducting and reporting of the work described in the article. Maria João Nabais Sá wrote the first draft of this manuscript. All the authors critically revised the original draft manuscript for intellectual content and approved the version submitted for publication.

Maria João Nabais Sá made the clinical diagnosis, requested and interpreted the necessary diagnostic procedures, conducted the biochemical investigation, is in charge of the follow-up of the patient, performed the family screening and counselling, collected the protocol baseline and historical and follow-up clinical data of the patient and performed and selected the corresponding photographs, included in Fig. 1a of this manuscript.

Lúcia Lacerda supervised the diagnostic biochemical and molecular studies of the peroxisomal biogenesis disorder and was responsible for their interpretation and reporting.

Esmeralda Martins, Vasco Miranda and Miguel Coutinho did the necessary diagnostic procedures, namely, the biochemical investigation, ophthalmologic evaluation and audiological evaluation, respectively, are in charge of the follow-up and treatment of the patients and collected the protocol baseline and historical and follow-up clinical data of the patient.

Vasco Miranda performed and selected the photographs included in Fig. 1b of this manuscript.

Júlio C. Rocha and Manuela F. Almeida are in charge of the nutritional follow-up and treatment of the patient.

Carla Carmona conducted the psychomotor development assessment of the patient and designed the bar chart included in Fig. 1c.

Rita Ferreira, Sara Pacheco and Isaura Ribeiro performed the biochemical assays and were responsible for their interpretation and reporting. Isaura Ribeiro performed and selected the photograph included in Fig. 2 of this manuscript.

Francisco Laranjeira performed the molecular analysis of the *PEX1* gene and was responsible for its interpretation and reporting.

Ana Maria Fortuna supervised the clinical and laboratory content of this manuscript, as Head of the Department of Medical Genetics, Centro de Genética Médica Dr. Jacinto de Magalhães/Centro Hospitalar do Porto, Porto, Portugal.

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RESEARCH REPORT

Mucopolysaccharidosis (MPS) Physical Symptom Score: Development, Reliability, and Validity

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Abstract *Objectives*: We quantified medical signs and symptoms to construct the Physical Symptom Score (PSS) for use in research to assess somatic disease burden in mucopolysaccharidoses (MPS) to track disease and monitor treatments. We examined scoring reliability, its concurrent validity with other measures, and relationship to age in MPS type I.

Methods: Fifty-four patients with MPS I (36 with Hurler syndrome treated with hematopoietic cell transplant and 18 with attenuated MPS I treated with enzyme replacement therapy), ages 5 to 18 years, were seen longitudinally over 5 years. The summation of frequency and severity of signs of specific organ involvement, surgeries, and hydrocephalus drawn from medical histories comprise the PSS. We examined relationship to age and to daily living skills (DLS) from the Vineland Adaptive Behavior Scale and physical quality of life from the Child Health Questionnaire (CHQ) for each group.

Results: The PSS was associated with age in both groups, indicating increase in disease burden over time. The PSS was significantly negatively associated with DLS ($r = -0.48$) and CHQ ($r = -0.55$) in the attenuated MPS I but not in the Hurler group.

Conclusions: The association of somatic disease burden with physical quality of life and ability to carry out daily living skills suggests that the PSS will be useful in the measurement of disease and treatment effects in the attenuated MPS I group. Earlier treatment with transplant and differing parental expectations are possible explanations for its lack of association with other outcomes necessitating an adaptation for Hurler syndrome in the future.

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Competing interests: None declared

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Introduction

Patients with mucopolysaccharidoses type I (MPS I) have medical problems in many organ systems (Neufeld and Muenzer 2001; Clarke and Heppner 2002; Muenzer et al. 2009). They are often severe and likely contribute significantly to their health-related quality of life (QOL) and adaptive status. However, there is no single score that can summarize their disease burden for clinical research purposes. Quantification of these problems will assist in understanding how medical problems contribute to other outcome measures. We have developed a Physical Symptom Score (PSS) to measure disease burden based on the severity and frequency of symptoms in relevant organ systems; we have assessed its scoring reliability and validated it with quality of life and daily living skills measures.

MPS I is one of the lysosomal storage disorders which is an autosomal recessive multi-organ system disease caused by a deficiency of the enzyme, alpha-L-iduronidase (Neufeld and Muenzer 2001; Hopwood and Morris 1990), with an incidence of 1:100,000 live births (Neufeld and Muenzer 2001; Meikle et al. 1999; Moore et al. 2008). The phenotypes of MPS I disorder are historically classified into three syndromes: severe Hurler, milder disorder Scheie, and the intermediate disorder Hurler–Scheie. Hurler–Scheie and

Scheie syndromes also have been referred collectively as attenuated MPS I and cannot be easily differentiated diagnostically using clinical, biochemical, or molecular criteria (Neufeld and Muenzer 2001; Clarke and Heppner 2002; Pastores et al. 2007; D'Aco et al. 2012). Hearing difficulties, corneal clouding, organomegaly, skeletal or orthopedic abnormalities, cardiorespiratory problems, central nervous system problems like hydrocephalus with or without shunt placement, other neurological problems such as carpal tunnel syndrome, headache, cognitive impairment, and sometimes cervical cord compression impede functionality on a day-to-day basis and contribute to disease burden. These physical signs and symptoms together with increased urinary excretion of GAG (glycosaminoglycan), and absent or deficient alpha-L-iduronidase enzyme activity are observed in all forms of MPS I (Terlato and Cox 2003; Scott et al. 1995; Beesley et al. 2001), but no method exists to accurately summarize phenotypic disease burden in retrospective or prospective research studies. Genotype–phenotype correlations have been established to a limited degree in Hurler syndrome and even more limited in the attenuated forms (Pastores et al. 2007; Terlato and Cox 2003; Scott et al. 1995; Beesley et al. 2001; Bertola et al. 2011; Ahmed et al. 2014a). Standard of care treatments such as hematopoietic cell transplant (HCT) (Peters et al. 1996; Peters et al. 1998; Souillet et al. 2003; Staba et al. 2004) in Hurler syndrome and enzyme replacement therapy (ERT) (Kakkis et al. 2001; Wraith et al. 2004) for the attenuated syndromes ameliorate some but not all symptoms. This scale will provide a summary score that could be used to assess the overall impact on disease burden of treatments over time.

Our goal is to establish a quantified measure of disease burden by creating a summative scale of the history of medical problems in multiple organ systems. We hypothesize that it will be associated with age, with the ability to carry out daily living skills (DLS), and with quality of life (QOL) outcomes in MPS I. The latter measures will provide information about concurrent validity.

Methods

Subjects

Subjects with MPS I were drawn from four centers contributing to a natural history study "Longitudinal Studies of Brain Structure and Function in MPS Disorders," NCT01870375 of the Lysosomal Disease Network (Rare Disease Clinical Research Network-RDCRN). Inclusion criteria were (1) age range of 5 to 18 years, (2) availability of medical data to create PSS score at each visit, and (3) completion of either Vineland Adaptive Behavioral Scales (VABS), Second Edition, or Child Health Questionnaire Parent Form 50 (CHQ-PF50), for at least one visit. Each patient had between one and five visits over 5 years.

Procedures

Development of the PSS Scale

Medical and treatment histories, as gathered from interviews with patients or parents of patients and review of medical records, were recorded on case report forms developed for the longitudinal study. When available all clinical files and medical records of patients were reviewed. A scoring system was devised to quantify abnormalities in four medical domains: skeletal/orthopedic, vision, hearing, and cardiorespiratory. These data were scored by a system of frequency and severity of the organ system involvement (Ahmed et al. 2014b). The scoring system also quantified the patient's number of surgical procedures using general anesthesia, and the absence or presence of hydrocephalus with or without shunt placement. Medical records were examined together with historical information for each domain. For example, to score the skeletal/orthopedic domain, we would collect appropriate information from the physician medical encounters and comments on symptoms such as limited range of motion, hip dysplasia, kyphosis, etc., and determine the frequency of these symptoms. Because we could not always determine the severity, each symptom was categorized as present or absent. Each of the 6 domains was scored 0 (absent) to 3 (severe). The specific criteria for coding each domain are provided in Table 1. Total summary scores were calculated by totaling each domain score. The range of total summary scores could be from 0 to 18.

Reliability

To investigate the inter-rater scoring reproducibility, 30 MPS I patients were chosen in a random order and independently scored by an expert who is involved in MPS research and previously was a health professional (E.R.) and compared with the PSS scoring of the author (A.A.). Inter-rater reliability is reported on the total score based on history and medical record review. All the items were scored in a similar manner according to the MPS-specific Physical Symptom Scale by both raters (Table 1).

Validation Measures

We compared the PSS with physical summary (PhS) measure from the Child Health Questionnaires Parent Form 50 (CHQ-PF50) and the daily living skills (DLS) domain from Vineland Adaptive Behavioral Scales (VABS), Second Edition. These two scales were selected because they have embedded measures of functional outcomes of somatic burden of

Table 1 MPS-specific physical symptom scale

Feature	Score	Description
Skeletal/orthopedic	0	No orthopedic symptom
	1	1–2 symptoms
	2	3–4 symptoms
	3	5–6 symptoms or cord compression
Skeletal/orthopedic symptoms include limited range of motion, kyphosis, scoliosis, hip dysplasia, knock knee, and high arch foot		
Vision	0	No visual impairment or symptom
	1	Mild corneal clouding or glaucoma or cataract
	2	Moderate corneal clouding or both glaucoma and cataract
	3	Severe corneal clouding or retinal degeneration
Hearing	0	None
	1	Mild hearing loss
	2	Moderate hearing loss
	3	Severe hearing loss
Cardiorespiratory	0	None
	1	1–2 cardiac or respiratory symptoms
	2	3–4 symptoms or presence of sleep apnea
	3	5–6 symptoms or history of cardiac surgery
Cardiac and respiratory symptoms include murmur, hypertension, valve disease, cardiac surgery, chronic nasal discharge/obstruction, tonsils/adenoids, respiratory infection/reactive airway disease, and sleep apnea		
Hydrocephalus	0	Absent
	1	Hydrocephalus without shunt
	2	Hydrocephalus with shunt
	3	Revision of shunt
Number of surgeries	0	No surgery
	1	Less than 4 surgeries
	2	4 to 8 surgeries
	3	More than 8 surgeries
Total score = 0–18		

disease (Physical Summary Measure of Quality of Life and Daily Living Skills), allowing for concurrent validation. Ideally, a measure such as the HAQ (Health Assessment Questionnaire) (Pastores et al. 2007) might be used to validate the PSS; however, normative data is not available for children, and the HAQ was not selected as a measure for the longitudinal study for the same reason.

The Child Health Questionnaire Parent Form 50 (CHQ-PF50) is a 50-item, parent-completed questionnaire designed to measure children's general functional health and/or health-related quality of life from parent's perspective (Landgraf et al. 1996). The CHQ-PF50 has been validated in multiple settings and various childhood disease groups and normed in a large national study of children from 5 to 18 years of age. We used the *T*-scores (where $M = 50$ and $SD = 15$) for the physical summary (PhS) measure. Lower scores on the CHQ indicate greater impairment in functioning. This measure was chosen because it reflects the impact of disease burden on the perception of how it affects physical quality of life.

Vineland Adaptive Behavioral Scales (VABS), Second Edition, is an observer-rated measure of personal and social skills needed for everyday living (Sparrow et al. 2005). This adaptive level of functioning measures along four broad domains (communication, daily living skills (DLS), socialization, and motor skills) and is designed for assessing individuals between ages of 0 and 90 years. The daily living skills domain is a measure of practical skill of self-care, care of home, and community participation. We used the standard scores (where $M = 100$ and $SD = 15$) of parent-reported DLS for comparison. Lower scores on the DLS indicate greater impairment in functioning. This measure was chosen because it reflects the impact of disease burden on the day-to-day functioning of the patient.

Table 2 Patient descriptives; values presented are mean (SD) or *N* (%) where indicated

Covariate	Hurler ($N = 36$)	Attenuated ($N = 18$)
Male	18 (50.0%)	9 (50.0%)
Age at first visit (years)	9.53 (3.64)	11.70 (4.49)
Age at treatment (years)	1.42 (0.77)	7.57 (5.02)
Time on treatment (years)	8.11 (3.83)	4.13 (2.74)
PSS score at first visit	9.39 (2.05)	7.89 (2.76)

Statistical Analysis

Descriptive statistics were tabulated separately for attenuated and Hurler forms of MPS I. These included the mean and standard deviation for continuous variables and frequency for categorical variables. Correlations were estimated with generalized estimating equations using normalized covariates with a working independence correlation structure, which corresponds to Pearson's product moment correlation coefficient (Diggle et al. 2002). Confidence intervals and P-values were based on robust variance estimation to account for the correlated nature of longitudinal measurements. All analyses were conducted using R v3.1.1 (R Core Team 2014).

Results

The attenuated group was slightly older than the Hurler group (Table 2). The age at treatment and years from treatment were also different for the two groups due to differences in standards of care for the age of diagnosis and timing at each treatment. All of the Hurler patients underwent hematopoietic cell transplantation, and all of the attenuated patients were on enzyme placement therapy only.

Reliability: Inter-rater reliability of the PSS scoring was good (intra-class correlation [ICC] = 0.99).

In Table 3 both groups showed a significant correlation with age such that older patients showed more disease burden in both groups (Fig. 1). Of those with multiple visits, no patient showed a decrease and several showed an increase in PSS: 6/22 (27%) for the Hurler patients and 3/11 (27%) for the attenuated patients.

In the attenuated group, PSS was negatively associated with the PhS from the CHQ (Fig. 2) and DLS from the Vineland (Fig. 3) such that lower scores for quality of life and daily living skills are associated with higher PSS, indicating more functional impairment. PSS was not significantly associated with PhS from CHQ and DLS from Vineland in the Hurler group.

Discussion

We have constructed a somatic disease severity score to reflect the degree of disease burden based on a multicenter longitudinal cohort of Hurler and attenuated (Hurler–Scheie and Scheie) forms of MPS I patients. Our goal was to design an MPS-specific physical symptom scale to quantify somatic disease burden for comparison with other outcome measures and for use in clinical trials.

Our analysis supports the scoring reliability of this measure for both MPS I groups and the validity of the PSS for attenuated MPS I patients. We have presented evidence for the association of PSS with age in both MPS I groups. In both groups, the percent increase of PSS was the same.

We know that despite ERT treatment, patients with attenuated forms of MPS I continue to undergo surgeries for orthopedic problems, cervical cord compression, and shunt placement for hydrocephalus. However, a good treatment response would be avoiding further decline. To be most useful, treatments would need to start early, before substantial progression (i.e., high PSS).

The PSS provides a shorthand measure of these accumulated problems. We found that these problems are associated with difficulties in carrying out activities of daily living and physical quality of life in the attenuated MPS I patients. While other measures such as the HAQ, a direct measure of disability, might also provide concurrent validation, the advantage of the VABS and the CHQ is that they both have normative data for children. The association of physical handicap with activities of daily living is consistent with previous reports that on a direct measure of physical performance, MPS I patients have difficulties in conducting daily functional activities (Haley et al. 2006).

Table 3 Correlation of PSS with Hurler and attenuated groups

Variable	Group	Correlation (95% CI)	*P*-value
Age	Hurler	0.39 (0.20, 0.55)	<0.001
Age	Attenuated	0.58 (0.31, 0.76)	0.001
CHQ-PhS	Hurler	−0.14 (−0.36, 0.09)	0.235
CHQ-PhS	Attenuated	−0.56 (−0.76, −0.25)	0.001
Vineland DLS	Hurler	−0.11 (−0.31, 0.11)	0.328
Vineland DLS	Attenuated	−0.48 (−0.70, −0.18)	0.003

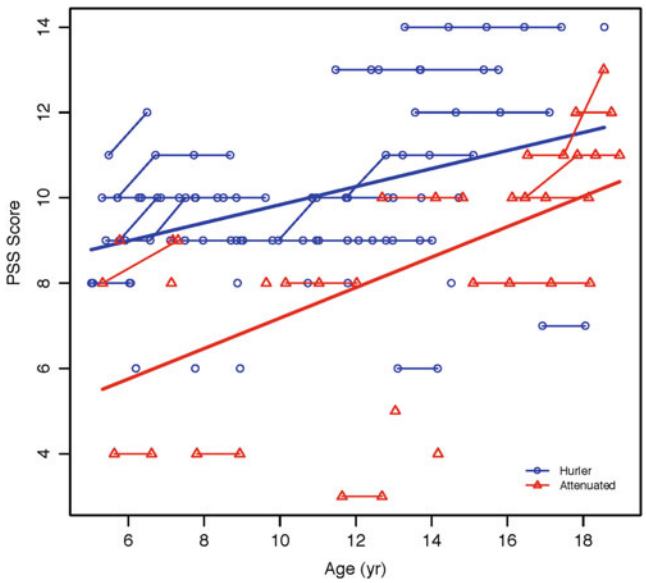

Fig. 1 Association of PSS with age among Hurler and attenuated MPS I groups

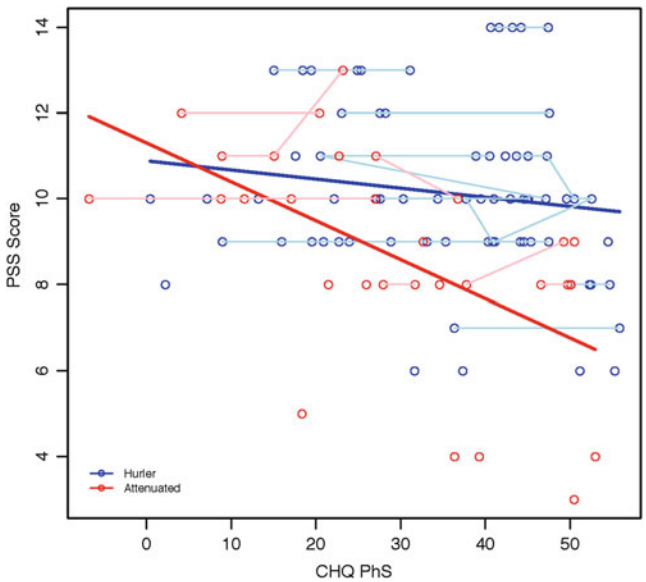

Fig. 2 Association of PSS with CHQ-PhS among Hurler and attenuated MPS I groups

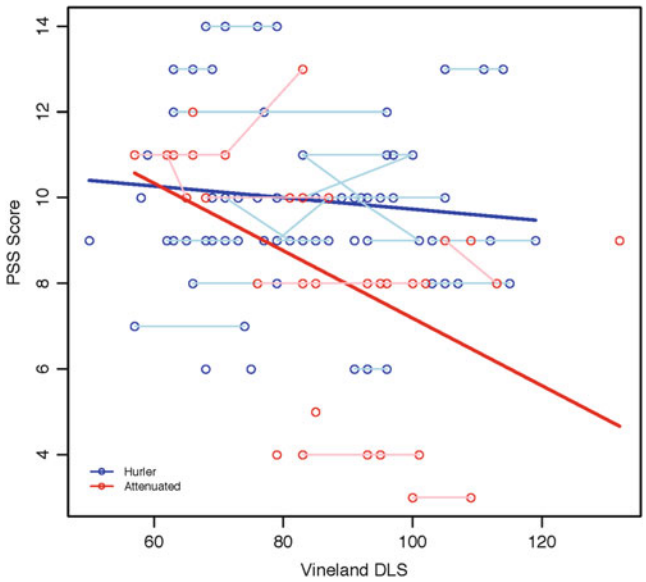

Fig. 3 Association of PSS with Vineland DLS among Hurler and attenuated MPS I groups

Correlation with such a measure in future studies might provide additional validity for the PSS.

