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Contents

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RESEARCH REPORT

The Acid Sphingomyelinase Sequence Variant p.A487V Is Not Associated With Decreased Levels of Enzymatic Activity

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Abstract Rare loss-of-function mutations in the sphingomyelin phosphodiesterase 1 (*SMPD1*) gene are known to dramatically decrease the catalytic activity of acid sphingomyelinase (ASM), resulting in an autosomal recessive lysosomal storage disorder known as Niemann-Pick disease (NPD) type A and B. In contrast to the general low frequency of those deleterious mutations, we found a relatively high frequency for the proposed type B NPD variant c.1460C>T (p.A487V) in our sample of 58 patients suffering from Major Depressive Disorder. We therefore investigated the biochemical consequences of this variant more closely. Our in vivo data derived from blood cell analyses indicated cellular ASM activity levels in the normal range. The secreted ASM activity levels in blood plasma were slightly lower, but still above those levels reported for type B NPD patients. In vitro expression studies of this ASM variant in different cell lines confirmed these results, showing cellular and secreted enzymatic activities equivalent to those of wild-type ASM and similar expression levels. Thus, we conclude that the ASM variant c.1460C>T (p.A487V) is not a rare missense mutation but an *SMPD1* sequence variant that yields a protein with functional catalytic characteristics.

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Introduction

An inherited deficiency of acid sphingomyelinase (EC 3.1.4.12; ASM) is the cause of autosomal recessive sphingolipidosis, known as Niemann-Pick disease (NPD) type A [MIM: 257200] or B [MIM: 607616] (Brady et al. 1966). Type A NPD is a severe infantile neurodegenerative disease that results in death within 3 years of life. It is also characterised by massive hepatosplenomegaly and psychomotor deterioration. In contrast, type B NPD does not display neurological involvement, but is a visceral form featuring hepatosplenomegaly and affecting the reticuloendothelial and respiratory system. Type B NPD patients can survive into adulthood (Dardis et al. 2005). The birth prevalence is estimated to be in the range of 0.5 per 100,000 (Pinto et al. 2004), with the exception of the Ashkenazi Jews, in whom the birth prevalence is approximately 3 per 100,000 (Schuchman and Miranda 1997). In addition to type A and type B, intermediate NPD phenotypes have been observed (Pavlu-Pereira et al. 2005; Wasserstein et al. 2006), leading to a proposed alternative classification system that includes four NPD types (Harzer et al. 2003).

Currently, up to 100 missense mutations in the sphingomyelin phosphodiesterase 1 gene (*SMPD1;* GenBank Accession Number NM_000543.4), which codes for ASM, are listed in the Human Gene Mutation Database. Novel *SMPD1* mutations are usually identified from patients presenting clinical symptoms of NPD and reduced cellular ASM activity (L-ASM, lysosomal) in cultured skin fibroblasts. Putative disease-causing mutations are subsequently identified by sequencing. The secreted form of ASM in blood plasma (S-ASM) can be diagnostically used and helps to distinguish between NPD patients (S-ASM activity below 7% of normal activity; range 0.18–7%) and NPD carriers (below 41% of normal activity; range 8–41%) (He et al. 2003). There exist validated genotype–phenotype correlations, which are based on the residual cellular ASM activity levels. Residual L-ASM activity under 5% is supposed to be indicative of NPD type A (Desnick et al. 2010).

Patients suffering from Major Depressive Disorder (MDD) display significantly higher levels of ASM activity in their peripheral blood cells compared to controls, and the severity of depression and ASM activity levels are positively correlated (Kornhuber et al. 2005). To analyse the impact of sequence variations on ASM activity levels in the context of MDD, we conducted a re-sequencing analysis of *SMPD1* in MDD patients. Among the identified missense variations was the single nucleotide polymorphism (SNP) c.1460C>T (rs141641266) that codes for the amino acid substitution p.A487V. This variant is a proposed type B NPD mutation and was first described in a patient with a less severe form of type B NPD characterised by organomegaly and mild pulmonary involvement (Simonaro et al. 2002).[1] Within a recent exome sequencing project, the frequency of this variant was 0.4%. To clarify the biochemical consequences of the proposed type B NPD variant c.1460C>T/p.A487V, we used in vivo and in vitro approaches. The cloning of identified mutations into expression vectors and the overexpression of the mutant proteins in cultured cells provide a powerful method to determine the impact of a particular mutation on ASM activity (Dardis et al. 2005; Desnick et al. 2010; Jones et al. 2008). To date, however, most of the proposed ASM missense mutations have not been verified by expression studies, which are necessary to provide conclusive evidence of their disease-causing loss-of-function status.