In contrast to the attenuated group, while showing an increase with age, the PSS was not associated with decreased daily living skills or quality of life among Hurler patients. There are several possible factors. As the majority of problems occurred in these patients at the time of HCT, the elapsed time since the occurrence of these symptoms is long. Because they occurred earlier, they may not be as handicapping, the symptoms are more stable, or they may have learned to live with their disease burden. Further work is necessary to adapt the PSS for children with Hurler syndrome for it to be a useful measure of late effects.

For the attenuated MPS I group, this measure of disease burden can be both a marker of long-term treatment efficacy and tracking individual domains of somatic involvement in disease progression. In MPS II, effects of PSS were documented in a previous publication where it was found that for every PSS point, a measure of variability in attention is decreased by 12 points (p value <0.001) in mild mucopolysaccharidosis type II (Yund et al. 2015).

It should be noted that this scale is not meant to be a clinical tool. It is a summary of the patient's history of medical problems. Thus, it does not define specific approaches to the severity or method of obtaining medical data. Because this scale is based on medical records, it relies on many physician assessments, which may not use the same criteria to judge a sign of MPS. Thus, while the scoring may be reliable, we are basing this scoring on the judgment of physicians who may differ in the criteria they use or their data collection methods. This is similar to the problem encountered in registry data. Pastores et al. note that registries suffer from a lack of standardization of assessment and data collection methods (Pastores et al. 2007). It should be noted that multiple sites enter data to a registry, but in this case, single well-trained personnel are compiling data for the PSS, eliminating one source of variation.

In conclusion, a reliable MPS I-specific Physical Symptom Score (PSS) has been developed which may be useful in future investigations of the effects of treatment and disease progression. The association of somatic disease burden with outcomes such as age, physical quality of life, and ability to carry out daily living skills supports the validity of this measure in attenuated MPS I patients. Research needs to be carried out to adapt the measure so it is more sensitive in children with Hurler syndrome. Future investigations will also focus on other correlates such as emotional and social status, genotypes, biomarkers, and MRI markers of the brain.

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Synopsis of the Article

A reliable, validated MPS-specific Physical Symptom Score (PSS) has been developed that assesses somatic disease burden and used to evaluate treatment outcomes and disease progression for research purposes in the attenuated group of MPS I.

Compliance with Ethics Guidelines

Contributions of Individual Authors

- A. Ahmed wrote this article and contributed pertinent aspects of the planning, conduct, and reporting of the work described in the article, provided scientific expertise, and created the PSS.
- K Rudser performed the data and statistical analysis and edited the manuscript.
- A. Kunin-Batson scored and advised regarding the CHQ-PF50 and edited the manuscript.
- K Delaney managed subject recruitment, scored DLS from Vineland Adaptive Behavioral Scales (VABS), Second Edition, and provided expertise about Vineland.
- C. Whitley recruited and managed the LDN (Lysosomal Disease Network) study as a P. I. from which these subjects were selected and ensured their cooperation with study protocol.
- E. Shapiro provided scientific expertise and co-wrote the manuscript.

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Conflict of Interest

Elsa G. Shapiro declares that she has received funds as a consultant from Genzyme-Sanofi, BioMarin, ArmaGen, REGENXBIO, and Shire.

Kathleen Delaney declares that she has received funds as a consultant from Shire, ArmaGen, BioMarin, and REGENXBIO.

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Alia Ahmed declares that she has no conflict of interest.

Kyle Rudser declares that he has no conflict of interest.

Alicia Kunin-Batson declares that she has no conflict of interest.

The authors confirm independence from the funders of this research; the content of the article has not been influenced by the funders.

Ethics board approval: This study was approved by the University of Minnesota Institutional Review Board: Human Subjects Committee.

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.

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

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RESEARCH REPORT

Energy Expenditure in Chilean Children with Maple Syrup Urine Disease (MSUD)

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Abstract *Introduction*: Maple syrup urine disease (MSUD) is an autosomal recessive disorder caused by a blockage of branched-chain keto acid of BCAA (branched-chain keto acid dehydrogenase, BCKDH) leading to neurological damage induced by accumulation of leucine and metabolites. MSUD expenditure and energy requirement information is limited.

Objective: To determine if basal/total energy expenditure (BEE/TEE) is comparable between different determination methods and if values agree with recommendations of energy in MSUD children, and whether they relate to nutritional status.

Methods: Case-control study between MSUD (n = 16) and healthy children (n = 11) aged 6–18 years. Current nutritional status, physical activity level, body composition by DEXA and BEE/TEE by indirect calorimetry (BEEr) and predictive equations (FAO/WHO/ONU – WHO – and Schofield) were assessed; STATA 2013 ($p < 0.05$).

Results: When comparing the energy expenditure variables, there was no significant difference between groups. Moreover, compared to BEEr, equations underestimate according to BEE WHO and Schofield, respectively (P = 0.00; 0.02). The WHO equation had lower average calorie difference, greater concordance correlation and association with indirect calorimetry compared to the Schofield equation for both groups, being the best predictor of the BEE for MSUD group.

Conclusion: Energy recommendations for MSUD children are according to energy expenditure; thus the use of WHO equation is a clinically and statistically feasible tool for its determination.

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Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive disorder caused by a blockage of branched-chain keto acid of BCAA (branched-chain keto acid dehydrogenase, BCKDH), resulting in the accumulation of branched-chain amino acids: valine, isoleucine, and leucine (VIL). High levels of leucine in the blood and brain and its keto acid (α-ketoisocaproic (αKIC)) cause metabolic decompensation. Worldwide incidence is 1:185,000 and is 1:60,000 in Latin America (Colombo et al. 2010; Kaye et al. 2006; Knerr et al. 2012; Morton et al. 2002; Strauss et al. 2010; Zinnanti and Lazovic 2011; Cornejo 2004; Cremer et al. 1982).

The classic form of MSUD has less than 2% residual enzyme activity. Clinical symptoms begin at the fifth day of life in terms of poor suck, food refusal, unexplained drowsiness, and coma. Later, additional symptoms appear, such as autonomic dysregulation, respiratory distress, apnea, bradycardia, hypothermia, axial hypotonia with episodes of distal hypertonia that could lead to opisthotonus, cerebral edema with bulging fontanelle, and a characteristic odor of burnt sugar. If this disease is not diagnosed and treated early in lifetime, severe neurologic damage or death can occur (Colombo et al. 2010; Kaye et al. 2006; Knerr et al. 2012; Morton et al. 2002; Strauss et al. 2010; Zinnanti and Lazovic 2011; Cornejo 2004; Cremer et al. 1982).

Treatment consists of a leucine-restricted diet, eliminating all foods of animal origin. Thus, to facilitate proper protein synthesis, prevent protein catabolism, restore energy homeostasis, and promote anabolism, it is necessary to deliver BCAA-free medical foods (Knerr et al. 2012; Robinson and Drumm 2001).

Information about energy supply, energy expenditure (EE), and daily energy requirements in children with MSUD is limited. However, it is an important area of study, as the information is necessary to maintain metabolic balance and proper growth in MSUD children. Some authors have proposed hypercaloric intakes, while others have argued that a relatively low energy intake is required for normal growth and development (Bodamer et al. 1997; Barja 2011; Hauser et al. 2011; Ruiz et al. 2007).

Some clinical studies have evaluated basal and total energy expenditure (BEE and TEE) with indirect calorimetry and estimated using various predictive equations to determine the level of agreement between them and thus improve clinical care. However, the failure of predicting BEE and TEE for children with inborn errors of metabolism (IEM) has been reported, because equations are based on age, weight, and height of healthy reference populations (Bodamer et al. 1997; Barja 2011; Hauser et al. 2011; Ruiz et al. 2007; Quirk et al. 2010). In order to extrapolate to populations with pathology, adjustments are required, illustrating the need for continued study (Hauser et al. 2011).

In relation to IEM, studies were performed in children with methylmalonic acidemia (MMA), propionic acidemia (PA), and phenylketonuria (PKU) measuring BEE via indirect calorimetry and using standard predictive equations (Quirk et al. 2010; Feillet et al. 2000). Overall, there is great variability in the results since in organic acidemia different predictive equations overestimate BEE, in contrast to PKU children in which BEE is underestimated (Quirk et al. 2010; Feillet et al. 2000).

Inconsistent results were found when using standard predictive equations for estimating BEE of some diseases. Equations may not be reliable enough to determine the expenditure and energy requirements in children with MSUD. Assessment of BEE and TEE requires using indirect calorimetry and estimating with predictive equations to establish the degree of agreement between these two methods and to accurately adjust energy intake of children. The aims of this study were to determine whether the recommended daily energy intake for children with MSUD is in line relative to basal and total energy expenditure evaluated using different methods and if energy expenditure is related to nutritional status.

Methods

We conducted a case–control study among a group of Chilean children between 6 and 18 years of age with MSUD ($n = 16$) and a control group ($n = 11$) matched by age, gender, and nutritional status. All participants signed informed consent, approved by the Ethics Committee of Instituto de Nutrición y Tecnología de Alimentos (INTA), University of Chile.

The study group was composed of MSUD children in medical and nutritional follow-up at INTA and able to follow directions. The control group was composed of healthy children of workers of this center who had no acute or chronic illnesses.

Nutritional status was assessed using WHO 2007 growth standards (WHO 2006).

Weight and height were measured in the Frankfurt position in light clothing with no shoes, using a digital scale and stadiometer (Seca), precision 0.1 kg and 0.1 cm, respectively.

Waist circumference (WC) was measured using an automatic adjustable tape (maximum of 182 cm) with a sensitivity of 0.1 cm (Fernández et al. 2004; Barrera 2013).

Indicators: Body mass index (BMI) *z*-score, height-for-age (H/A) *z*-score, and WC percentile (PCTL) were determined (Pizarro et al. 2004).

Body composition: Fat mass (FM) and fat-free mass (FFM) were evaluated using Lunar Prodigy Encore 2007 X-ray absorptiometry (DEXA) (software version 11,30,062).

Intake assessment: We conducted 24-h dietary recalls for 2 weekdays and 1 weekend day. Daily energy intake was assessed using the amino acid analyzer program, averaging respondent's days.

Assessment of physical activity (PA) was determined using a validated questionnaire that assessed usual PA for a child or adolescent during the week (Monday through Friday), consisting of five categories: (1) daily hours lying down, (2) daily hours of sedentary activities, (3) number of blocks walked per day, (4) daily hours of recreational outdoor activities, and (5) weekly hours of scheduled exercise or sports. Each category has a score of 0–2, so that the total score ranges from 0 to 10. Sedentary/light PA was considered less than 5 points; moderate activity 5–6 and vigorous was defined as 7 or more points. Physical activity factor (PAF) was identified by gender and age (Godard et al. 2008; Burrows et al. 2008; OMS 2010; FAO/WHO/UNU Expert 2001, 2005).

BEE was measured with indirect calorimetry (BEEr) and estimated by two standard predictive equations. The TEE was calculated from data obtained from BEE by applying the PAF (FAO/WHO/UNU Expert Consultation 2001). A canopy indirect calorimetry (SM-2900, model 2900, Sensormedics Metabolic cart, Yorba Linda, CA, USA) was used after participants fasted for 8–10 h and 30 min of mental and physical rest in a comfortable atmosphere and temperature (Bodamer et al. 1997; Ferrannini 1988; Rodríguez et al. 2002). We also determined BEE using two standard predictive equations. The FAO/WHO/UNU 2001 by age, gender, and actual body weight (BEE WHO) and Schofield 1985 based on height and weight were used (BEE Schofield) (Table 1) (FAO/WHO/UNU Expert 2001,

Table 1 Standard predictive equations for children ages 3–18 years

	Standard predictive equations	
Basal energy expenditure	FAO/WHO/UNU 2001	Schofield based on weight and height, 1985
Men		
3–10	22.706 × W + 504.3	19.59 × W + 1,303 * H + 414.9
10–18	17.686 × W + 658.2	16.25 × W + 1,372 * H + 515.5
Women		
3–10	20.315 × W + 485.9	16.969 × W + 1,618 * H + 371.2
10–18	13.384 × W + 692.6	8.365 × W + 4,65 * H + 200.0

Source: (Shimizu-Fujiwara et al. 2012; Rodríguez and Pizarro 2006)
*Actual weight (kg) and height (cm)

Table 2 Characteristics of study groups

Description	MSUD ($n = 16$)	CONTROL ($n = 11$)	P*
Age (years)	12.8 (±3.3)	13.0 (±3.5)	0.86
Gender			0.82
Men	5 (31%)	3 (27%)	
Women	11 (69%)	8 (73%)	
Anthropometry			
Weight (kg)	37.3 (±10.3)	41.5 (±13.0)	0.36
Height (cm)	140.2 (±13.6)	141.6 (±15.6)	0.81
zBMI	−0.03 (−1.49 to 1.04)	0.42 (±0.52)	0.10
zT/E	−1.58 (−3.42 to 0.04)	−1.4 (−0.21to −2.33)	0.69
Waist circumference (cm)	66.1 (±7.5)	73 (49.2–73)	0.20
Body composition			
Fat mass (kg)	11.57 (±4.54)	13.97 (±5.73)	0.21
Fat-free mass (kg)	25.2 (±7.45)	26.39 (±7.31)	0.62

*$p < 0.05$, *T*-Student/Wilcoxon

2005; Schofield 1985). All measurements and questionnaires were conducted by a nutritionist.

Statistical analysis: The Shapiro–Wilk test was performed to determine whether the variables had normal distributions; if they did, they were presented using parametric tests. Results were expressed as means and standard deviations; otherwise, nonparametric tests were used (medians, interquartile ranges). Comparison tests (*T* test/Mann–Whitney), associations (Pearson/Spearman correlation and logistic regression), and the level of agreement (Bland–Altman test) were performed. A P value <0.05 was considered significant. The database was created in Microsoft Excel 2010, and analyses were performed using STATA 13 (StataCorp 2011 College Station, Texas).

Table 3 MSUD children description

Description	MSUD <7 days diagnosis ($n = 5$)	MSUD >7 days diagnosis ($n = 11$)	P*
Gender (*n*)			
Male	1	4	
Female	4	7	
Leucine at diagnosis (μmol/L)	690 (±207)	1,939 (±884)	0.00
IQ			
Verbal	85 (±11)	69 (±22)	
Motor	81 (±19)	61 (±16)	
Total	82 (±16)	63 (±20)	
Psychometric diagnosis (*n*)			
High average (111–119)	–	1	
Average (90–110)	2	1	
Low average (80–89)	2		
Borderline (70–79)	–	1	
Mild impaired (55–69)	1	3	
Moderately impaired (40–55)	–	4	
Severe impaired (≤40)	–	1	
Motor disability (*n*)	0[a]	6[a]	
Level of physical activity (*n*)			
Sedentary/light	4	10	
Moderate	1[b]	1	
Nutrition			
Oral	5	8	
Gastrostomy	–	1	
Oral + gastrostomy	–	2	

[a] One child with mild motor disability
[b] Hyperactivity
*$p < 0.05$, *T*-Student

Results

Table 2 describes general characteristics and anthropometric indicators of study groups. Both groups had normal nutritional status according to BMI *z*-score with a tendency toward lower height (H/A *z*-score ≤0). According to WC, 25% of children with MSUD and 18% of controls had a slight tendency to be underweight (<25 PCTL). We found no significant differences between groups in relation to body composition, FFM and FM. For descriptive purposes only, the study group was divided according to age at

Table 4 Comparison of total and basal energy expenditure between groups

	MSUD ($n = 16$)	CONTROL ($n = 11$)	P*
BEEr[a,b] (kcal/day)	1,353 (185)	1,489 (853–1,678)	0.52
TEEr[b] (kcal/day)	2,032 (351)	2,204 (502)	0.30
BEEe OMS[a,b] (kcal/day)	1,269 (866–1,472)	1,261 (181)	0.51
TEEe OMS[b] (kcal/day)	1,809 (298)	2,006 (336)	0.12
BEEe Schofield[a,b] (kcal/day)	1,248 (871–1,483)	1,231 (166)	0.49
TEEe Schofield[b] (kcal/day)	1,791 (292)	1,957 (306)	0.17

[a] Mann–Whitney test (Wilcoxon rank-sum test)
[b] *T*-Student
*$p < 0.05$

diagnosis (before and after 7 days of life), as this determines important features such as IQ, feeding route, and neurological and motor impairments (Table 3).

The average energy and protein intake was calculated, and no significant difference was found between the study group (1,621 kcal/day, 78.9 g/day) and control group (1,512 kcal/day, 54.9 g/day). However, the average uptake of leucine differed significantly by group, with MSUD children having 484 mg/day compared to controls with 4,338 mg/day.

Comparing variables of energy expenditure, no significant differences between groups were found (Table 4). Both predictive equations underestimated the basal energy expenditure as to BEEr in both groups ($P = 0.00$ and 0.02) (Table 5). The BEE WHO and Schofield underestimated the MSUD group by 9.5% and 10.4%, respectively, and 7.5% and 9.4% for the control group, respectively.

Regarding indirect calorimetry, the WHO equation had lower average calorie difference, greater concordance correlation coefficient, and association than the Schofield equation for each group (Table 5; Fig. 1 and 2).

In the study group, real and estimated BEE was significantly correlated with BMI *z*-score, WC, and FFM and TEE related to WC and FFM. The control group had similar associations, except no relationship was found between BEE and BMI *z*-score ($P < 0.05$). Using logistic regression, FFM explained 81% of BEEr for MSUD children ($P < 0.001$), while FFM and WC explained 95% ($P = <0.001$) and 92% ($P = <0.001$) of the BEE computed using the WHO and Schofield equations, respectively. For the control group, FFM explained 87% of BEEr ($P = 0.05$), while FFM and WC explained 94% of the BEE computed using the WHO equation. We found no significant predictors of BEE estimated using the Schofield equation.

The majority of the MSUD children (88%) were sedentary/light PA, with only two children (12%) reporting a moderate level. In the control group, 55% were in the sedentary/light category and 45% in the moderate ($P = 0.05$) (Table 6).

Discussion

Scientific evidence relating expenditure and daily energy requirements in MSUD children is limited, yet there are recommendations on energy intake for long-term monitoring of these children, whereas it is essential to promote energy balance and maintain proper metabolic control in order to avoid metabolic decompensation and promote weight–height growth and normal development (Lewis et al. 2001).

Despite finding inconsistencies between reported energy intake and energy expenditure measured by indirect calorimetry, which could be explained by underreporting and/or weaknesses of the data collection tool used, evaluation and assessment in this study allowed us to determine that for children with MSUD daily energy intake is recommended according to their basal and total energy expenditure.

Calculation of the BEE in children with MSUD would be underestimated by predictive equations used; however, in contrast to the inconsistency of the results reported in other IEM, our results indicate that the WHO equation can be used as an alternative method for BEE determination, if the final value is adjusted in calories (+133 kcal). The equation can provide clinically reliable data and can encourage the promotion and maintenance of energy and metabolic balance in MSUD children (Hauser et al. 2011; Quirk et al. 2010; Feillet et al. 2000).

It has been reported that factors including anthropometric variables such as height, BMI, body composition, and physical activity may contribute to the variation of the BEE in healthy individuals (Marugan de Miguelsanz et al. 2011). Groups were comparable in terms of these variables ($P < 0.05$); thus, it can be noted that the elements that determine BEE in healthy children may similarly affect children with MSUD.

For healthy reference populations, FFM explains 60–80% of the variance in BEE and is closely related to protein intake. This was demonstrated in the fact that no difference was found in body composition (FM and FFM) between the study and control groups and that FFM explained 81% of the variance in BEE for MSUD children.

It was confirmed that intake of high biological value protein (intact) in children with MSUD is restricted and 85.5% of it comes from the BCAA-free medical formula (synthetic protein) (Hauser et al. 2011; Marugan de Miguelsanz et al. 2011; Shimizu-Fujiwara et al. 2012).

Table 5 Level of agreement for predictive equations regarding actual basal energy expenditure

	MSUD							CONTROL						
		Difference BEE			Bland–Altman				Difference BEE			Bland–Altman		
	BEE (kcal/day)	% Adequacy	Kcal	*P**	r^a	CCC[b]	X[c]	BEE (kcal/day)	% Adequacy	Kcal	*P**	r^a	CCC[b]	X[c]
Indirect calorimetry	1,353							1,489						
OMS	1,269	94.5	74	0.00	0.786	0.607	−133	1,261	84.7	228	0.02	0.848	0.682	−120
Schofield	1,248	93.4	89		0.780	0.570	−145	1,231	82.7	258		0.787	0.563	−150

[a] Association level
[b] Concordance correlation coefficient
[c] X = average difference in kcal
*$p < 0.05$, Mann–Whitney test (Wilcoxon signed-rank test)

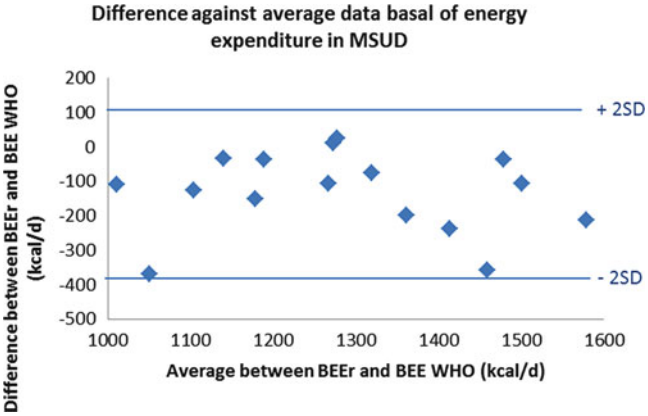

Fig. 1 Difference against average data of BEE (actual and estimated by WHO) in MSUD children

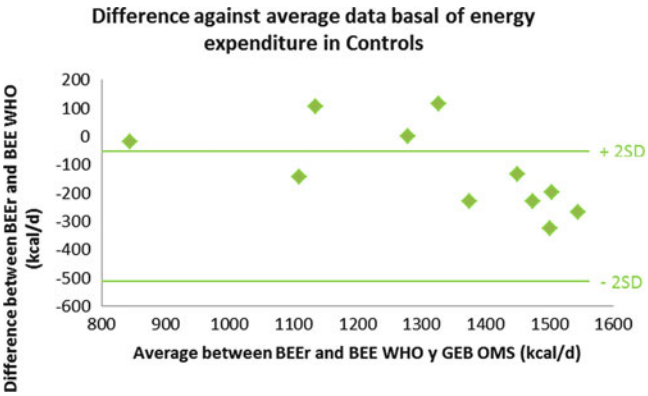

Fig. 2 Difference against average data of BEE (actual and estimated by WHO) in control children

Protein restriction and its quality have been described as possible causes of stunting in children with MSUD, and some research has found that this type of diet may affect BEE variability (Lewis et al. 2001; Nishimoto et al. 2012; Sentongo et al. 2000). However, it is important to highlight that in this group, the BEE did not correlate with any of the macronutrients nor did it relate to protein source.

In this study, both groups had stunted children, which suggest that the disease alone is not the decisive factor of linear growth. There may also be a genetic component because the Chilean population, in general, is smaller in size compared to international anthropometric standards. Considering this aspect, and that no association was established with the BEE, no adjustment by this variable was required as discussed in other studies (Rodríguez and Pizarro 2006).

To our knowledge, no previous studies have described PA in this specific group. In our sample of children with MSUD, some are less likely to exercise due to late diagnosis (>7 days old) with metabolic decompensation, which caused motor disabilities. According to the National Survey of Physical Activity and Sport Habits conducted in 2012, Chile's population lacks adequate PA habits. As a country, Chile has the second highest percentages of inactivity at the international level, which is reflected in the study group analyzed in this study (Deportes 2012). In our sample of MSUD and control children, 88% and 55%, respectively, were sedentary.

Table 6 Level of physical activity by group

	MSUD ($n = 16$)	CONTROL ($n = 11$)	P*
Physical activity factor (PAF)[a]			**0.0531**
Sedentary/ light	14 (88%)	6 (55%)	
Moderate	2 (12%)	5 (45%)	

[a] n (%)
*$p < 0.05$, T-Student

It is important to mention some limitations of the study that may attenuate the importance of the results obtained. First, sample size determines the variability of the data and our sample was small. Another limitation was the failure to obtain an assessment of pubertal development, a known predictor of BEE in these stages. Regarding the latter, it is noteworthy that the groups split into smaller and older than 10 years (MSUD <10 years, $n = 4$; >10 years, $n = 12$; CONTROL <10 years, $n = 2$; >10 years, $n = 9$) to evaluate energy expenditure in prepubertal and pubertal stage, finding intragroup differences in children with MSUD that would change the results found for children under 10 years; however, the size of these subsamples does not allow for statistical significant conclusions; thus, determining the effects on the BEE of this population is pending for a future study.

The high percentage of children with MSUD in Chile assessed in our sample is a strength of the study (55%) also and that it is pioneering study in the evaluation of these variables and the results obtained. Future studies should be conducted to improve the method of PA evaluation using direct assessment, which has greater validity and precision (Rodríguez and Terrados 2006). Additional studies could also clarify and explore the course of the disease in different stages of life.

Conclusion

For children with MSUD, energy recommendations are in accordance with energy expenditure. Our results indicate

that the predictive equation, FAO/WHO/UNU 2001, reliably estimates energy expenditure for MSUD children between 6 and 18 years and that it provides adequate energy prescription for achieving the goals of treatment of this disease such as promote anabolism, proper growth, and development and maintain energy and metabolic balance in all stages of development.

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Laboratory of Genetics and Metabolic Diseases – Thesis project.

Compliance with Ethics Guidelines

Conflict of Interest

Karen Campo, Gabriela Castro, Valerie Hamilton, Juan Francisco Cabello, Erna Raimann, Carolina Arias, and Verónica Cornejo declare that they have no conflict of interest.

Informed Consent

All procedures were in accordance with the ethical standards of the Instituto de Nutrición y Tecnología de Alimentos – INTA – ethics committee and with the Helsinki Declaration, the Nüremberg Code of 1975, and the regulations for ethics committees of the University of Chile. Informed consent was obtained from all participants.

Details of the Contributions of Individual Authors

1. Karen Campo: First author responsible for planning, conducting and executing the study, data analysis, and reporting of the work described in the article.
2. Gabriela Castro: Helped plan the study and reviewed the work described in the article.
3. Valerie Hamilton: Helped plan the study and reviewed the work described in the article.
4. Juan Francisco Cabello: Reviewer.
5. Erna Raimann: Reviewer.
6. Carolina Arias: Reviewer.
7. Verónica Cornejo: Mentor and Chief of Laboratory.

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RESEARCH REPORT

News on Clinical Details and Treatment in PGM1-CDG

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Abstract Phosphoglucomutase 1 deficiency has recently been reported as a novel disease that belongs to two different classes of metabolic disorders, congenital disorders of glycosylation (CDG) and glycogen storage diseases.

This paper focuses on previously reported siblings with short stature, hypothyroidism, increased transaminases, and, in one of them, dilated cardiomyopathy (DCM). An intronic point mutation in the PGM1-gene (c.1145-222 G>T) leads to a complex alternative splicing pattern and to almost complete absence of PGM1 activity.

Exercise-induced muscle fatigue, chest pain, and rhabdomyolysis persisted into adulthood. Fainting occurred during the first minutes of strong exercise due to glucose depletion and serum heart troponin was increased. A second wind phenomenon with an improvement in exercise capacity after some minutes of training was observed. Regular aerobic training improved fitness and helped to avoid acute damage. DCM improved during therapy.

Glycosylation deficiency was most prominent in childhood. Glycosylation improved with age and further improved with oral galactose supplementation even in adulthood. Optimal improvement of glycosylation-dependent phenotypes should be achieved by early and permanent galactose treatment.

However, in case of mutations in ZASP, DCM can develop as a consequence of impaired binding of PGM1 to the heart-specific isoform of ZASP, independently of overall glycosylation efficiency. Thus, even if mutations in PGM1 impair the function of the ZASP-PGM1 complex, supplementation of galactose cannot be expected to restore that function. Therefore, knowledge of PGM1 deficiency in a patient should prompt surveillance of early signs of DCM and specific treatment if necessary.

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Abbreviations

CDT	Carbohydrate-deficient transferrin
DCM	Dilated cardiomyopathy
ER	Endoplasmic reticulum
FS	Fractional shortening
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IGF-1	Insulin-like growth factor 1
LVIDD	Left ventricular internal dimension in diastole

PGM1	Phosphoglucomutase 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TF	Transferrin
ZASP	Z-band alternatively spliced PDZ-motif protein

Introduction

N-glycosylation is a common cotranslational modification of proteins and starts with the preassembly of oligosaccharides on dolichol, a lipid anchor in the ER membrane. The preassembled oligosaccharide is transferred en bloc by oligosaccharyltransferase to glycosylation consensus sites of the nascent protein. The glycoprotein is transferred to the Golgi by vesicular transport where the glycans are further processed. Congenital disorders of glycosylation (CDG) are a group of metabolic disorders affecting the synthesis and attachment (CDG-I) or the processing (CDG-II) of the glycan (Marquardt and Denecke 2003).

PGM1 deficiency is a novel treatable metabolic disorder and combines glycosylation abnormalities found in CDG-I and CDG-II (Tegtmeyer et al. 2014). Phosphoglucomutase functions in glycolysis and glyconeogenesis by catalyzing interconversion of glucose-1-phosphate and glucose-6-phosphate. Therefore, the liver is unable to maintain a normal glycemia, and muscles are not able to use glycogen as an effective energy source.

The clinical phenotype of PGM1 deficiency ranges from hypoglycemia, hepatopathy with elevated transaminases, cardiomyopathy, exercise intolerance, muscle weakness, rhabdomyolysis, growth retardation, hypogonadotropic hypogonadism to uvula bifida and/or cleft palate (Tegtmeyer et al. 2014).

The two patients described in this paper are siblings, a boy and a girl, mentioned as 5.1 and 5.2 in our previous publication (Tegtmeyer et al. 2014). Both show the typical clinical PGM1 phenotype. We present a more detailed analysis of the molecular defect, the biochemical consequences, and new observations on the effect of age on PGM1 deficiency-related glycosylation efficiency and discuss the specific treatment of DCM.

Materials and Methods

Patients

The parents of the patients are 2nd cousins (see Supplemental Fig. 1), healthy, and of normal height (mother: 165 cm, 60th percentile; father: 170 cm, 17th percentile; http://iea.de/perz/).

Patient 1, a boy, was born at term with normal body weight and length. After birth, a soft cleft palate was noted and closed by surgery 2 months later. Lumbar lordosis and disproportionate dwarfism were diagnosed at age 4, and disproportionate growth persisted to adulthood with an upper/lower body segment ratio of 1,43, i.e., more than 4 SD above the mean (Tanaka et al. 2004). At age 7, mild dilated cardiomyopathy was noticed. In recent years, during the first two minutes of strong exercise, he repeatedly experienced chest pain, breathing difficulties, and a feeling of nearly fainting. These symptoms disappeared with prolonged exercise and could be ameliorated by training. Three episodes of rhabdomyolysis with red urine were reported but only when strong exercise was started after a training pause of several weeks.

In the past, ravenous appetite sometimes occurred, and at the age of 12, a low glycemia (after overnight fasting) was found (2.8 mmol/l). The patient and his sister report a high consumption of milk products.

Currently, at the age of 25, the patient's height is 166 cm (<3rd percentile) with a normal body weight of 76 kg. The echogenicity of the liver is increased with reduced portal vein flow of 13 cm/s (reference mean ± SD = 20.4 ± 4.7 cm/s). Echocardiography showed a slightly enlarged left ventricle (LVIDD 60 mm, reference range is 41.5–57 mm, see supplement) with good systolic function (FS 31%, reference range (±2 SD) males 26.9–47.3%).

Patient 2, a girl, was born after 40 weeks of pregnancy with a length of 49 cm and a body weight of 3570 g. Immediately after birth, a Pierre–Robin sequence (with cleft hard and soft palate) and preauricular tissue were noted. A ventricle septum defect disappeared within the first year of life.

At the age of 18 months, dilated cardiomyopathy was diagnosed with a shortening fraction of 12% (Fig. 1). Captopril and metildigoxin increased the fractional shortening to the lower end of the normal range (Fig. 1). Metildigoxin was continued, and 18 month later, cardiac function was stable. However, in primary school, the cardiological situation got worse. Endomyocardial biopsies at 7.4 years did not show signs of acute or chronic myocarditis or virus infection, by histology/immunohistology and molecular biology. Storage diseases such as hematochromatosis, amyloidosis or M. Fabry were excluded. Some glycogen accumulation was shown by PAS staining in patient 2 but not patient 1. Cardiomyocytes with a considerable variety in size and hyperchromatic, pleomorphic nuclei and interstitial fibrosis were observed in patient 2 (Supplemental Fig. 2a–c). Electron microscopy of the biopsy revealed vacuolization, reduction in myofibrils, and fibrosis (Fig. 1a, b). Cardiac catheter examination disclosed mitral, tricuspid, and aortic valve insufficiency. She was listed for cardiac transplantation. After adding

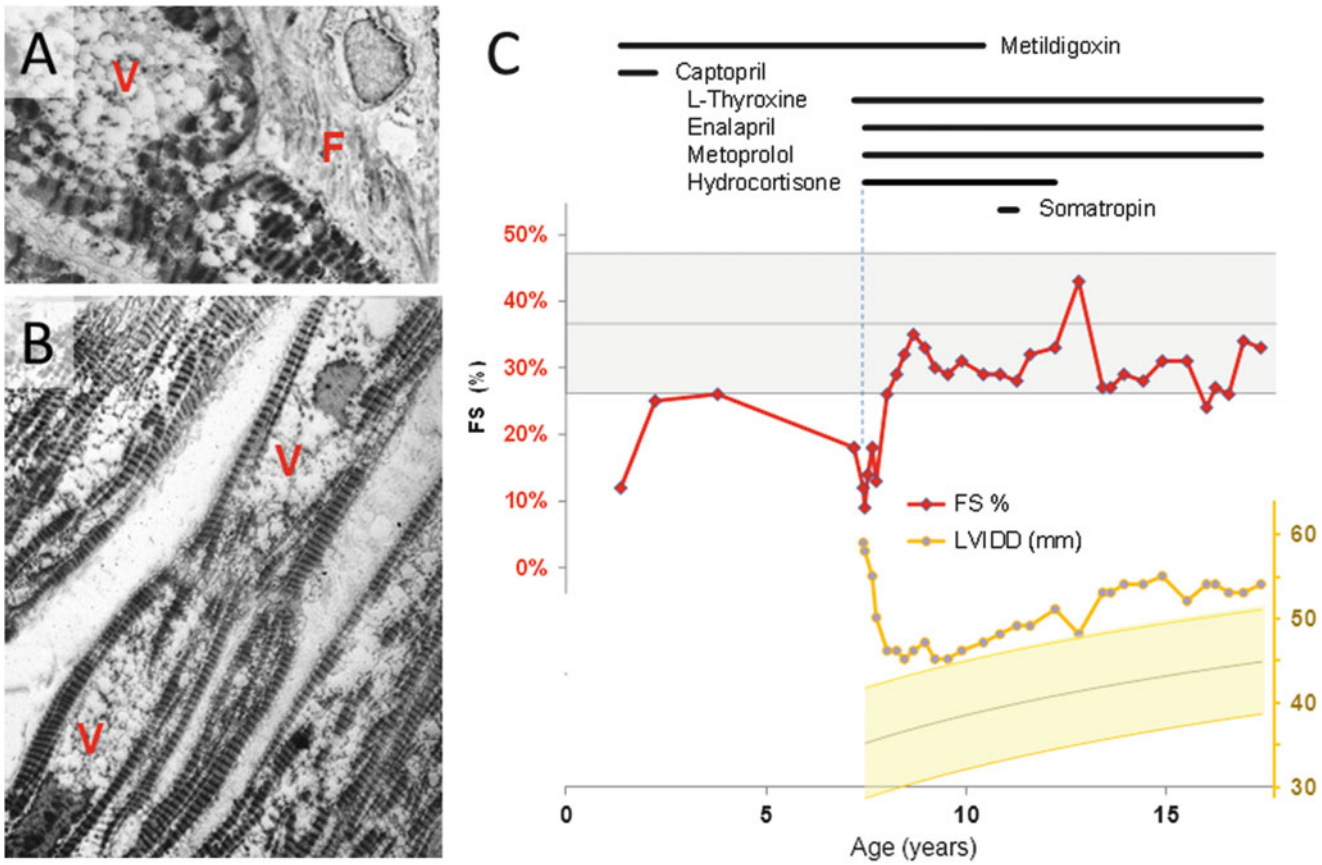

Fig. 1 Dilated cardiomyopathy in patient 2. (**a**, **b**) Electron microscopy demonstrating vacuolization (*red* "V"), fibrosis (*red* "F") and reduction in myofibrils. (**c**) Drug therapy and course of DCM. Reference range for fractional shortening (FS; *gray shaded*, mean over all age classes ±2 SD: 26.1–47.2%) was obtained from 48 females aged 8.1–17.8 (Punn et al. 2012). Dilated cardiomyopathy is represented as left ventricular inner diastolic diameter (LVIDD); a reference range (*yellow shaded*, mean ± 2 SD) for LVIDD was determined as described in supplemental material

L-thyroxin (for correction of hypothyroidism) and at 7.5 years enalapril, metoprolol, and hydrocortisone to her medication, she showed an enormous improvement, getting back to normal range of FS within 7 month (Fig. 1). However, LVIDD still was above the 2 SD range and in following years developed at such higher level in parallel to the normal growth of the left ventricle (Fig. 1).

Early dwarfism was recognized (Supplemental Fig. 3; however, not disproportionate, in contrast to her brother), and kyphoscoliosis. Currently, at the age of 21, the patient's height is 150 cm (<3rd percentile) and weight is 51 kg (between 10th and 25th percentiles). Fasting hypoglycemia was found at several moments (e.g., 1.6 mM at 2 years).

Growth hormone was given to both patients for a short period of time (see Supplemental Fig. 3). Growth hormone deficiency had been excluded, but a reduced growth hormone sensitivity due to hypoglycosylation was assumed, with low IGF-1 (0.4 μg/ml at 7 years, reference range 0.5–3 μg/ml).

For additional patient details, see Supplement.

IEF (Isoelectric Focusing) and SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

IEF and SDS-PAGE of serum TF were performed as described (Niehues et al. 1998).

HPLC (High-Performance Liquid Chromatography)

HPLC of carbohydrate-deficient transferrin (CDT) was performed as described (Biffi et al. 2007).