In this study, we clearly showed using in vivo and in vitro approaches that the ASM variant c.1460C>T/p.A487V is not associated with decreased levels of enzymatic activity, but displays functional enzymatic characteristics.

[1] According to Schuchman et al. (1991) numbered as A485V, here numbered according to GenBank Accession Number NM_000543.4 as A487V. The difference is due to the polymorphic region coding for the signal peptide of ASM.

Materials and Methods

Ethics Statement

The collection of blood samples was approved by the local Ethics Committees and conducted in concordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Subjects

For the initial re-sequencing analysis, 58 patients suffering from MDD according to ICD-10 criteria and treated at the University Hospital Erlangen, Germany, were included. For further sequencing analyses, samples from 420 patients with affective disorders and 422 control samples were provided by the German Research Network on Depression and Suicidality. Diagnosis was confirmed by common psychiatric procedures. Additional control samples were collected and analysed for sequence variations and ASM activity levels at the University Hospital Erlangen, Germany.

Genotyping

Genomic DNA was isolated from whole blood using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). The sequence variant c.1460C>T was amplified by polymerase chain reaction (PCR) using the oligonucleotides 5′-CCCCAGATGTCTTCCTACCCC-3′ and 5′-TTCCCCTATCCCACCAACTCC-3′ and cycle sequenced on an Applied Biosystems 3730 DNA Analyzer using the same primers.

Overexpression of ASM Variants

A wild-type ASM transcript was amplified from RNA by PCR and cloned into the FLAG-N2 expression vector to create an in-frame C-terminal fusion of ASM with the 16 amino acid FLAG-tag (Rhein et al. 2012). The variants A487V and P325A were generated by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). ASM variants were transfected into HeLa cells by the calcium phosphate precipitation procedure (Rhein et al. 2012). MDCK cells were transfected using polyethylenimine (Ehrhardt et al. 2006). Cells were harvested 48 h after transfection and lysates were analysed.

Western Blot Analysis

Transfected cells were lysed in RIPA buffer and analysed for protein concentration using the BCA assay (Bio-Rad, Munich, Germany). Total protein (10 µg) was separated by 10% SDS gel electrophoresis and transferred to a PVDF-membrane (Millipore, Schwalbach/Ts, Germany). Blots were incubated with a primary mouse monoclonal anti-FLAG-antibody (1:1,000; Sigma-Aldrich, Munich, Germany), with a primary mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:200,000; Millipore, Schwalbach/Ts, Germany) and with a secondary goat anti-mouse IgG antibody, coupled to horseradish peroxidase (1:10,000; Dianova, Hamburg, Germany). Detection was performed using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Munich, Germany) and visualised on a high-sensitivity camera device (Fluor-S-Max, Bio-Rad, Munich, Germany) (Rhein et al. 2012).

In Vitro Determination of ASM Activity

L-ASM and S-ASM activity from primary samples and from cell lysates was determined using the fluorescent substrate BODIPY-C12-sphingomyelin (Invitrogen, Darmstadt, Germany). The method was validated and described in detail in Reichel et al. (2011). Briefly, 2 µl of cell lysate (typically corresponding to 0.5–2 µg of protein), 10 µl of serum-free conditioned medium or 1.5 µl blood plasma were added to 116 pmol of fluorescent substrate in sodium acetate buffer (200 mM sodium acetate pH 5.0, 500 mM NaCl and 0.2% NP-40) in a total volume of 100 µl. For the measurement of S-ASM activity, sodium acetate buffer was supplemented with 0.5 mM $ZnCl_2$. After incubation for 0.5 to 4 h at 37 °C, the fluorescent product ceramide and uncleaved substrate were extracted by the addition of 250 µl chloroform/methanol (2:1, v/v). Following vortexing and centrifugation, the lipid phase was concentrated in a SpeedVac vacuum concentrator and spotted on silica gel 60 plates (Macherey-Nagel, Düren, Germany). Ceramide and sphingomyelin were separated by thin layer chromatography using chloroform/methanol (4:1, v/v) as a solvent and quantified on a Typhoon Trio scanner (GE Healthcare, 488 nm excitation and 520 nm emission wavelengths, 280 V, 100 µm resolution) with QuantityOne software (Bio-Rad, Munich, Germany). Measurements of all samples were performed in triplicates. For overexpression studies, each variant was transfected in triplicates within one experiment and all experiments were repeated at least once. The enzymatic activity of ASM was calculated as the rate of hydrolysis of sphingomyelin to ceramide per hour and per mg of protein in the cell lysate sample (pmol/mg/h).