Cell Culture

A skin biopsy was taken from the patients after informed consent of the parents. Fibroblasts were cultured in Dulbecco's modified Eagle medium (Sigma), and 10%

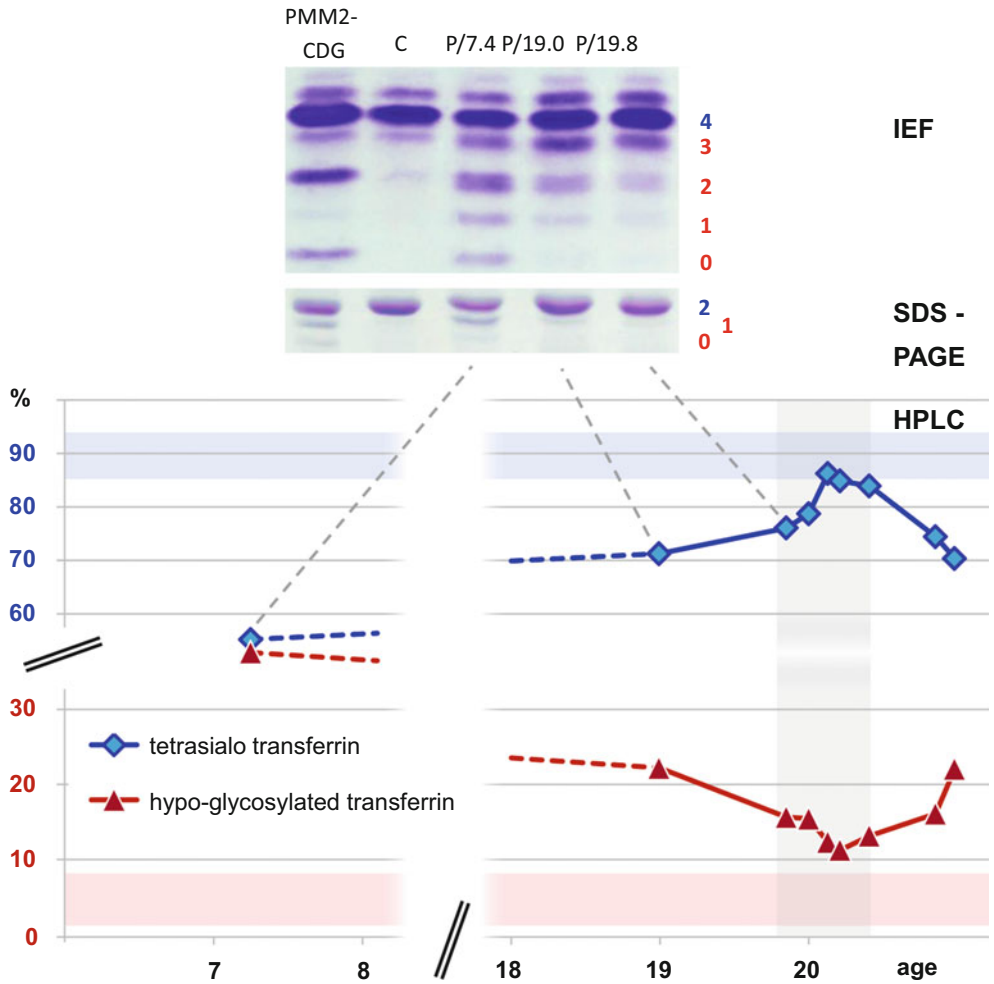

Fig. 2 Age dependency of glycosylation efficiency and galactose treatment effect in patient 2. Isoelectric focusing and SDS-PAGE of serum transferrin of a PMM2-CDG patient, healthy control (C) and patient 2 ("P/7.4": patient 2 at age 7.4 years). The numbers 0–4 at IEF indicate transferrin isoforms with four, three, two, one, or no sialic acid, while in SDS-PAGE 0, 1, and 2 indicate transferrin isoforms with 0, 1, or 2 occupied glycosylation sites. *Lower panel*: quantification by HPLC. *Horizontal blue* and *red shading* indicate the reference ranges for tetrasialotransferrin and hyposialotransferrin, respectively (Grundahl et al. 2012). Doubleband of patients disialotransferrin, known as "C2" variant (Helander et al. 2001), is explained in the Supplement. Note change from strong glycosylation deficiency at young age (7.4 years) to milder glycosylation deficiency as adult (19.0 years) in all panels (also in her brother; not shown). Further improvement in glycosylation during galactose treatment is shown in the *gray-shaded* region around age 20. Galactose treatment consisted of 1 g/kg/day galactose powder dissolved in water and divided into 5 doses. Transferrin isoform relative concentrations were obtained by HPLC. Hyposialotransferrin is the sum of isoforms with 0–3 sialic acids (*red line*). Tetrasialotransferrin normalizes under therapy (*blue line*; pentasialotransferrin not shown)

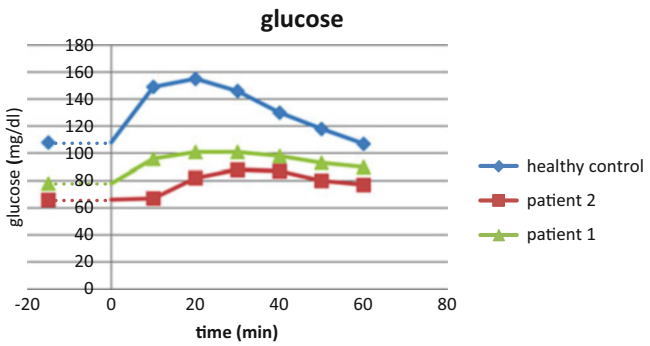

Fig. 3 Glucagon test. Baseline glycemia was determined 15 min before the test. Glucagon injection at timepoint 0 was followed by determination of glycemia every 10 min

fetal calf serum (Invitrogen, Darmstadt, Germany) and 1% L-glutamine-penicillin-streptomycin (PAA) were added.

Cell Isolation

Fibroblasts (80–90% confluence) were washed with phosphate buffered saline (PBS) and trypsinized. Cell pellets were washed in PBS and after centrifugation for 5 min at room temperature put in HEPES–sucrose buffer (0.2 M sucrose, 0.02 M HEPES pH 7.3) containing protease inhibitors (complete, Roche; 10 µg/ml pepstatin A, 10 µg/ml PMSF).

Gene and Protein Analysis

For detailed mutation analysis of PGM1 and transferrin, see supplemental data. Also read the supplement for the Western Blot description.

Functional Tests: Glucagon Test and Galactose Kinetics

Blood samples were taken after an overnight fast to determine the initial values. For the glucagon test, 1 mg GlucaGen (Novo Nordisk Pharma GmbH, Mainz, Germany) was injected intravenously, and blood samples were taken every 10 min for 1 h. The kinetics of blood glucose has been tested.

Determination of galactose kinetics in whole blood was performed as described in Tegtmeyer et al. (2014). Galactose powder was supplied by falcento AG, Kreuzlingen, Switzerland

Results

Analysis of Serum TF Glycosylation

Reduced glycosylation of transferrin in our patients, including an unusual mixture of CDG-I and -II patterns, has been discussed earlier (Tegtmeyer et al. 2014). Subsequently, we discovered that both patients showed considerably improved glycosylation with increasing age. HPLC quantification demonstrates that hyposialylated transferrin isoforms (i.e., specifically those with two, one, or no sialic acid) are clearly further reduced by galactose treatment (Fig. 2). Tetrasialotransferrin increased to 87 % under galactose (normal range: 85.6–94% [mean ± 2 SD, Grundahl et al. 2012]). After stopping galactose supplementation, glycosylation efficiency returned to pretreatment values. For quantification of transferrin isoforms by HPLC of patient 1, see Supplemental Table 1.

Mutation analysis

The patients mutation in *PGM1* at g.55020 from G>T (c.1145-222 G>T) creating a new splice site has been described by Tegtmeyer et al. (2014). While the normal donor splice site at exon 7 is not mutated, nevertheless the new splice site is used instead with high preference in splicing to exon 8 acceptor. However, beyond the published result, while, at first glance sequencing data looked somewhat noisy, a more detailed analysis demonstrated, that two new splice variants containing additional intron sequences occur (Exon 7a or Exon 7b, see Supplemental Fig. 4). While real-time quantification had demonstrated patients' total PGM1-mRNA level about 2% of normal (Tegtmeyer et al. 2014), we demonstrate here that the regular transcript in fact is reduced to about 1/400, i.e., 0.25% of normal control. Details of the transcript analysis, the effect of nonsense-mediated decay, and quantification of the correctly spliced transcript are given in the supplementary material (Supplemental Figs. 4 and 5). Western blot does not show protein at all (Tegtmeyer et al. 2014). Corresponding to low mRNA level, only less than 0.3% of normal PGM1 protein was expected.

Glucagon Test and Galactose Kinetics

Glucagon test and galactose kinetics were performed with patients and a healthy control. A diagnostic glucagon test was performed in order to check gluconeogenesis and to reveal abnormalities compared to our healthy control. The patient's fasting glycemia was lower than in our healthy control (healthy control: 108 mg/dl, patient 2: 66 mg/dl and patient 1: 78 mg/dl), and glucose concentrations reached a lower level and showed a delayed reaction compared to our healthy control (Fig. 3). Since patients with a PGM1 deficiency cannot efficiently obtain glucose from their glycogen stores, the increase of blood glucose is probably mainly due to gluconeogenesis.

Discussion

PGM1 deficiency is a novel treatable metabolic disorder which combines CDG and glycogenosis. Regular supplementation of oral galactose shows a remarkable biochemical improvement of glycosylation, and complex carbohydrates stabilize blood glucose (Tegtmeyer et al. 2014). We here report further data on the sib pair 5.1/5.2 of that publication. In particular, we observed that the serum TF hypoglycosylation was more pronounced in childhood than in adulthood (Fig. 2). A similar phenomenon of

pronounced hypoglycosylation in early childhood that improved over time and could be further improved by galactose supplementation has been observed in UDP-galactose transporter deficiency (Dörre et al. 2015). It can be argued that the growing organism has a higher demand for glycosylation of all those proteins of the growing body, while the adult needs to keep steady state only and limited substrate allows higher percentage of normal glycosylated proteins in steady state. Thus, for CDG that lead to reduced speed of glycan production, one might generally expect a stronger hypoglycosylation in children than in adults.

Improved glycosylation should also improve IGF1 function and growth (Tegtmeyer et al. 2014). In addition, mutations in *XYLT1* have been shown to cause short stature (Schreml et al. 2014). The encoded protein xylosyltransferase (XT1) is necessary to start proteoglycan production with the common linker: protein-Ser-O-Xyl-Gal-Gal-GlcA, i.e., requiring two galactose molecules. Thus, PGM1 deficiency causing low levels of activated galactose may promote short stature also via inefficient proteoglycan production. It is not clear yet how much different pathways contribute to short stature. It may be possible that different degree of CDG-I vs. CDG-II component and total glycosylation efficiency, as seen in both patients, depending on age and galactose supply (Fig. 2), affect the pathways differently. Such differences may also be related to the degree of disproportionate growth. In addition to galactose, puberty-adjusted growth hormone or rhIGF-1 therapy may further improve growth (Mauras et al. 2000). It has been demonstrated in more recent literature that the typical growth spurt during puberty is associated with a doubling of GH amplitude and corresponding increase in IGF-1. Most significant effects using therapeutic GH for treatment of short stature were obtained when this normal doubling of GH was also mimicked in therapy by increasing GH to 0.7 mg/kg b.w. per week (Mauras et al. 2000). Recently, it has been suggested that early installation of rhIGF-1 therapy may efficiently improve growth in another congenital disorder of glycosylation, PMM2-CDG (Miller et al. 2013). Therefore, early start of rhIGF-1 therapy and an increased dosing scheme during puberty may help to improve outcome.

The rationale for galactose treatment is that protein misglycosylation in PGM1 deficiency is caused by the decrease of UDP-galactose. Galactose supplementation provides additional galactose and fills up the UDP-Gal pool: Gal-1-P+ UDP-Glc→UDP-Gal+Glc-1-P (Tegtmeyer et al. 2014). Determination of galactose kinetics in whole blood was performed in order to see galactose absorption in a patient with PGM1 deficiency compared to a healthy control (Supplemental Fig. 6). Blood galactose was measured for patient 2 and healthy control during the test. A second galactose kinetic test was performed while under galactose treatment. After oral galactose consumption of 0.3 g/kg body weight, whole blood values of the control increased up to 0.7 mmol/l, while patient 2 only showed concentrations of up to 0.2 mmol/l. Lower galactose peak concentrations in the patient may result from reduced absorption in PGM1 deficiency or from a stronger first pass effect in the liver so that an increase of galactose in the blood can almost not be measured. A permanent galactose treatment might fill up pools, and indeed under this condition, a galactose bolus resulted in higher galactose concentrations.

The reported feelings of discomfort during exercise on a bike in untrained condition were not correlated to electrocardiogram changes. However, on a treadmill, perceived exertion up to a point of muscle fatigue, chest pain, and breathing difficulties and exercise even beyond that point were similar as in patients with myophosphorylase deficiency (MD, McArdle's disease). McArdle's disease patients develop a second wind after 6–9 min of continuous exercise which results from an increased capacity for oxidative phosphorylation and an increased availability of extramuscular fuels (Haller and Vissing 2002). Since phosphoglucomutase deficiency and McArdle's disease both block the production of glucose from glycogen, the same second wind mechanism might be assumed. In McArdle disease, where glycogen usage is impaired in muscles only (defect of myophosphorylase), but not in the liver, it has been shown that during exercise, muscles use more blood born glucose and liver fills up the blood glucose. In PGM1-patients, however, also the liver cannot quickly deliver glucose from glycogen, so that increased use of blood born glucose by PGM1-deficient muscles meets reduced possibilities of PGM1-deficient liver to stabilize blood glucose, resulting in acute reduction of blood glucose. Thus, there is strong evidence that hypoglycemia, which already may occur in PGM1-patients without doing strong exercise by simply overnight starving (Tegtmeyer et al. 2014), will be exaggerated by strong exercise and can explain the fainting experienced by our patient. Fainting did not occur on a cycle ergometer but only on a treadmill. Generally, heart rate is higher on a treadmill than with cycling (Tsintzas et al. 2003), and blood glucose falls in the first minutes on a treadmill even stronger than at cycling, causing a nearly fainting in our patients.

Dilated cardiomyopathy can result from mutations in the protein ZASP (Vatta et al. 2003). An association of mutations in the ZASP protein (exon 4, 6, or 10) and the binding of PGM1 was first described by Arimura et al. and suggested an involvement of a deficient ZASP-PGM1 interaction in the pathogenesis of dilated cardiomyopathy (Arimura et al. 2009). In patient 1, we could now demonstrate that under strong exercise on a treadmill that lead to rhabdomyolysis, also the marker for damage of the heart muscle, troponin I, increased to a level at the warning threshold for myocardial infarction (0.04 ng/ml, peak

extrapolated with value at timepoint of blood sampling – about 1 week after exercise – to the time after exercise). Severe DCM can be effectively treated with standard medication; however, in addition, improvements of citrate cycle by anaplerotic supplements and training, both improving oxidative energy production, are suggested to reduce dependency on PGM1-ZASP interaction and thus to reduce likelihood for developing clinical relevant DCM. Indeed, we now discovered that rhabdomyolysis occurred at a specific strong level of exercise but only if there was a training pause of several weeks, and the patient observed that regular (moderate) training had a protective effect.

Regular supplementation with oral galactose induced a considerable biochemical improvement of glycosylation. Early detection, e.g., with the diagnostic scheme for PGM1 deficiency created by Tegtmeyer et al. (2014), and early galactose supplementation can ameliorate some aspects of this disease. We discussed earlier that simply knowledge of PGM1 deficiency may allow to prevent some consequences, e.g., rhabdomyolysis and sudden death by guidance to avoid strong exercise. However, by regular surveillance, it will also be possible to detect signs of DCM development early and to initiate a specific therapy to prevent clinical obvious DCM.

Compliance with Ethics Guidelines

Author Contributions

Esther Schrapers did most of the experiments, retrieved the data from decades, analyzed data, drafted, and revised the manuscript.

Laura C. Tegtmeyer was involved in several experiments, e.g., galactose kinetics.

Gunter Simic-Schleicher discovered the patients and provided many data.

Volker Debus provided the data on DCM.

Janine Reunert supported and supervised experiments.

Sebastian Balbach was involved in glucagon test and galactose kinetics.

Karin Klingel did light and electron microscopy incl. interpretation.

Ingrid Du Chesne was involved in analysis of mutation.

Anja Seelhöfer supported detailed analysis of transcript.

Manfred Fobker analyzed the clinical chemistry data.

Thorsten Marquardt supervised the study, drafted, and revised the manuscript.

Stephan Rust supervised the study, supported detailed analysis of transcript, analyzed data, drafted, and revised the manuscript.

Thorsten Marquardt and Stephan Rust have contributed equally to the manuscript.

All authors contributed to and reviewed the manuscript.

Conflict of Interest

Esther Schrapers, Laura C. Tegtmeyer, Gunter Simic-Schleicher, Volker Debus, Janine Reunert, Sebastian Balbach, Karin Klingel, Ingrid Du Chesne, Anja Seelhöfer, Manfred Fobker, Thorsten Marquardt, and Stephan Rust 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. Informed consent was obtained from all patients for inclusion in the study.

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RESEARCH REPORT

Safety and Efficacy of Chronic Extended Release Cornstarch Therapy for Glycogen Storage Disease Type I

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Abstract *Background*: Glycogen storage disease type I (GSD I) causes severe hypoglycemia during periods of fasting since both glycogenolysis and gluconeogenesis are impaired. Primary treatment in North America consists of cornstarch therapy every 3–4 h. Waxy maize extended release cornstarch was introduced for maintaining overnight glucose concentrations, but no studies have assessed long-term safety and efficacy of the product.

Objective: To demonstrate the safety and efficacy of modified cornstarch in GSD I.

Design: An open-label overnight trial of extended release cornstarch was performed. Subjects with a successful trial (optimal metabolic control 2 or more hours longer than with traditional cornstarch) were given the option of continuing into the chronic observational phase. Subjects were assessed biochemically at baseline and after 12 months.

Results: Of the 106 subjects (93 GSD Ia/13 GSD Ib), efficacy was demonstrated in 82 patients (88%) with GSD Ia and 10 patients (77%) with GSD Ib. The success rate for extending fasting was 95% for females and 78% for males. Of the patients who entered the longitudinal phase, long-term data are available for 44 subjects. Mean duration of fasting on traditional cornstarch prior to study for the cohort was 4.1 and 7.8 h on the extended release cornstarch ($P < 0.001$). All laboratory markers of metabolic control have remained stable in the chronically treated patients.

Conclusion: Extended release cornstarch appears to improve the quality of life of patients with GSD I without sacrificing metabolic control. Avoiding the overnight dose of cornstarch should enhance safety in this population.

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
GSD	Glycogen storage disease

Introduction

The hepatic glycogen storage diseases are a group of inherited disorders characterized by the abnormal storage or release of glycogen (Wolfsdorf and Weinstein 2003). The inability to release glycogen as glucose during periods of fasting results in marked hypoglycemia. In glycogen storage disease type I (GSD I), hypoglycemia is severe since all endogenous glucose production is impaired, and the shunting of glucose-6-phosphate into alternative pathways results in the accumulation of uric acid, triglycerides, and lactate (Kishnani et al. 2014).

The primary goal of treatment for both GSD Ia and Ib is to maintain a normal blood glucose concentration to prevent neuroglycopenia and the associated acidosis by ameliorating the counter-regulatory response. In the early 1980s, uncooked cornstarch was introduced as a treatment for maintaining normoglycemia (Chen et al. 1984), and it has remained the mainstay of therapy in North America (Koeberl et al. 2007). While cornstarch has dramatically improved the quality of

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life and clinical outcome for patients with GSD I, it has a limited duration of action. A previous study suggests that it only prevents hypoglycemia for a median time of 4.25 h in children (Weinstein and Wolfsdorf 2002). All children must wake up in the middle of the night for therapy, and delayed administration of the cornstarch can be associated with development of hypoglycemia, seizures, neurologic injury, and death. Even as adults, almost all patients still require therapy every 4–5 h, and overnight administration of cornstarch is required in over 90% of patients to achieve optimal metabolic control (Weinstein and Wolfsdorf 2002).

We previously reported on the efficacy of an extended release cornstarch preparation for maintaining glucose concentrations during the overnight period (Correia et al. 2008). In 2009, the extended release waxy maize cornstarch Glycosade® (Vitaflo International Ltd, Liverpool, England) was approved in the United Kingdom for treatment of GSD I, and it was released as a medical food in the United States in 2012. While this extended release formulation has been used for several years, there have not been any publications reporting the long-term safety and efficacy of the product. We now describe our long-term, overnight experience with the extended release cornstarch in a cohort of patients with GSD I.

Subjects and Methods

Subjects

Patients followed by the University of Florida Glycogen Storage Disease Program with genetically proven GSD I were eligible to participate. Inclusion was limited to patients 5 years of age and older based upon the recommendations for use of the product in the United States. From 2012–2013, patients were given the option of trying the extended release cornstarch overnight. A total of 106 eligible subjects ranging from 5 to 60 years of age chose to participate. This cohort consisted of 93 subjects with GSD Ia (43 males/50 females), 13 with GSD Ib (7 males/6 females). Patients who clinically did well on the extended release formulation were given the option of participating in a longitudinal study. These studies were approved by the Institutional Review Board of the University of Florida, and informed consent and assent from the child, when under the age of 18, was obtained prior to enrollment in this study.

Study Design

The overnight challenge was performed using the protocol outlined by Correia et al. (2008). Since dosing standards for the extended release cornstarch have not been established, the dose of the product for the overnight challenge was calculated by replacing the carbohydrate load from traditional cornstarch with a comparable amount of the new waxy maize formulation. An in-dwelling intravenous catheter was placed, and hourly monitoring of glucose and lactate was performed using a YSI 2300 STAT Plus™ Glucose & Lactate Analyzer (YSI Incorporated, Yellow Springs, Ohio) until glucose concentrations fell to 70 mg/dL or patients fasted for 12 h. Lactate concentrations over 5 mmol/L were also established as criteria for ending the trial, but these stop criteria were never reached.

Following completion of the overnight trial, patients were given the option of staying on the product chronically during the overnight period. For inclusion in the follow-up safety and efficacy studies, the extended release cornstarch needed to be consumed at least 3 nights per week. All patients remained on their baseline cornstarch therapies throughout the day. Doses of both the daytime and overnight cornstarch preparations were titrated over the course of the year based upon home glucose monitoring as per standard GSD I care to maintain glucose concentrations over 75 mg/dL (4.2 mmol/L). The following laboratory data were collected at baseline and twelve months: aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, triglycerides, uric acid, total protein, and albumin.

Statistical Analysis

For the overnight trial, success was defined as maintaining glucose concentrations above 70 mg/dL at least 2 h longer than with traditional uncooked cornstarch with comparable markers of metabolic control. Statistical analysis was performed using paired two-tailed *t*-tests to determine whether there were differences between the metabolic markers before treatment and after 12 months. P values <0.05 were considered statistically significant.

Results

Overnight Challenge

A total of 106 patients (50 males/56 females) participated in the overnight challenge. Efficacy for extending fasting for 2 h beyond baseline was demonstrated for 88% patients (82/93) with GSD Ia and 77% (10/13) with GSD Ib. The success rate for extending fasting was 95% (53/56) for females and 78% (39/50) for males.

Long-Term Safety and Efficacy Follow-Up

Of the initial 106 patients who underwent the overnight fasting challenge, 77 patients chose to be followed as part of

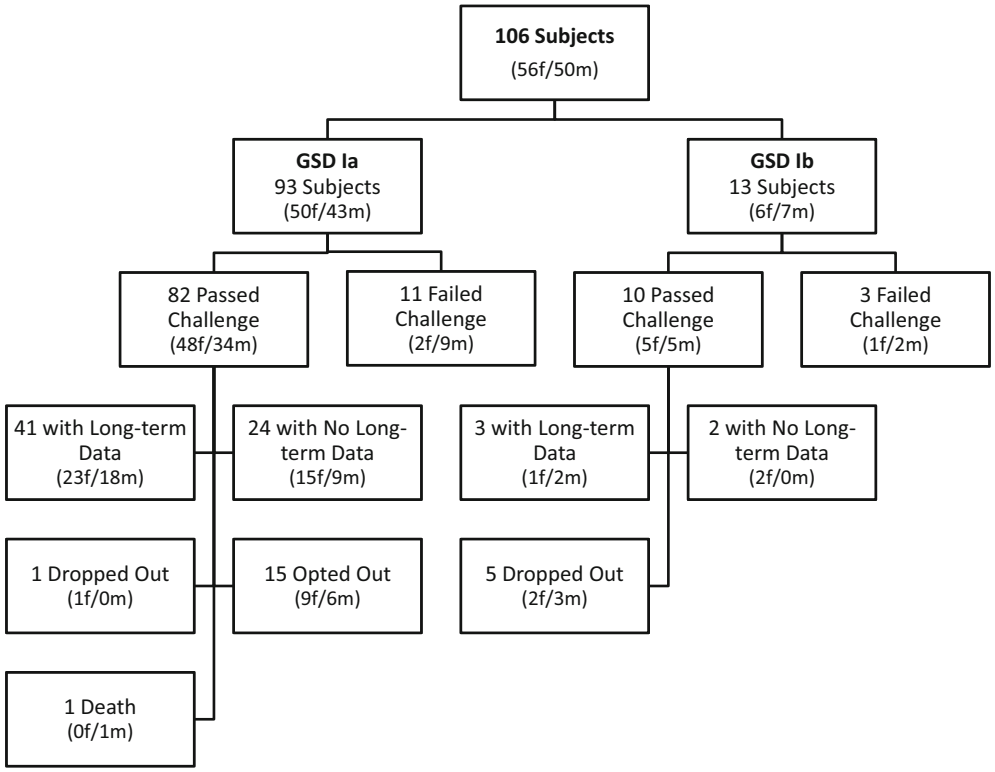

Fig. 1 Outcome of participants who attempted waxy maize cornstarch

a protocol aimed at determining long-term efficacy and safety of the extended release cornstarch (67 GSD Ia, 10 GSD Ib). Of the subjects who entered the longitudinal phase of the study, long-term data are available for 44 subjects; 26 subjects remained on the waxy maize cornstarch, but long-term data could not be obtained; 6 subjects dropped out of the study (1 GSD Ia and 5 GSD Ib) due to discontinuation of the experimental product; and 1 person with GSD Ia died during the study after missing the waxy maize dose and going all night with no therapy. Fourteen subjects were not included in the chronic phase of the study after failing the initial overnight challenge (11 GSD Ia, 3 GSD Ib), and 15 subjects who passed the overnight challenge opted not to participate in the longitudinal phase (Fig. 1). Gastrointestinal intolerance or exacerbation of inflammatory bowel disease and hypoglycemia were the reasons provided by the GSD Ib patients who dropped out, while worsening metabolic control was the primary reason for withdrawing in the GSD Ia population. Figure 2 shows the total duration of fasting increase on waxy maize compared with uncooked cornstarch. The mean duration of overnight fasting on traditional cornstarch prior to the study for the cohort was 4.2 and 7.8 h on the extended release cornstarch for both the GSD Ia and Ib populations ($P < 0.001$ for all types; Figs. 3 and 4). All laboratory markers of metabolic control have remained stable in the chronically treated patients (Tables 1 and 2).

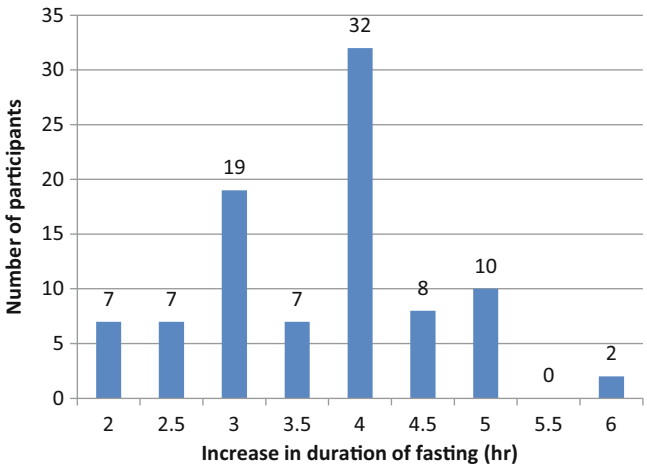

Fig. 2 Duration of fasting increase on waxy maize cornstarch compared with uncooked cornstarch (mean = 3.7 ± 0.9 h)

Discussion

Prior to the description of continuous glucose therapy for treatment of GSD I, most patients with glycogen storage disease did not survive (Crigler and Folkman 1977). With the introduction of cornstarch therapy in 1982, the prognosis for children and adults with GSD I improved, but dosing every 3–5 h has been required to achieve optimal metabolic

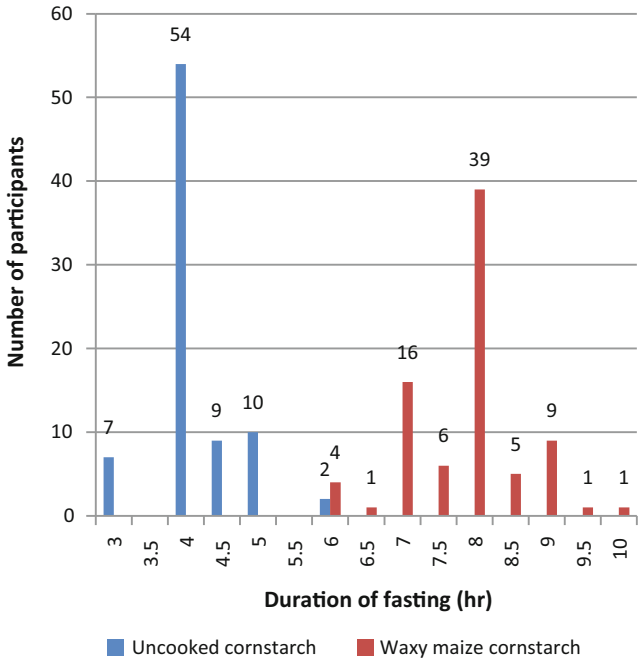

Fig. 3 Duration of fasting with uncooked cornstarch (mean = 4.2 ± 0.5 h) and waxy maize cornstarch (mean = 7.9 ± 0.8 h) for glycogen storage disease type Ia

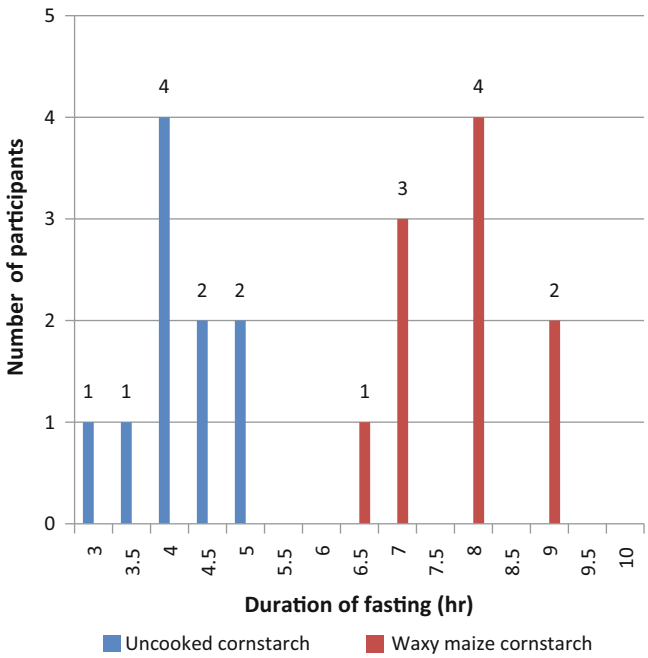

Fig. 4 Duration of fasting with uncooked cornstarch (mean = 4.2 ± 0.6 h) and waxy maize cornstarch (mean = 7.8 ± 0.9 h) for glycogen storage disease type Ib

control (Weinstein and Wolfsdorf 2002). Extended release waxy maize cornstarch was introduced in 2009 as the first new treatment for the hepatic GSDs, but the product was approved as a medical food based solely upon short-term studies (Bhattacharya et al. 2007). Long-term complications in GSD I can be delayed or prevented with achievement of optimal metabolic control (Beegle et al. 2015; Minarich et al. 2012; Tsilianidis et al. 2013; Wang et al. 2011). It is therefore imperative that outstanding metabolic control is maintained, and it is reassuring that the biochemical studies have remained stable in this cohort chronically treated with the new therapy.

As with traditional cornstarch, therapy with the extended release formulation must be individualized. Not all patients responded well when challenged with the extended release formulation. In fact, 10 (9 GSD Ia/1 GSD Ib) patients developed hypoglycemia within 5 h during the initial challenge, and therefore initiation of the therapy is recommended in an in-patient setting where close monitoring can occur. GSD Ia patients that failed typically were in the pubertal growth spurt. Active inflammatory bowel disease in both GSD Ia and Ib was also found to be associated with treatment failure, but it is not clear if this was due to abnormal absorption or increased metabolic demand. Exacerbations of inflammatory bowel disease with increased stool frequency and worsening inflammatory markers were the primary cause of treatment failure and discontinuation of the waxy maize cornstarch in the GSD Ib population. The increase in gastrointestinal problems contributed to the worse outcome in GSD Ib when compared with the GSD Ia population.

It is important to note that none of the published studies to date have investigated use of the extended release cornstarch in children under 5 years of age. Hypoglycemia in younger children has been associated with increased neurologic sequelae (Flykanak-Gantenbein 2004), and use in this vulnerable population cannot be recommended until a study has been performed. Similarly, there have been very limited data published to date on daytime use of the waxy maize cornstarch (Bhattacharya et al. 2015). Formal investigation of daytime use is warranted since higher energy demands during the day may not be adequately covered with the slower release therapy.

The extended release cornstarch appears to improve quality of life without sacrificing metabolic control. Since the middle-of-the-night dose of cornstarch is avoided, fewer episodes of overnight hypoglycemia may result and should improve compliance in teenagers and young adults who historically have struggled with transitioning to independence (Storch et al. 2008). While the therapy appears to be safer than traditional cornstarch, administration of the product is critical, and missed therapy can have dire consequences as occurred with the death of a 13-year-old young man followed as part of this study. His death occurred after he missed his overnight dose of the waxy maize therapy, and he experienced severe hypoglycemia the next morning which led to seizure and ultimately his

Table 1 Glycogen storage disease type Ia metabolic markers before and after extended release cornstarch therapy

	Pre-therapy [mean ± SD ($n = 41$)]	Pre-therapy median	Pre-therapy range	Post-therapy (12 months) [mean ± SD ($n = 41$)]	Post-therapy median	Post-therapy range	*P*-value
AST (U/L)	29.7 ± 10.9	26	16–65	32.0 ± 17.7	29	9–97	0.43
ALT (U/L)	27.0 ± 17.0	22	7–89	31.9 ± 25.9	24	7–153	0.23
Cholesterol (mg/dL)	179.6 ± 42.5	177	95–285	187.1 ± 43.8	188	99–279	0.18
Triglycerides (mg/dL)	265.2 ± 147.3	235	77–732	293.8 ± 166.8	272	66–745	0.28
Uric acid (mg/dL)	6.2 ± 1.7	6.1	3.2–10.5	6.2 ± 1.7	6	3.1–10.5	0.96
Protein (g/dL)	7.2 ± 0.5	7.2	6.0–8.1	7.2 ± 0.4	7.2	6.6–8.9	0.98
Albumin (g/dL)	4.4 ± 0.3	4.3	3.7–5.0	4.4 ± 0.3	4.4	3.5–5.1	0.31

Table 2 Glycogen storage disease type Ib metabolic markers before and after extended release cornstarch therapy

	Pre-therapy [mean ± SD ($n = 3$)]	Pre-therapy median	Pre-therapy range	Post-therapy (12 months) [mean ± SD ($n = 3$)]	Post-therapy median	Post-therapy range	*P*-value
AST (U/L)	19.0 ± 5.6	18	14–25	18.3 ± 4.9	16	15–24	0.69
ALT (U/L)	8.7 ± 3.8	7	6–13	8.7 ± 0.6	9	8–9	1.00
Cholesterol (mg/dL)	98.3 ± 4.5	98	94–103	113.5 ± 21.9	114	98–129	0.40
Triglycerides (mg/dL)	95.7 ± 43.0	88	57–142	78.0 ± 24.0	78	61–95	0.17
Uric acid (mg/dL)	8.4 ± 1.1	8.6	7.2–9.4	7.1 ± 0.6	6.8	6.7–7.7	0.30
Protein (g/dL)	8.1 ± 0.6	8.1	7.5–8.7	7.5 ± 0.4	7.45	7.2–7.7	0.64
Albumin (g/dL)	4.0 ± 0.5	4.1	3.5–4.4	3.9 ± 0.4	4.1	3.5–4.2	0.42

demise. There is no difference between missed doses of traditional therapy and the extended release therapy, but the longer duration between doses increases the risk. The higher cost of the new therapy must also be considered as the extended release formulation is about 15 times more expensive than regular cornstarch (approximately $8.00 per night compared with $0.56 per night for regular cornstarch). However, an emergency department visit due to a missed overnight cornstarch dose would be more expensive than several years of the extended release formulation.

In conclusion, patients benefited from the extended release cornstarch by avoiding the overnight dose while maintaining metabolic control. Future studies are warranted, however, on daytime use and in younger children. Even with this reassuring experience, patients should continue to be followed closely until more experience has been gained with this therapy.

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Compliance with Ethics Guidelines

Conflict of Interest

Katalin M. Ross, Laurie M. Brown, Michelle M. Corrado, Tayoot Chengsupanimit, Latravia M. Curry, Iris A. Ferrecchia, Laura Y. Porras, Justin T. Mathew, and David A. Weinstein 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.

Details of the Contributions of Individual Authors

Katalin Ross designed the research study, conducted the research, and contributed to the data collection, data analysis, writing of the manuscript, and approval of the final content of manuscript.

Laurie Brown conducted the research, data analysis, data collection, writing of the manuscript, and approval of the final content of manuscript.

Michele Corrado designed the research study and data analysis and contributed to the data collection, writing of the manuscript, and approval of the final content of manuscript.

Tayoot Chengsupanimit contributed with the data analysis, writing of the manuscript, and approval of the final content of manuscript.

Latravia Curry contributed to the data collection, data analysis, writing of the manuscript, and approval of the final content of manuscript.

Iris Ferrecchia contributed to the data collection, data analysis, writing of the manuscript, and approval of the final content of manuscript.

Laura Porras contributed to the data collection, data analysis, writing of the manuscript, and approval of the final content of manuscript.

Justin Mathew contributed with the statistical analysis, writing of the manuscript, and approval of the final content of manuscript.

David Weinstein designed the research study, conducted the research, and contributed to the data collection, data analysis, writing of the manuscript, and approval of the final content of manuscript.

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CASE REPORT

Normal Neurodevelopmental Outcomes in PNPO Deficiency: A Case Series and Literature Review

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Abstract Pyridox(am)ine 5′-phosphate oxidase deficiency results in an early-onset neonatal encephalopathy that can be fatal if not detected and treated early. The condition is rare, can result in preterm delivery, and can mimic hypoxic ischemic encephalopathy. Thus, suspicion of the diagnosis, appropriate investigations, and therapeutic trials with pyridoxal-5′-phosphate are often delayed. In this paper we report four cases of pyridox(am)ine 5′-phosphate oxidase deficiency, two of whom are siblings. Three were treated with pyridoxal-5′-phosphate in the first few days of life and the fourth within the first month. One of the siblings was electively treated from birth until a diagnosis was secured. Our cases demonstrate that early diagnosis and treatment can be associated with normal neurodevelopment in childhood. We suggest that a low threshold for investigating for pyridox(am)ine 5′-phosphate oxidase deficiency and electively treating with pyridoxal-5′-phosphate is considered in any neonate with encephalopathy, including those with presumed hypoxic ischemic encephalopathy in whom the degree of encephalopathy is not expected from perinatal history, cord gases and/or neuroimaging.

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Introduction

Pyridoxal-5′-phosphate (PLP), the active form of vitamin B6, is a cofactor for over 100 different metabolic reactions integral to normal cellular health and maintenance (Hoffmann et al. 2007; Clayton 2006). Examples of metabolic processes requiring PLP as a cofactor include the synthesis of nucleic acids, haemoglobin, sphingomyelin and other sphingolipids; synthesis of neurotransmitters such as dopamine, norepinephrine and gamma-aminobutyric acid (Clayton 2006); and the metabolism of some amino acids and glycogen (Clayton 2006).