Web Resources

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/.
Human Gene Mutation Database, http://www.hgmd.org/.

Results

High Frequency of ASM Sequence Variation c.1460C>T in the General Population

In a re-sequencing analysis of *SMPD1* in 58 patients suffering from Major Depressive Disorder, we identified an unexpected high frequency of the SNP c.1460C>T ($n = 3$; allele frequency 2.6%). To determine whether the c.1460C>T variant is specific for patients suffering from MDD, we analysed another 998 DNA samples, 420 from patients with affective disorder and 578 from controls. Overall, nine (allele frequency 1.1%) and eight (0.69%) carriers of the T-allele were found among the patients and controls, respectively, indicating that the unexpected high frequency of the c.1460C>T variant is not associated with MDD.

Carriers of c.1460C>T Do Not Display Decreased Levels of ASM Activity

Next, we analysed the in vivo ASM activity of c.1460C>T carriers in a subgroup of the control sample ($n = 156$). Within this subgroup, two individuals carried the c.1460C>T variation (allele frequency 6.4%). Based on the analyses of He et al. (2003), we hypothesised that carriers of proposed NPD mutations should display lower S-ASM activity. The S-ASM activity in the individuals' blood plasma was determined using a previously established enzymatic assay (Reichel et al. 2011). The S-ASM activities of all 156 individuals were used to establish a mean S-ASM activity level (196 ± 67 pmol/ml/h, $n = 156$). The S-ASM activity of carrier #1 was 80% of the mean S-ASM activity, and 42% activity was observed for carrier #2. We also determined the L-ASM activity in isolated lymphocytes, although the informative value of this activity level for the identification of NPD patients and carriers might be weak (Wenger et al. 1977). Carrier #1 exhibited an L-ASM activity of 62% and carrier #2 exhibited an L-ASM activity of 119% relative to the established mean L-ASM activity level (1.85 ± 0.62 pmol/mg/h, $n = 156$). Thus, only one of the two c.1460C>T carriers displayed a lower ASM activity, but this level was still above the level indicative of NPD carrier

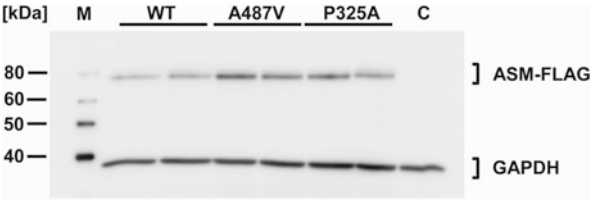

Fig. 1 Western blot analysis of transiently expressed wild-type ASM and ASM variants. Cells transfected with constructs of wild-type ASM and the ASM variants p.A487V and p.P325A produced proteins with the predicted sizes. Lysates of transfected HeLa cells were analysed by Western blotting 48 h after transfection. ASM-FLAG variants were detected using an anti-FLAG antibody. Each second lane comprises the indicated variant with an additional common SNP for control reasons. The cloning vector served as a negative control (C). GAPDH was used as a loading control. M indicates the size marker.

Table 1 L-ASM activities of transiently expressed ASM variants in HeLa and MDCK cells

	HeLa cell lysates		MDCK cell lysates	
Construct	ASM activity (pmol/mg/h)	% Wild-type activity	ASM activity (pmol/mg/h)	% Wild-type activity
Wild-type ASM	24,579	100	35,679	100
p.A487V	26,014	106	34,229	95
p.P325A	915	1	5,778	1
Mock control	1,230		5,536	

Table 2 S-ASM activities of transiently expressed ASM variants in HeLa and MDCK cells

	HeLa cells		MDCK cells	
Construct	ASM activity (pmol/ml/h)	% Wild-type activity	ASM activity (pmol/ml/h)	% Wild-type activity
Wild-type ASM	4,061	100	6,658	100
p.A487V	4,973	123	6,420	96
p.P325A	186	4	398	1
Mock control	40		0	

status as defined by the analysis of He et al. (2003). The other carrier exhibited ASM activity levels within a normal range.