Evidence to support the protean importance of PLP is demonstrated in clinically and biochemically significant manifestations of the B6 deficiency disease states. The classic examples are the inborn errors of metabolism (IEM) associated with B6 synthesis, pyridoxine-dependent epilepsy (PDE, OMIM 266100) and pyridox(am)ine 5′-phosphate oxidase (PNPO) deficiency (OMIM 610090). The role of PLP depletion in these disease processes occurs

via varied mechanisms. PDE is caused by mutations in the *ALDH7A1* gene that encodes the enzyme antiquitin, which is involved in cerebral lysine catabolism (Stockler et al. 2011). A block at the level of antiquitin leads to the accumulation of piperideine-6-carboxylic acid (P6C), which inactivates PLP via a Knoevenagel condensation.

PNPO deficiency is a rare autosomal recessive IEM. To date there have been <40 cases reported in the medical literature (Mills et al. 2005, 2014; Hoffmann et al. 2007; Bagci et al. 2008; Ruiz et al. 2008; Schmitt et al. 2010; Veerapandiyan et al. 2011; Ware et al. 2014; Plecko et al. 2014; Porri et al. 2014). The initial clinical reported phenotype of PNPO deficiency included prematurity, early-onset neonatal encephalopathy and seizures that are resistant to conventional anticonvulsants and pyridoxine. Those who have survived the neonatal period have had significant neurodevelopmental disorders in the form of ongoing seizures, developmental delay and microcephaly (Mills et al. 2005; Hoffmann et al. 2007; Bagci et al. 2008; Ruiz et al. 2008). As is often the case with rare diseases, the clinical phenotype expands as more cases are diagnosed with time. Three subgroups of patients have recently been proposed: (1) patients with neonatal onset seizures responding to PLP, (2) patients with infantile spasms responsive to PLP and (3) patients with seizures starting under 3 months of age responding to pyridoxine (Mills et al. 2014). In this paper we report four cases of PNPO deficiency, two of whom are siblings, who all demonstrate normal neurodevelopmental outcomes. We discuss the importance of distinguishing PNPO deficiency from other more common neonatal encephalopathies, such as hypoxic ischaemic encephalopathy (HIE).

Case Reports

Table 1 provides a summary of the four cases while salient features of the four are expanded in the reports below.

Case 1

Cases 1 and 2 are siblings and have been reported previously with respect to genotype-phenotype correlation (Mills et al. 2014). By 1.5 h of age, case 1 had developed status epilepticus and severe encephalopathy was recognised. He required intubation and ventilation and received loading doses of phenobarbitone, phenytoin and midazolam without seizure control being established. A trial of oral PLP 100 mg TDS was commenced at 40 h of age, after iv pyridoxine failed to result in clinical or EEG change. Prior to PLP administration, the EEG demonstrated a burst suppression pattern; in the 12 h following PLP administration, the EEG showed ongoing ictal events, the majority of which manifested clinically as multifocal erratic myoclonic jerks. EEG improvement was seen by 3 days of treatment with PLP; seizures and neonatal encephalopathy progressively resolved. A withdrawal of PLP was attempted at 4 months of age; however, seizures recurred when PLP was weaned to a dose of 50 mg once daily. Subsequently a PNPO gene mutation was confirmed, and PLP was continued at doses of up to 50 mg/kg/day. Seizures occurred only infrequently, typically at trough times in PLP dosing (usually in the early morning or if delays in dose administration). On follow-up at 4.5 years of age, case 1 is developing normally. A formal Bayley Scale of Infant and Toddler Development (Bayley-III) and Preschool Language Scale (Fourth Edition) were administered at 2.6 years of age. Low average range scores were obtained on the cognitive and adaptive behaviour scales; average scores were obtained for language, gross and fine motor scales; and high average scores were obtained for social-emotional scales. Formal clinical neurological and developmental examination is normal.

Case 2

Case 2 is the younger sibling of case 1. Her mother had taken pyridoxine as part of a pregnancy multivitamin during the pregnancy (2.6 mg/day pyridoxine) and PLP during the last 3 days of the pregnancy. The infant was administered PLP from birth as a precaution given her sibling's diagnosis. No seizures or abnormal neurological behaviour was noted. An EEG, done at 24 h of age, was normal.

Initial PLP dosing was 25 mg TDS (30 mg/kg/day). This dose was changed to four times a day at 3 months of age to facilitate administration of PLP at the same times as her sibling. An EEG at this time showed periods of focal slowing with sharp waves overlaid, in a posterior distribution. Case 2 has had two seizures to date, the first at 10 months corrected age and the second at 11 months corrected age. Formal clinical neurodevelopmental assessment at 2.5 years of age shows her progress to be within age-appropriate parameters and her neurological examination is normal.

Case 3

Case 3 has been reported previously with respect to genotype-phenotype correlation (Mills et al. 2014). Her mother had taken pyridoxine during the pregnancy as part of a multivitamin supplement. Seizures were first diagnosed at 4 weeks of age, although more subtle events may have occurred prior to this. Trials of pyridoxine at 4 weeks and phenobarbitone, phenytoin and oxcarbazepine failed to control seizures. An EEG at 4 weeks of age was normal;

Table 1 Summary of clinical features and investigations

	Case 1[a]	Case 2[a]	Case 3	Case 4
Current age	4.5	2.5	7.5	11
Heritage	Caucasian	Caucasian	Caucasian	Caucasian
Neonatal				
Gestation	37/40	34/40	38/40	38/40
Mode of delivery	emLUSCS	emLUSCS	SVD	SVD
Foetal distress	Nil	Reduced foetal movement and non-reassuring CTG	Nil	Nil
APGAR	5^1, 8^5	9^1, 9^5	7^1, 9^5	8^1, 9^5
Irritability	Extreme	Nil	Nil	Nil
Neonatal encephalopathy	Yes	No[b]	No	Yes
Neurological examination	Hypotonic	Normal	Normal	Normal
Growth parameters	BW 3.4 kg	BW 2.4 kg	BW 2.6 kg	BW 3.4 kg
	L 50 cm	L 46 cm	L 48 cm	L 53.5 cm
	HC 34 cm	HC 30.2 cm	HC 31.5 cm	HC 33 cm
Seizure semiology				
Age of seizure onset	1.5 h of age	10 months[b]	4 weeks	24 h of age
Myoclonus	Yes	No	No	Yes
Status epilepticus	Yes	No	Yes	Yes
Ictal eye movements	Yes (from infancy)	No	Yes	Yes
Focal clonic	No	No	Yes	Yes
Initial EEG pattern	Burst suppression	Normal	Rhythmic slowing in posterior regions, with frequent focal seizures	Normal
MRI	Normal	NP	Normal	Normal
Pretreatment CSF studies[c]				
Homovanillic acid	343	NP	576	1,063
	RR 330–1,300 nmol/L		RR 330–1,300 nmol/L	RR 310–1,100 nmol/L
3-*O* methyl DOPA	438 RR <300 nmol/L	NP	111 RR <300 nmol/L	NP
5-HIAA	395	NP	362	838
	RR 200–1,160 nmol/L		RR 200–1,160 nmol/L	RR 150–800 nmol/L
HVA/5-HIAA ratio	0.9 RR 1.5–3.5	NP	1.6 RR 1.5–3.5	1.2
Tetrahydrobiopterin	57 RR 40–110 nmol/L	NP	55 RR 40–110 nmol/L	NP
Total neopterin	8 RR 7–65 nmol/L	NP	7 RR 7–65 nmol/L	NP
Folate	153 RR 50–180 nmol/L	NP	63 50–180 nmol/L	NP
Glycine	9	NP	1.9	9
	RR 4.7–8.1 μmol/L		RR 4.7–8.1 μmol/L	RR 0.7–14.7 μmol/L
Threonine	60	NP	49.6	51
	RR 12.6–18.6 μmol/L		RR 12.6–18.6 μmol/L	RR 22.5–52.6 μmol/L
Phenylalanine	12 RR 5.8–7.6 μmol/L	NP	2.8 5.8–7.6 μmol/L	NP
Urine VMA	11.1	NP	4.2	Not detected

(continued)

Table 1 (continued)

	Case 1[a]	Case 2[a]	Case 3	Case 4
	RR <10 mmol/mol creatinine		RR <10 mmol/mol creatinine	
Response to B6	No	No treatment	No	No
PLP				
Age at first treatment	40 h	At birth	8 weeks	28 days
Initial dose	−30 mg/kg/day in three divided doses	−30 mg/kg/day in three divided doses	40 mg TDS	100 mg/kg/day
Current dose	50 mg/kg/day	50 mg/kg/day	~45 mg/kg/day	50–60 mg/kg/day
Current dose interval	Q6H	Q6H	Q6H	4 hourly with no dose overnight
***PNPO* sequencing**	c.737 C>T/c.737 C>T (p.P213S/p.P213S)	c.737 C>T/c.737 C>T (p.P213S/p.P213S)	c.98A>T/c.98A>T (p.D33V/p.D33V)	c.98A>T/c.246delT (p.D33V/p.(Leu83Trpfs*17))
Development				
Gross motor skills	Age appropriate	Age appropriate	Age appropriate	Age Appropriate
Fine motor skills	Age appropriate	Age appropriate	Age appropriate	Age appropriate
Speech and language	Mild delay in expressive language (clarity)	Age appropriate	Mild delay in expressive language (clarity)	Age appropriate
Social skills	Age appropriate	Age appropriate	Age appropriate	Age appropriate
Formal Psychometric testing				
Age of assessment	Bailey-III, 3 years	[d]	[d]	[d]
Cognition	Low average	[d]	[d]	[d]
Language	Average	[d]	[d]	[d]
Motor	Average	[d]	[d]	[d]
Social-emotional	High average	[d]	[d]	[d]

[a] Siblings
[b] Was treated with PLP from birth
[c] Cases 1, 2, 3 and 4 performed in different laboratories, hence variant reference ranges
[d] Formal psychometric testing not performed but normal developmental assessments by paediatric neurologist
BW birth weight, *L* length, *HC* head circumference, *NP* not performed, *RR* reference ranges. *LUSCS* lower uterine segment caesarean section, *CTG* cardiotocogram, *SVD* spontaneous vaginal delivery

however, by 6 weeks of age, the EEG demonstrated periods of slowing and frequent intermixed multifocal sharp waves. A further EEG at 8 weeks of age, when seizures had changed to include tonic events with vocalisation, showed periods of electrodecrement with ongoing multifocal epileptiform discharges.

PLP (40 mg TDS) was commenced at 8 weeks of age with cessation of seizures and normalisation of the EEG. Occasional break through seizures occurred, related to periods of febrile illness, vomiting or medication refusal. At 20 months of age, a trial of PLP withdrawal for 24 h resulted in a significant increase in seizures and deterioration in the EEG. The current dose of PLP is 100 mg five times a day (~45 mg/kg/day). Neurodevelopmental assessments were normal throughout, with the exception of minor delays in expressive language development, affecting clarity of speech. Case 3 is now school age (7.5 years), is progressing well with no concerns in relation to her learning and is considered advanced in her reading ability for her age.

Case 4

Details of the initial presentation of case 4 have been reported previously (Hoffmann et al. 2007; Schmitt et al. 2010; Sudarsanam et al. 2014). Intractable neonatal seizures occurred, with a number of different seizure types seen – abnormal eye movements, abnormal smiling, multifocal myoclonic jerks, focal clonic seizures and spasms associated with screaming and irritability. He was treated with multiple different anticonvulsants and pyridoxine, before being treated with PLP at 28 days of age. This was associated

with immediate resolution of seizures. A trial of withdrawal of PLP at 8 months of age was associated with recurrence of encephalopathy, and PLP has subsequently been continued (doses ranging from 50 to 100 mg/kg/day in divided doses). However, at age 2 years, as a result of concerns about hepatotoxicity (Sudarsanam et al. 2014), lower PLP doses were given more frequently (on a 4 hourly basis including an overnight dose) and the total daily dose was decreased to 50–60 mg/kg/day. During infancy, episodes of encephalopathy were relatively frequent and often associated with febrile illnesses, but after 18 months of age these became less frequent and abated with careful management of PLP dosage and timing of administration. He currently receives frequent doses of PLP (4 hourly during the day); however, the overnight dose has been able to be omitted. At 11 years of age, he is academically advanced in school, particularly in mathematics and music. His neurological examination is normal.

Discussion

Neonatal encephalopathies are uncommon, with an incidence of 9 in 1,000 live births worldwide (Graham et al. 2008). While HIE is the most common cause of a neonatal encephalopathy, numerous genetic conditions can masquerade as HIE; prime examples are the IEM associated with vitamin B6 responsiveness. Other IEM that can masquerade as HIE in the newborn period broadly include (a) disorders of neurotransmitter metabolism, e.g. nonketotic hyperglycinaemia (OMIM 605899); (b) disorders of energy metabolism, e.g. biotinidase deficiency (OMIM 253260); and (c) biosynthetic defects, e.g. the congenital disorders of glycosylation (Van Hove and Lohr 2011).

Early reports have suggested that the common clinical features in the reported PNPO cases thus far include (1) neonatal encephalopathy, seizures resistant to multiple anticonvulsants (Mills et al. 2005; Schmitt et al. 2010), (2) burst suppression EEG pattern (Veerapandiyan et al. 2011), (3) non-responsiveness to pyridoxine (Clayton 2006), (4) complete or partial responsiveness to PLP (Pearl et al. 2013), (5) prematurity (Veerapandiyan et al. 2011) and (6) neonatal lethality if the diagnosis is not suspected and PLP administered (Khayat et al. 2008). Early diagnosis and treatment with PLP have been linked with improved neurodevelopmental outcomes (Hoffmann et al. 2007; Plecko et al. 2014) with more recent reports supporting that normal neurodevelopmental outcomes can occur (Khayat et al. 2008; Mills et al. 2014; Plecko et al. 2014). The four cases reported in this paper reinforce these observations, with case 2 being the only known patient to have been treated as a preventative measure from birth, until it was confirmed by genetic testing whether she had PNPO deficiency or otherwise.

Mothers of cases 2, 3 and 4 took multivitamins containing pyridoxine during the pregnancy. Maternal supply of B6 has been postulated to modify the clinical phenotype in infants with PNPO deficiency (Mills et al. 2014). It is likely, in the four current cases, that maternal treatment in pregnancy, prompt early consideration of PNPO deficiency in the differential diagnosis, early administration of PLP and excellent tertiary level supportive neonatal care have all contributed to the normal developmental and neurological outcomes that occurred. Expression studies involving the D33V and the R225H/C mutations demonstrate sufficient residual enzyme activity to allow synthesis of PLP from pyridoxine (Mills et al. 2014), which may result in higher foetal levels of PLP with maternal pyridoxine supplementation in pregnancy. Case 2 is unique in the literature; her elder brother presented with a neonatal encephalopathy in the first few hours of life. Case 2 was treated in utero with maternal supplementation of pyridoxine containing multivitamins and electively supplemented with PLP immediately after her premature delivery, in the delivery suite. She was identified as having an abnormal interictal EEG at 3 months of age, but did not have any neurological or developmental signs or symptoms until she experienced a seizure at 10 months of age. She is the first case, to the best of our knowledge, that has been treated presymptomatically in utero, with this likely modifying her age of presentation.

In the presence of clinical suspicion of PNPO deficiency, the results of molecular and/or biochemical investigation should not defer a therapeutic trail of PLP. Elevated urinary vanillyllactate can serve as a marker for PNPO deficiency, but the sensitivity and specificity of this test in neonates with seizures are unclear at this stage (Clayton 2006). Cerebrospinal fluid (CSF) studies, collected via lumbar puncture, may demonstrate aberrations in amino acids and neurotransmitters reflecting the role of PLP as a cofactor for their respective enzymatic metabolism, e.g. elevated serine, glycine (consequent of defective glycine cleavage system) and threonine (threonine dehydratase) and decreased 5-hydroxyindoleacetic acid and homovanillic acids with elevated 3-methoxytyrosine (due to decreased L-AADC function) (Goyal et al. 2013; Clayton 2006). However, normality in these biochemical parameters cannot completely exclude PNPO deficiency, as patients have been reported with normal CSF amino acids and neurotransmitter values (Hoffmann et al. 2007; Bagci et al. 2008). Case 1 in our cohort supports this observation and highlights the importance of a clinical trial of PLP in neonates with

encephalopathy. It is possible to measure CSF concentrations of PLP; however, low levels are not diagnostic for B6-related seizure disorders and there are age-related variations (Footitt et al. 2011; Albersen et al. 2012; Ormazabal et al. 2008). The evolution of next-generation sequencing (NGS) over recent years has proven beneficial for clinicians dealing with clinical phenotypes with genetic heterogeneity. NGS is a valuable diagnostic tool for disorders with encephalopathy; however, if PNPO deficiency is suspected, early treatment trial with pyridoxine and/or PLP is an important practical approach while awaiting NGS results to become available.

The treatment of PNPO deficiency requires frequent administration of PLP. Our patients demonstrate exquisite sensitivity to PLP dose and timing of administration in preventing seizures. The dosage required to achieve this varied with age and body weight. PLP dosages of 10 mg/kg every 6 h have been utilised to control seizures (Clayton 2006). A consistent feature within our cohort is breakthrough seizures occurring at times of dosage interruption/delay. This was most commonly observed at times of intercurrent vomiting illnesses. Case 1 in particular has demonstrated remarkable dosage sensitivity; a delay in the delivery of any dose through the day by even 20 minutes can lead to a seizure, which often is associated with apnoea. A similar time-sensitive requirement for PLP dose administration has been reported once before (Hoffmann et al. 2007). The requirement for 4–6 hourly medication delivery, with the ever-present fear of breakthrough seizures and encephalopathy, results in a significant burden for the families. Cases 1 and 4 have utilised rectal delivered PLP in cases of seizure emergencies and at times of reduced oral intake, e.g. fasting for a surgical procedure or during an intercurrent vomiting illness.

The normal neurodevelopmental outcomes in the cases reported here emphasise the importance of early consideration of PNPO deficiency in the assessment of any neonate presenting with seizures and/or encephalopathy. Infants with PNPO deficiency can be born preterm and may have foetal distress with lactic acidosis, factors that are also associated with HIE. It is important that clinicians have a low threshold for investigating for PNPO deficiency and electively treating with PLP, in any case of presumed HIE in whom the degree of encephalopathy is not fully explained by perinatal history, cord gases and/or neuroimaging.

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Compliance with Ethics Guidelines

Conflict of Interest

Joshua Hatch, David Coman, Peter Clayton, Philippa Mills, Sophie Calvert, Richard Webster and Kate Riney declare that they have no conflicts 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.

Author Contributions

Dr Joshua Hatch has driven the manuscript development.

Professor David Coman is a metabolic physician involved in the care of patients 1–3 and has coordinated the manuscript development and design.

Professor Peter Clayton is a metabolic physician who has provided invaluable clinical advice on all four patients and has been involved in the manuscript development.

Dr Philippa Mills has provided valuable advice regarding the molecular pathogenicity of the mutations identified and has been involved in the manuscript development.

Dr Sophie Calvert is a paediatric neurologist, providing neurology care for case 3, and has been involved in the manuscript development.

Dr Richard Webster is a paediatric neurologist, providing neurology care for case 4, and has been involved in the manuscript development.

Dr Kate Riney is a paediatric neurologist, providing neurology care for cases 1 and 2, and has been involved in the manuscript development.

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RESEARCH REPORT

Screening for Attenuated Forms of Mucopolysaccharidoses in Patients with Osteoarticular Problems of Unknown Etiology

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Abstract *Introduction*: The mucopolysaccharidoses (MPS) are a group of 11 inborn errors of metabolism (IEM) which are part of the lysosomal storage diseases (LSDs). The MPS are multisystemic conditions that affect the entire body, with variations in the clinical presentation, having specific treatments available depending on the type of MPS. Nearly all MPS disorders compromise the osteoarticular system in different ways, and virtually all patients have abnormal urinary excretion of glycosaminoglycans (GAGs). MPS are rare diseases that are underdiagnosed due to health-care professionals' lack of awareness, to poor access to screening and diagnostic methods, and to their extensive clinical heterogeneity. Attenuated forms may occur, which can make diagnosis of MPS even more difficult.

Methods: This study was conducted prospectively from March 2012 to January 2014 and included 55 patients at rheumatology and/or orthopedic services in Porto Alegre, Brazil. The screened patients presented with articular manifestations with no defined etiology. These patients were screened by quantitative and qualitative assessment of urinary GAGs.

Results and Discussion: Among the 55 cases investigated, one 15-year-old patient exhibited increased urinary GAG excretion; this patient was subsequently diagnosed with an attenuated form of MPS II, which was previously undetected.

Conclusion: Although the proportion of patients with MPS identified in the study sample was small (1/55), this study shows that these diseases are underdiagnosed and that systematic screening can help identify patients who may benefit from specific treatments already available for several MPS types.

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Introduction

The mucopolysaccharidoses (MPS) are inborn errors of metabolism involving the glycosaminoglycans (GAGs), which are formed by sugar chains bound to a protein core structure and are a major component of the connective tissue matrix. Deficiencies in the enzymes involved in GAG degradation lead to the accumulation of GAGs in the lysosomes, particularly in organs such as the liver, spleen,

heart, and cornea, among others, and result in marked osteoarticular impairment (Neufeld and Muenzer, 2001).

With the exception of MPS II (Hunter syndrome, inherited recessive X-linked mode), all MPS are autosomal recessive. Currently, there are 11 different enzyme deficiencies known to result in MPS. MPS are rare conditions, affecting an estimated 1 in 22,000 individuals (Poorthuis et al. 1999; Meikle et al. 1999). Currently, enzyme replacement therapy is available for 3 MPS types (I, II, and VI) and in development for 4 other types (IIIA, IIIB, IVA, and VII).

The clinical manifestations of each condition vary greatly, which may be related not only to the varied underlying genotypes but also to other poorly understood factors (Giugliani 2012). Because of the clinical heterogeneity of these conditions, early diagnosis is most likely for patients who exhibit severe phenotypes, and diagnosis may be delayed in the attenuated forms (Hendriksz 2011).

Stiffness occurs in patients with all types of MPS, except MPS IV, which is associated with ligament laxity. In cases of attenuated disease, specialists in rheumatology and orthopedics are often sought before a diagnosis is reached because of the common osteoarticular problems in MPS patients, which are often the first symptoms to appear. These specialists should be prepared to recognize and diagnose MPS or rule out a MPS diagnosis and refer the patient to an appropriate management. However, recognition and diagnosis can be a challenge because the attenuated forms of MPS manifest with subtle and/or nonspecific symptoms. Incorrect diagnoses common to these cases include autoimmune diseases, muscular dystrophies, connective tissue disease, osteogenesis imperfecta, polymyositis, dermatomyositis, polyneuropathy, fibromyalgia, rheumatoid arthritis, scleroderma, juvenile idiopathic arthritis, spondyloarthritis, Legg–Calve–Perthes disease, and other systemic rheumatic conditions including "growing pain" (Hendriksz 2011). One study performed by Cimaz et al. (2009) showed that only 13% of pediatric rheumatologists and 19% of rheumatologists were able to detect MPS when clinical cases of an 8-year-old girl and a 23-year-old woman were presented, respectively, in which both had classic symptoms and articular manifestations of MPS.

The goal of the present study was to evaluate the frequency of MPS in a sample of rheumatology and orthopedic patients with joint complaints of unknown etiology after a typical clinical and laboratory investigation was performed.

Materials and Methods

This study was conducted prospectively from March 2012 to January 2014 in rheumatology and orthopedic outpatient clinics in Porto Alegre, Brazil, and was previously approved by the research ethics committee of Hospital de Clinicas de Porto Alegre (university hospital).

This study had a cross-sectional design, and the main inclusion criterion was the presence of osteoarticular manifestations (usually articular pain, joint stiffness, joint contractures, noninflammatory increased articular volume, secondary osteoarthrosis) that had unknown etiology after a typical clinical and laboratorial investigation was performed. In addition to a known etiology, the presence of inflammatory arthropathy or primary osteoarthrosis was also considered an exclusion criterion.

The study included 55 patients with symptoms that could not be explained by any diagnosis. Data were collected from these patients. Despite available for many patients, X-ray results were not considered as inclusion or exclusion criteria and were not analyzed in this study.

From all patients, a random urine sample was collected, which was evaluated for measurement of the excretion of total GAGs using a quantitative di-methylene blue colorimetric assay (De Jong et al. 1992) and electrophoresis for qualitative evaluation of the GAG species (Pennock 1976; Cappelletti et al. 1979).

Results and Discussion

Of the 55 patients included in the study, 28 were male and 27 were female, with ages ranging from 3 to 21 years (mean age of 9 years). All patients reported discomfort or joint pain, and 2/3 of the patients also complained of stiffness.

The results of the urinary GAG analysis allowed us to identify six cases with increased GAG excretion when reference values were adjusted for age. In five of these cases, the results could not be confirmed in a second sample, and the qualitative analysis of the GAG species was normal; thus, a MPS diagnosis was ruled out. False positives were more frequently observed in childhood, when urinary GAG excretion is relatively higher (data not shown). This 10% false positive rate was considered acceptable because, as in the present study, additional tests are available to confirm each diagnosis. We should point out that false negatives may occur and that more refined tests should be performed when a specific clinical suspicion of MPS is raised, especially of MPS III or MPS IV, even if the result of the screening is negative.

In one case, an increase in urinary GAGs was observed along with an altered qualitative pattern of GAG species. This patient, a 15-year-old male, was referred to the Pediatric Orthopedics Service with joint limitation in his hands and a limitation of extension in his fingers. An evaluation of GAG

excretion indicated a urine level of 147 μg/mg creatinine (reference value for this age, 13–59 μg/mg creatinine). The electrophoresis analysis showed an abnormal pattern of urinary GAGs in the presence of dermatan sulfate and heparan sulfate. As this pattern may be indicative of MPS I, II, or VII, further diagnostic tests were performed to analyze the activities of the specific enzymes deficient in these three MPS types (alpha-iduronidase, iduronate sulfatase, and beta-glucuronidase, respectively).

Iduronate sulfatase levels within the leukocytes of this patient were lower than normal (1.3 μmol/h/mg protein compared with the reference range of 31–110 μmol/h/mg). This result, coupled with the normal activity of another sulfatase (arylsulfatase B), ruled out the possibility of multiple sulfatase deficiency and confirmed the diagnosis of Hunter syndrome (MPS II).

Although our study included only 55 patients, the finding of a positive MPS case can be considered quite significant as the frequency of MPS is estimated to be 1 in 22,000 in the general population (Poorthuis et al. 1999; Meikle et al. 1999).

The attenuated forms of MPS have less systemic involvement than the classical forms, but over time, the osteoarticular manifestations can be significant. Joint pain, joint limitation, mild bone deformities, and necrosis of the femoral head can occur. Because of these symptoms, the demand for rheumatology and/or orthopedic services is common in these cases, making the patients treated by these specialists target groups for the detection of attenuated forms of MPS (Hendriksz 2011).

The history of the diagnosed patient indicated that he weighed 3.2 kg and was 49.0 cm long at birth, which is considered normal for babies born at term. The patient was the child of healthy, non-consanguineous parents with no known family history of MPS. At 2 months of life, the patient began to suffer from recurrent infections of the upper airways, which became increasingly less frequent until the age of 6, when the infections became rare. As recorded in this study, his weight at age 15 was 43.4 kg, and his height was 1.56 m, both of which are below average for his age. A closer examination revealed the presence of a winged scapula, palpable liver 5 cm below the costal margin, and a slightly infiltrated face. The results of additional tests, such as spirometry, resting electrocardiogram, pulmonary function tests, abdominal ultrasound, and a 6-minute walk test, were normal for his age. Together with the biochemical results, these findings established the clinical diagnosis of a non-neuronopathic (attenuated) form of MPS II, a condition for which enzyme replacement therapy with idursulfase is indicated and may positively modify the natural history of the disease in this patient.

Conclusion

Although the proportion of patients with MPS identified in this study sample was small (1/55), this study indicates that underdiagnosis of these diseases can occur and that systematic screening in patients with articular changes of unknown etiology may help to identify patients with MPS. This screening, which could be performed in a random urine sample, could be included in the evaluation work-up and requested directly by the rheumatologist or orthopedic surgeon. If clinical concerns remain, the referral to a genetic/metabolic specialist could be considered. Diagnosed patients may have access to available treatment measures, and their families may benefit from genetic counseling, prenatal diagnosis, and the early detection of new cases.

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Synopsis

As attenuated forms of mucopolysaccharidoses may be difficult to identify, selective screening in patients with osteoarticular disease of unknown etiology may help to identify affected cases who could benefit from the specific therapies already available for several MPS types.

Contributions of the Individual Authors

TCRS interviewed the patients, obtained informed consent, collected the samples, and drafted the first version of the manuscript. CFMS, PL, MP, and IS referred patients for inclusion in the study, provided clinical information, and reviewed the final version of the manuscript. FB and MB performed the laboratory tests needed for screening and diagnosis and reviewed the final version of the manuscript. RG supervised the project, provided guidance in all steps, and revised the final version of the manuscript.

Compliance with Ethics Guidelines

Competing Interest Statement

RG received travel grants, speaker honoraria, and/or investigator fees from Actelion, Amicus, BioMarin, Genzyme, Shire, and Synageva; all other authors have no competing interests to disclose.

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Details of Ethics Approval

This study was approved by the Institutional Review Board (GPPG/HCPA) in 17/07/2012, with the number 11-0557.

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RESEARCH REPORT

Causes of Death in Adults with Mitochondrial Disease

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Abstract *Introduction*: Mitochondrial diseases are a clinically, biochemically and genetically heterogeneous group of disorders with a variable age of onset and rate of disease progression. It might therefore be expected that this variation be reflected in the age and cause of death. However, to date, little has been reported regarding the 'end-of-life' period and causes of death in mitochondrial disease patients. For some specific syndromes, the associated clinical problems might predict the cause of death, but for many patients, it remains difficult to provide an accurate prognosis.

Aims: To describe a retrospective cohort of adult mitochondrial disease patients who had attended the NHS Highly Specialised Services for Rare Mitochondrial Diseases in Newcastle upon Tyne (UK), evaluate life expectancy and causes of death and assess the consequences for daily patient care.

Methods: All deceased adult patients cared for at this centre over a period of 10 years were included in the study. Patient history, data on laboratory findings, biochemical investigations and genetic studies were analysed retrospectively.

Results: A total of 30 adult mitochondrial patients died within the time period of the study. The main mitochondrial disease-related causes of death in this patient cohort were respiratory failure, cardiac failure and acute cerebral incidents such as seizures and strokes. In almost half of the patients, the cause of death remained unknown. Based on our study, we present recommendations regarding the care of patients with mitochondrial disease.

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Introduction

Mitochondrial Function and Mitochondrial Disease

Mitochondria are cytoplasmic organelles that undertake key metabolic functions, prime of which is the generation of adenosine triphosphate (ATP), the principal energy source of the cell. This process of ATP production, known as oxidative phosphorylation (OXPHOS), takes place via the mitochondrial respiratory chain, a series of four multi-subunit enzymatic complexes (I–IV) that are coupled with the mitochondrial ATP synthase (complex V) and located in the inner mitochondrial membrane. Despite the fact that many metabolic processes take place in mitochondria, the term mitochondrial disease is generally reserved for clinical phenotypes associated with primary impairment of OXPHOS. Mitochondrial diseases can arise from mutations in the small, circular, double-stranded, maternally inherited mitochondrial DNA (mtDNA) or as a result of mutations in nuclear genes intimately linked to mitochondrial function (McFarland et al. 2010). One consequence of this complex

genetic arrangement is that the phenotypic diversity of mitochondrial disease is enormous (DiMauro and Schon 2003). Generally, tissues with high energy demands such as the brain, liver and muscle are most likely to be affected (Smeitink et al. 2006), but clinical manifestations vary from single organ (e.g. optic neuropathy) to multisystem involvement. However, central nervous system disease with, for example, ataxia, dementia, spasticity, behaviour abnormalities, seizures or movement disorders is common (Morava et al. 2006a, 2010).

Given the heterogeneous disease manifestations, the clinical course and long-term outcomes of affected patients vary widely. Onset of mitochondrial disease in early childhood is in most cases multisystem and often rapidly progressive (McFarland and Turnbull 2009), whereas in the adult population, 'classic mitochondrial syndromes' such as Leber's hereditary optic neuropathy (LHON); myoclonic epilepsy with ragged red fibres (MERRF); mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS); maternally inherited diabetes and deafness (MIDD); and chronic progressive external ophthalmoplegia (CPEO) are more often encountered and associated with variable clinical courses. In Kearns–Sayre Syndrome (KSS), caused by either a large-scale deletion or complex mtDNA rearrangements, and MELAS, a clinical phenotype associated with the m.3243A>G mutation (*MTTL1* (MIM 590050)), life expectancy is often markedly reduced due to cardiac conduction block (Kearns–Sayre Syndrome) and progressive cardiomyopathy (MELAS) (Ashizawa and Subramony 2001; McFarland and Turnbull 2009). In contrast, in some 'milder' genotypes, such as those leading to CPEO, reduced life expectancy has not been described.

Although mitochondrial disease is one of the commonest metabolic and neuromuscular disorders, estimating the exact prevalence has been complicated by the diversity of phenotypes, genotypes and their loose association. Studies in North East England found that in an adult population, 9.2 per 100,000 people have clinically manifest mtDNA disease and a further 16.5 per 100,000 children and adults are at increased risk of developing mtDNA disease (Schaefer et al. 2008). One particular mutation, m.3243 A>G, was found to have an overall point prevalence of 16.3/100,000 of the adult population in Northern Finland (Majamaa et al. 1998).

Mortality in Mitochondrial Disease

The mortality of patients with mitochondrial disease is likely to be influenced by the age of the patient and the underlying genotype, but the extensive phenotypic diversity complicates the study of this aspect of disease. As described above, several mitochondrial syndromes present with skeletal muscle weakness, affecting especially the proximal muscles of the hip and shoulder girdle. This myopathy may eventually also affect respiratory muscles causing respiratory failure, a need for ventilatory support and a markedly reduced life expectancy (McFarland et al. 2010). Respiratory failure appears to be a relatively common cause of death in mitochondrial disease (Arpa et al. 2003).

Smaller cohorts of mitochondrial patients have been reported with cardiopulmonary failure, status epilepticus, aspiration pneumonia and pulmonary embolism as the underlying cause of death (Klopstock et al. 1999). Other papers focus on the cause of death in specific patient groups (Coenen et al. 1999; Wortmann et al. 2007; Morava et al. 2006b; de Vries et al. 2007; Majamaa-Voltti et al. 2008). Currently, there is little data available on mortality in larger cohorts of mitochondrial patients and especially the adult patient group. We have attempted to address this shortfall by performing a retrospective review of the adult population of mitochondrial disease patients who attended the NHS Highly Specialised Services for Rare Mitochondrial Diseases at the Royal Victoria Infirmary in Newcastle upon Tyne, UK. We assessed mean survival rates, causes of death, genotypes, phenotypes and, where available, diagnostic information from skeletal muscle biopsy. In particular, we wanted to identify any potentially avoidable deaths and learn how we might better individualise patient care.

Methods

We performed a retrospective study in all deceased adult and mitochondrial disease patients diagnosed by the Mitochondrial Disease Clinical and Diagnostic Service in Newcastle upon Tyne, UK, who died between 1 Jan 2000 and 31 Dec 2009. In this 10-year period from a total adult patient population of 380 patients, with histochemical and/or biochemical evidence of a respiratory chain deficiency (with or without a known underlying genetic defect), we evaluated 30 deceased patients above the age of 18 years.

All medical files, including correspondence with all involved medical specialists and family doctors, death certificates, postmortems and other reports related to the patients' death, of these 30 patients, were gathered and analysed retrospectively. Relevant information regarding onset of disease, symptoms, clinical investigations (including DNA analysis, muscle biopsy and mutation load) and all available information referring to the death of the patient was systematically recorded in a database. Inclusion criteria were death in the presence of an ascertained diagnosis of mitochondrial disease, established by muscle biopsy or DNA analysis. Figure 1 provides an overview of this cohort of mitochondrial disease patients including data on the common mutations.

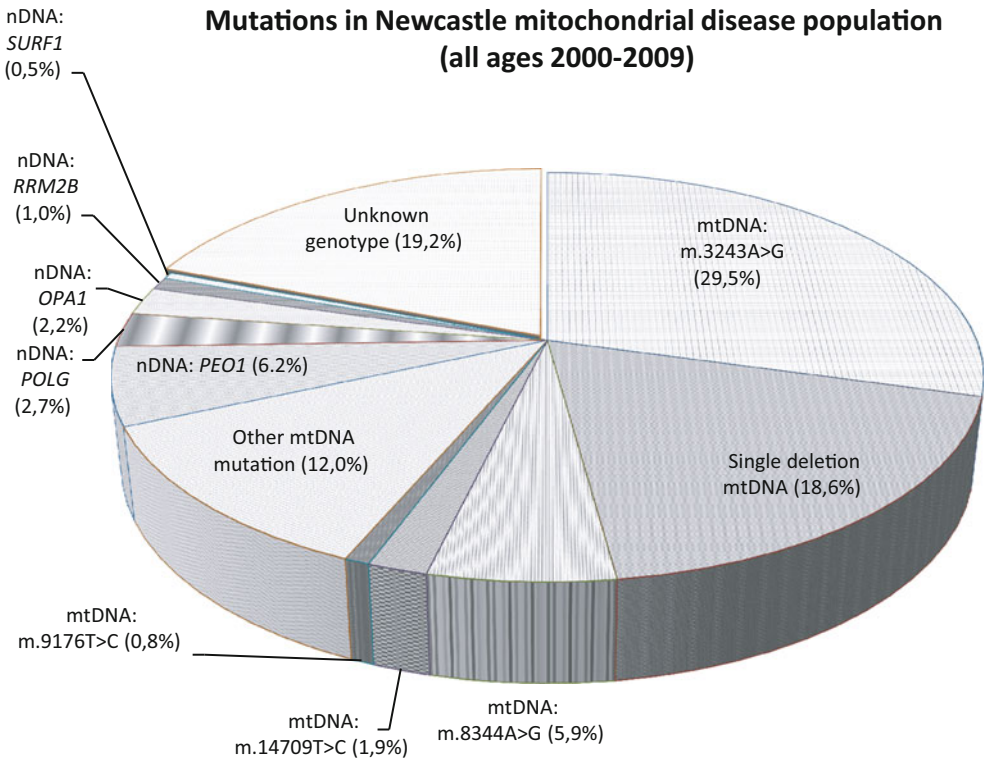

Fig. 1 Mutations in the Newcastle mitochondrial disease population. This figure shows cumulative epidemiological data regarding common mutations diagnosed in a 10-year time period in the Newcastle mitochondrial patient cohort

Results

In the time period studied, our centre has largely been treating adult patients. The deceased adult patient group consisted of a heterogeneous mix of 30 patients with mitochondrial disease (see Table 1).