ASM Variant p.A487V is Catalytically Active Upon Transient Overexpression

To further test the significance of the proposed NPD mutation c.1460C>T, we performed overexpression studies in cultured cells. As a control condition, we analysed the type B NPD mutation c.973C>G, which codes for the amino acid substitution p.P325A, in parallel. Variant cDNA plasmids were generated by site-directed mutagenesis and transiently overexpressed in HeLa and MDCK cells. Western blot analyses showed that the FLAG-tagged ASM variants were translated into proteins of the predicted sizes that were expressed to a similar extent as the wild-type ASM protein (Fig. 1). Overexpression of wild-type ASM in HeLa and MDCK cells increased the L-ASM activity in the cell lysates by 20-fold and 6-fold, respectively, and the S-ASM activity in the conditioned medium by 100-fold and 500-fold over endogenous levels, respectively. Overexpression of the p.P325A ASM variant did not increase the ASM activities above the endogenous levels, a result that is consistent with the notion that p.P325A is a loss-of-function mutation contributing to the development of NPD. In sharp contrast, overexpression of the ASM variant p.A487V resulted in ASM activities similar to that of wild-type ASM (Tables 1 and 2).

Discussion

In this study, we analysed the properties of the proposed type B NPD mutation c.1460C>T (p.A487V) in detail. Contrary to expectations, the classification of variant c.1460C>T is debatable. Our analysis revealed an in vitro ASM activity that was comparable to the activity of wild-type ASM and almost normal S- and L-ASM activities in two carriers. In comparison, the proposed type B NPD missense mutation c.973C>G

(p.P325A) (Simonaro et al. 2002), which was found in one patient suffering from MDD in our study, resulted in a severely reduced in vitro ASM activity. This result is in line with the classification as a deleterious mutation. It is still possible that c.1460C>T is associated with a defect that does not take effect under the chosen in vitro conditions, as reported for assays using the artificial sphingomyelinase substrate 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine (Harzer et al. 2003). In addition, the high frequency of c.1460C>T carriers in our sample argues against the notion that c.1460C>T acts as an NPD mutation. However, we cannot exclude the possibility that the NPD allele frequency is underestimated in the general population. Consulting bioinformatic mutation prediction tools, our claim is further supported. The sequence variation c.1460C>T is supposed to be a polymorphism by Mutation Taster (Ramensky et al. 2002), neutral by SNAP (Bromberg and Rost 2007) and benign by PolyPhen-2 (score 0.014 out of 1.00) (Adzhubei et al. 2010).

What might be learned from these conflicting results? Considering the critical consequences that may arise from the classification, further analyses are warranted. It has to be taken into account that c.1460C>T was originally identified in an NPD patient as a proposed second defective allele. In some cases, c.1460C>T might be associated with another yet unknown polymorphism. Thus, either the effect of c.1460C>T could be modulated by a second site variant or the variant c.1460C>T could be acting as a surrogate marker for a more critical mutation in *SMPD1*. The existence of such a second marker is still speculative, and deeper investigations should be conducted to clarify this issue. The findings reported here, however, stress the importance of validating potential type A and B NPD mutations by various methodical approaches to ensure that they are deleterious mutations.

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Author Contributions

Conception and design: CR, MR, JK; Analysis of data: CR, JN, CM, PZ, MR; Interpretation of data: CR, JN, CM, PZ, MR; Drafting of article: CR, MR; Revising manuscript: JN, CM, PZ, UH, CH, RM, HJM, JK; Intellectual input: MA, UH, CH, RM, HJM, JK

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RESEARCH REPORT

The Changing Face of Infantile Pompe Disease: A Report of Five Patients from the UAE

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Abstract *Objective*: We aim to present our experience with infantile Pompe disease with focus on the impact of availability of treatment on awareness, diagnosis, and management of such patients.

Method: Case – review study of patients diagnosed with infantile Pompe disease and literature search.

Results: We identified five cases of infantile Pompe disease. The first was diagnosed by muscle biopsy; all others were diagnosed by enzyme assay on peripheral blood lymphocytes or dried blood spot. There was no determination of the CRIM status on these patients. Two have died at a much later age than the reported median age of death for untreated cases. One died very early at 2 months of age with severe cardiomyopathy and had received only one dose of enzyme replacement therapy (ERT). The remaining two surviving patients are siblings: the younger was diagnosed by prenatal ultrasound screening and started on ERT at 24 h of age; she is the youngest treated patient in our case series.