MELAS

Eight patients were diagnosed as having a MELAS phenotype (patients 1–8). The onset of disease was in late childhood or adolescence, and the first symptoms were in most cases muscle weakness, headaches or epileptic seizures. All eight patients had the *MTTL1* (m.3243A>G) mutation. Tissue-specific mutation levels varied widely in this group. In most of the muscle biopsies, COX-deficient and red ragged fibres (RRF) were seen. The mean and median ages of death were, respectively, 42.5 and 38.75 years (age range 22 to 78 years). Patient 1 died because of accidental drowning. Suicide was considered but was difficult to confirm. He had been stable for a long time following his good recovery from a number of severe stroke-like episodes. In 3 of the MELAS (m.3243A>G) patients, death due to a cardiac cause was described (patients 3, 4 and possibly 8). Patient 3 was known to have hypertrophic cardiomyopathy and suffered from recurrent chest infections and respiratory muscle weakness. Since he failed to attend clinic, evaluation data from the year prior to his death were not available, but his clinical condition seemed stable before then. He died from cardiac arrest after hyperglycaemia and ketoacidosis after an initial respiratory tract infection. Patient 4 had asymptomatic hypertrophic cardiomyopathy from the age of 30 years. Severe biventricular failure, developing 9 months before death, was treated with cardiac medication (including a beta blocker, diuretic and angiotensin-converting enzyme (ACE) inhibitor). Regular cardiac evaluation was performed, with no deterioration noticed two weeks prior to death. Defibrillator placement had been considered but was rejected given his associated comorbidity and the risk of sudden cardiac death being considered low. The patient died of cardiac arrest, most likely tachyarrhythmic, after a seizure. Post-mortem examination revealed a mildly enlarged heart with mild dilatation of both ventricles. Patient 7 was severely affected by mitochondrial disease. During the last months of her life, she deteriorated quickly, both physically and mentally, and eventually died of multi-organ failure. In 4 of the MELAS patients, the precise cause of death was not known (patients 2, 5, 6 and 8). Some 5 months prior to the death of patient 8, an echocardiogram identified severely reduced left ventricular function, but as he remained asymptomatic, follow-up was planned for 6 months. Prior

Table 1 Epidemiologic, genetic, clinical and biochemical characteristics of adult mitochondrial disease patients from the Newcastle cohort, deceased from 2000 to 2009

						Muscle biopsy specimen				
No.	Sex	Year of birth	Diagnosis	Age at onset of symptoms	DNA analysis	Hist	Complex def	Level of mutant DNA (%)	Age at death (yr)	Cause of death
1	M	1985	MELAS	9–10	*MTTL1* m.3243A>G	NK	NK	BL: 59 UEC: 89	22	Drowning incident
2	F	1977	MELAS	16–19	*MTTL1* m.3243A>G	COX- RRF	NK	BL: 27 UEC: 77	28	NK
3	M	1973	MELAS	5	*MTTL1* m.3243A>G	COX-	I, IV	BL: NRD UEC: 37 H: high (PM)	34	Ventricle fibrillation possibly due to hyperglycaemia/ ketoacidosis after RTI
4	F	1967	MELAS	18	*MTTL1* m.3243A>G	COX- RRF	NK	BL: 33 UEC: 21	36	Cardiac arrest after seizure
5	F	1959	MELAS	21	*MTTL1* m.3243A>G	COX- RRF	NK	BL: 20 MU: 80	41/42	NK
6	F	1960	MELAS	21	*MTTL1* m.3243A>G	NK	NK	BL: 23 (PM) H: 72 (PM) MU: 87 (PM)	42	NK
7	F	1945	MELAS	26	*MTTL1* m.3243A>G	COX- RRF	NK	BL: 5 UEC:72 H: 85 (PM) MU: 75 (PM)	59	Multi-organ failure
8	M	1930	MELAS	NK	*MTTL1* m.3243A>G	COX- RRF	I	UEC: 30 MU: 29	78	NK; possibly cardiac failure after MI
9	M	1978	CPEO+ CM MM DM Deafness	14	*MTTL1* m.3243A>G	COX- RRF	NK	UEC: 96 MU: 71	30	NK; possibly cardiac failure
10	M	1961	CPEO+ MIDD MM CNSI Dysphagia Dementia	28	*MTTL1* m.3243A>G	COX-	NK	BL: 14 UEC: 60 MU: 69	43	Inhalation of gastric contents
11	M	1963	Kearns–Sayre	15	MTdel5Kb	COX- RRF	None	CB: 27 (PM)	40	Respiratory failure due to RTI after surgery for hip fracture (ataxia)

12	M	1952	Kearns–Sayre	15	MTdel5Kb	COX-RRF	I, IV	NP	56	Cardiopulmonary and renal failure, deterioration after RTI
13	M	1934	Kearns–Sayre	NK	MTdel5Kb	COX-	NK	NP	66	Respiratory failure
14	F	1934	CPEO+ MM CNSI Dysphagia Neuropathy Deafness	NK	Mult. del.	COX-RRF	NK	NP	73	Sepsis due to pneumonia after MI
15	F	1935	DOA+ CPEO MM Ataxia Dysphagia Deafness	<18	*OPA1* c.1635C>G	COX-RRF	I	NP	71/72	NK
16	M	1949	CPEO+ MM CNSI Ataxia Neuropathy Deafness OA	NK	*POLG* c.1399G>A	COX-RRF	None	NP	50	NK
17	F	1954	CPEO+ CNSI MM Ataxia Neuropathy Dysphagia	17	MTdel5Kb	COX-RRF	I, IV	BL: low	53	NK
18	M	1936	CPEO Dysphagia	5	Mult. del.	NP	NP	NP	63	NK
19	M	1937	CPEO+ CNSI Ataxia Neuropathy Dementia MM	NK	MTdel5Kb	COX-RRF	IV	NP	65	Upper GI bleed due to varices (alcoholic chronic liver disease)
20	M	1946	CPEO+ CNSI Parkinsonism Dementia MM	NK	*POLG* c.2542G>A c.3311C>G (compound heterozygous)	COX-(PM) RRF (PM)	NK	NP	59	NK

(continued)

Table 1 (continued)

No.	Sex	Year of birth	Diagnosis	Age at onset of symptoms	DNA analysis	Muscle biopsy specimen: Hist	Muscle biopsy specimen: Complex def	Level of mutant DNA (%)	Age at death (yr)	Cause of death
			Neuropathy Dysphagia							
21	F	1942	CPEO+ CNSI MM Ataxia Deafness Dysphagia	NK	Mult. del.	COX- RRF	NK	NP	66	Probably due to lung cancer
22	F	1964	MERRF	18	*MT-TK* m.8344A>G	NP	NP	BL: 92	41	NK; possible respiratory failure due to aspiration or epileptic seizure
23	F	1969	Strokes CNSI Ataxia (PM Leigh)	NK	*MT-ND5* (MIM 516005) m.13094T>C	NK	I, II (PM)	NP	34	Cardiac arrest after stroke or seizure
24	F	1976	CNSI Ataxia	20	*POLG* c.1399G>A c.2243G>C (compound heterozygous)	None	NK	NP	24	Respiratory failure due to RTI in terminal phase with reduced consciousness
25	M	1947	DM MM CNSI Ataxia IHD CM	NK	*MT-TE* (MIM 590025) m.14709T>C	NK	I,II,IV	BL: 100	55	Cardiac arrest due to MI after above knee amputation (thrombosed aortic bypass graft)
26	F	1931	MM Neuropathy DM IHD	<40	*MT-TE* m.14709T>C	NP	NP	NP	70	Respiratory failure due to RTI in bedridden patient
27	F	1951	MM CNSI Dystonia Ataxia Dysphagia	NK	*MT-TC* (MIM 590020) m.5816A>G	COX-	None	MU: 100	51	NK

28	M	1952	MM CM	12	*MT-ND6* (MIM 590020) m.14484T>C	COX- RRF	I,II,IV	NP	54	Respiratory failure due to RTI after period of increased muscle weakness
29	M	1935	Neuropathy Ataxia Dysphagia MM CNSI (cognitive decline)	<12	NK	COX-RRF	None	NP	36	NK
30	F	1936	DM CNSI Ataxia OA + RP Deafness Dementia	33	*MT-TS2* (MIM 590085) m.12258G>A	NP	NP	NP	72	NK

BL blood, *CB* cerebellum, *CM* cardiomyopathy, *CNSI* central nervous system involvement, *Complex def* complex deficiency, *COX* cytochrome *c* oxidase-negative fibre, *CPEO* chronic progressive external ophthalmoplegia, *DM* diabetes mellitus, *DOA* dominant optic atrophy, *F* female, *GI* gastrointestinal, *H* heart, *Hist* histology, *IHD* ischemic heart disease, *M* male, *MELAS* mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, *MERRF* myoclonic epilepsy with ragged red fibres, *MI* myocardial infarct, *MIDD* maternally inherited diabetes and deafness, *MM* mitochondrial myopathy, *MU* muscle, *Mult. del.* multiple deletions, *NK* not known, *No.* patient number, *NP* not performed, *NRD* not reliably detectable, *OA* optic atrophy, *PM* postmortem, *RRF* ragged red fibres, *RP* retinitis pigmentosa, *RTI* respiratory tract infection, *UEC* urinary epithelial cells

to further follow-up, he had a myocardial infarction with subsequent pulmonary oedema. Since his age at death was 78 years, the myocardial infarction may be, in part at least, age related.

Chronic Progressive External Ophthalmoplegia (CPEO)

In nine patients, a CPEO or 'CPEO-plus' syndrome was diagnosed (patients 9, 10, 14, 16–21). The underlying genetic causes were point mutations in *POLG* (MIM 174763) or *MTTL1*, large-scale single deletion of mtDNA or multiple deletions of mtDNA where the nuclear gene responsible remained unidentified. The majority of these patients presented with one or more of ptosis, ophthalmoplegia, myopathy or ataxia. The mean age of death was 55.8 years and the median 59 (age range 30 to 73 years). Patient 10, known to suffer from dementia and muscle weakness, died after inhalation of gastric contents though dysphagia had not been clinically identified prior to death. Patient 14 died of sepsis after pneumonia following a myocardial infarction at the age of 73 years. She did have cardiac medication and regular cardiac checkups. Patient 19 died of complications of chronic alcoholic liver disease and patient 21 died of lung cancer. In five patients, the cause of death was unknown. One of them, patient 20, showed symptoms of Parkinsonism.

Kearns–Sayre Syndrome

In our cohort, three male adult patients were diagnosed with Kearns–Sayre Syndrome (patients 11–13), caused by large-scale single deletions of mtDNA. Initial presentation in all three cases was with muscle weakness and ophthalmoplegia. All died of cardiopulmonary complications following respiratory tract infection at an age varying from 40 to 66 years.

MERRF

One patient (patient 22) was diagnosed with MERRF, *MTTK* (MIM 590060), and died suddenly at the age of 41 years from possible respiratory failure due to a seizure or aspiration. She had severe muscle involvement and was care dependent.

Heterogeneous Phenotypes

The other nine patients (patient 15, 23–30) were phenotypically heterogeneous and died between the ages of 24 and 72 years. The cause of death was unknown in 4 patients (patient 15, 27, 29, 30). The other five patients (patient 23–26, 28) died of cardiac and/or respiratory failure during a respiratory tract infection, myocardial infarction, stroke or seizure.

Overall, the mean and median ages of death in the total adult patient group ($n = 30$) were, respectively, 50.4 and 52 (interquartile range 29.25) years, and in 50% of patients, no cause of death was registered. The respiratory chain deficiencies identified in the adult patient group differed in all phenotype and genotype groups.

Discussion

In this study we aimed to review the causes of death in a large, well-characterised population of patients with confirmed mitochondrial disease attending the NHS Highly Specialised Services for Rare Mitochondrial Diseases in Newcastle (UK). In this cohort, the three adult patients with KSS died of respiratory failure. Although perhaps not a typical cause of death for KSS patients, respiratory infections often have fatal consequences in debilitated, immobile patients with severe respiratory muscle weakness and proximal myopathy irrespective of their aetiology (Benditt and Boitano 2013; Palmio et al. 2014). However, the risk of respiratory compromise might be anticipated to be higher in patients with mitochondrial disease where dysphagia and associated aspiration pneumonia are not an uncommon occurrence. Impaired cardiac function, identified in all three patients, may also have contributed to their death. Approximately 57% of KSS patients exhibit cardiac symptoms (Berenberg et al. 1977), and multiple cases of sudden complete heart block have been described in both adults and children (Welzing et al. 2009; Katsanos et al. 2002; Chawla et al. 2008).

In our MELAS patient group (patients 1–8), we predominantly found cardiovascular causes of death. This finding concords well with the published literature where cardiovascular or neurological diseases were found to account for 30% of immediate causes of death and 32% of underlying causes in m.3243A>G carriers (Majamaa-Voltti et al. 2008). One MELAS patient died of a drowning incident, possibly suicide. Chronic illness is a risk factor for developing mood disorders (Greden 2001), and several studies have indicated a biologically plausible relationship between mitochondrial disease and depression (Morava et al. 2010; Mancuso et al. 2013a). However, this was the only potential suicide recorded in the cohort, and it is therefore difficult to conclude that mitochondrial disease was responsible.

Next to cardiopulmonary failure and pulmonary embolism, respiratory muscle weakness and dysphagia have been reported as a cause of death in CPEO patients (Klopstock et al. 1999; Caballero et al. 2007; Smits et al. 2011), and this was the case in 2 of the CPEO patients reported here. Interestingly one of the CPEO patients showed symptoms of Parkinsonism, a rare and late-onset symptom in CPEO-plus syndromes (Hudson et al. 2007; Mancuso et al. 2004)

and an independent risk factor for developing dysphagia (Pfeiffer 2011); unfortunately the cause of death in this patient remains unknown.

The patient with MERRF syndrome died suddenly of possible respiratory failure due to a seizure or aspiration. Epilepsy is perhaps one of the most predictable causes of death in MERRF syndrome although overwhelming lactic acidosis and cerebral haemorrhage are also reported (Sanger and Jain 1996; Huang et al. 2002; Kato et al. 2006; Herrero-Martín et al. 2010; Mancuso et al. 2013b).

Cardiopulmonary failure appears to be one of the most frequent causes of death in mitochondrial disease patients. Cardiac involvement in adult mitochondrial disease patients, such as cardiomyopathy, has been reported with a prevalence up to 25% (Limongelli et al. 2010; Wahbi et al. 2010; Yilmaz et al. 2012). Early recognition of cardiac involvement is important to initiate appropriate therapeutic measures and may possibly prevent complications such as heart failure, arrhythmias and embolism (Finsterer and Kothari 2014). Subclinical myocardial abnormalities can already be seen in mitochondrial disease patients with two-dimensional strain echocardiography, while conventional echocardiographic findings did not show any signs of ventricular systolic dysfunction at all (Marcus et al. 2011). Likewise cardiac MRI is detecting abnormalities of cardiac function in mitochondrial disease patients that are not picked up by conventional echocardiography (Bates et al. 2013).

A few patients in this cohort died of causes not obviously related to mitochondrial disease (patients 19, 21), namely, alcoholic chronic liver disease and lung cancer. The impaired physical condition of many mitochondrial disease patients compared with the general population may be a contributing factor to the poor outcome of other, apparently unrelated diseases that mitochondrial disease patients encounter, but further work in this field is required.

Our patients were born between 1930 and 1985. National life expectancy data show a life expectancy of 58.7 years for males born in 1930 to 71.7 years for males born in 1985 and for females 63.0 years when born in 1930 to 77.4 years in 1985 (Sweet 2011). The age of death in this cohort varies widely, ranging from 22 to 78 years, with a mean age of death of 50.4 years, considerably less than expected on national statistics. Notably those with the CPEO phenotype and large-scale rearrangements/deletions of mtDNA died at younger age than patients with CPEO phenotype caused by other (point) mutations in mtDNA. The patient with MERRF syndrome died at the age of 41. The MERRF phenotype shows high clinical heterogeneity with generalized epilepsy being a negative prognostic factor (Mancuso et al. 2013a). This patient had very severe muscle involvement and was entirely dependent on others for her care. Given the small number of deceased patients in our clinic in the mentioned period and the additional high percentage of unknown causes of death, statements regarding causes of death in specific mitochondrial syndromes cannot be formulated.

In this cohort of patients with mitochondrial disease, a correlation between the degree of heteroplasmy in several tissues and the age of death could not be found, though the precise level of heteroplasmy at, or near, the time of death was known in only a small number of patients. The different complex deficiencies and the presence of COX negative and ragged red fibres also did not seem to relate to the age of death. Postmortem examination of patients 3 and 7, who suffered from hypertrophic cardiomyopathy and died of multi-organ failure, respectively, showed high levels of mutated mtDNA in cardiac tissue from both individuals.

Conclusion and Recommendations

Despite the relatively limited number of patients described, we can carefully draw some conclusions and propose recommendations for the daily care of mitochondrial disease patients. One of the striking findings in this study is the limited information reported about the time period preceding the death of our patients and even about the cause of death in a substantial part of our cohort. Some of the patients died in their home environment, receiving palliative care coordinated by their general practitioner without awareness of the involved tertiary specialists. Better communication between caregivers would aid the study of death in mitochondrial disease patients and probably subsequently improve the care for mitochondrial disease patients.

As a consequence of specific care required, most patients are seen periodically in tertiary-level specialist centres. Optimization of the information exchange between the multidisciplinary team members (specialist clinicians, nurses, paramedical therapists, primary care physicians) involved and the patient might also contribute to early recognition in changes in the medical condition of the patient. For example, the primary care physician should coordinate the patient care and the specialist centre should provide a point of contact for the patient. This communication is equally important in relation to end-of-life issues where effective management may avoid unnecessary intervention in the terminal phase of an illness and allow early institution of palliative care. It may also permit appropriate postmortem samples to be obtained, with minimal distress to relatives, and address the issue of the relatively low number of postmortems performed in our cohort. The timing of when to have ‘end-of-life discussions’ with patients and family will depend on a number of factors including the particular form of mitochondrial disease, the patient’s willingness to engage and the rate of

disease progression. The latter will be obvious in rapidly progressive disease, but much more difficult to determine in patients who experience gradual deterioration. A possible approach to objectify disease progression might be by regular review using functional quality scales, such as the adult (NMDAS) forms of the Newcastle Mitochondrial Disease Rating Scale (Schaefer et al. 2006). Objective evidence of deterioration might aid both the physician and patient in discussing future prognosis and planning.

The majority of causes of death in our cohort were cardiac related. This study endorses that early detection of cardiac involvement is important, as it is associated with a poor prognosis, and timely therapeutic intervention may improve both quality and quantity of life. New investigative modalities, such as cardiac magnetic resonance spectroscopy, to detect cardiomyopathy in the initial stages look promising and may permit an even earlier institution of therapy. In this light, we underscore the importance of an interdisciplinary approach and communication between the primary care physician and the cardiology team. Our study highlights that a common cause of death in mitochondrial disease is cardiopulmonary failure following respiratory tract infection. Groups at higher risk of respiratory infection, including those patients with dysphagia or immobility, may therefore benefit from antibiotic prophylaxis, earlier institution of gastrostomy feeding or regular chest physiotherapy.

Finally, in the cohort of mitochondrial patients described in this study, 8% died in a time period of ten years. In almost half of these patients, all of whom were attending a specialist mitochondrial centre, the cause of death remains unknown. If we hope to influence the progression and outcome of mitochondrial disease for these patients, then we need to take careful note of the cause of death and any contributing factors. Good communication between primary care physician and specialist clinicians following the death of patients is essential in reaching this goal.

Take-Home Messages

Mitochondrial disease can cause significant morbidity and mortality.

Mitochondrial disease is incurable and 'end-of-life' planning should be discussed with patients appropriately.

Compliance with Ethics Guideline

Conflict of Interest

Marlieke Barends and Lotte Verschuren declare that they have no conflicts of interest. Victoria Nesbitt is a Clinical Research Associate for the Medical Research Council Mitochondrial Diseases Patient Cohort Study, UK. Eva Morava is a guest editor for the *Journal of Inherited Metabolic Disease*. Doug Turnbull and Robert McFarland are both PIs on the same study.

Contributions of Individual Authors

Marlieke Barends and Lotte Verschuren conducted the collection, analysis and interpretation of data and drafted the article. Eva Morava contributed to the revision of the article. Victoria Nesbitt contributed to data collection and revision of the article. Doug Turnbull contributed to the conception and design of the study and to the revision of the article. Robert McFarland was responsible for the conception and design of the audit, interpretation of data and to the revision of the article; he is the guarantor for the article.

Human and Animal Rights and Informed Consent

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

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