Conclusion: The natural history of infantile Pompe disease is changing, so are the challenges of managing these infants in the post- ERT era. Currently, increased awareness and early access to therapy provide the best outcomes and incur the least shift of burden from mortality to morbidity.

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Introduction

Pompe disease (Glycogen Storage Disease II; OMIM# 232300) is an autosomal recessive lysosomal storage disorder caused by deficiency of acid α-glucosidase. The cardinal features of severe cardiomyopathy and muscle weakness represent the phenotype of infantile Pompe disease, with prediction of death at a median age of 7.7 and 6 months in Dutch and non-Dutch patients, respectively (Van den Hout et al. 2003) . Kishnani et al. reported on the impact of rhGAA on prolonging the survival of infants with Pompe disease (Kishnani et al. 2009), while Kanters et al. have demonstrated the substantial burden of disease both on the individual and the society in adults with Pompe disease (Kanters et al. 2011). There are emerging challenges with the prolonged survival of patients with infantile Pompe disease, with a potential shift of burden of illness from high mortality to high morbidity, given the current limitations in response to enzyme replacement therapy (ERT), particularly when started late. On the other hand, the clear efficacy of early treatment has shifted the focus of diagnostic window to the presymptomatic period, i.e., neonatal screening. According to the results of the Taiwan screening program, e.g., the newborn screening allowed initiation of treatment at an age of less than 1 month compared with the age of 3 to 6 months in infants in the control group (Chien et al. 2008).

We aim to review our case series of infantile Pompe disease in context of the evolving new natural history of this fascinating and challenging disease.

Methods

A case review study of all cases of infantile Pompe disease diagnosed and treated with enzyme replacement therapy

(ERT) with alglucosidase alfa, recombinant human GAA (rhGAA, Myozyme), at the dose of 20 mg/kg every 2 weeks between 2006 and 2011. Charts of all patients were reviewed and the following information collected: clinical presentation, diagnostic confirmation method, clinical course, complications, and outcomes.

Case Reports

Case 1: This UAE male infant of consanguineous marriage referred to our pediatric neurology service at 4 months of age for delayed development. He was found to have hypotonia, weakness, and hypertrophic cardiomyopathy. Diagnosis was confirmed by muscle biopsy. Weakness had progressed to −1/5 before ERT was started. Viral-induced respiratory failure occurred after his first infusion at 8 months of age; he became ventilator dependent. Despite significant cardiac response to ERT, muscle strength improvement was minimal. He continued on a biweekly ERT infusion and remained cognizant of his parents until he died at 5 years of age.

Case 2: This Bangladeshi male infant was referred to our pediatric neurology service at 9 months of age for motor delay. We found him to have hypotonia, hypertrophic cardiomyopathy, and motor delay. Infantile Pompe disease was confirmed by peripheral blood lymphocyte enzyme assay. ERT started at 11 months of age. He required no respiratory support. He had significant reduction in cardiac hypertrophy, improvement in muscle power, and acquisition of new motor skill. He had feeding problems that required G-tube placement. He continued on biweekly ERT and had occasional acute respiratory illness requiring hospitalization. He never required invasive ventilator support. His parents were excited at 18 months of age because he outlived his previous sibling who died of weakness of unknown cause (probably a missed case of infantile Pompe disease). He unexpectedly died in sleep at 2 years of age.

Case 3: This Palestinian female infant of consanguineous marriage presented at 2 months of age with massive hypertrophic cardiomyopathy and severe cardiac failure. She also had profound hypotonia and mild hepatomegaly. Pediatric neurology consult was requested to rule out Infantile Pompe disease. Diagnosis was confirmed by dried blood spot enzyme assay (α-glucosidase at pH 3.8 was 0.23 nmol/spot*21 h, reference range 1.5–10 nmol/spot*21 h). She received one dose of ERT, but died from cardiac failure before receiving her second dose.

Case 4: This UAE male of consanguineous marriage was referred by a pediatric cardiologist for neurological assessment of possible infantile Pompe disease at 4 months of age after diagnosing for hypertrophic cardiomyopathy. He had significant hypotonia, mild hepatomegaly, and CPK elevation. ERT was started after confirming his diagnoses by dried blood test enzyme assay. He was evaluated in another center abroad on parental choice, and genetic testing confirmed his homozygous mutation as well as carrier state of both his parents (exact mutation not available in our records). His cardiomyopathy improved and his left ventricular mass index normalized with ERT, but he continued to have some gross motor delay, and he suffered most significantly from dysphagia; requiring G-J tube placement. He had repeated respiratory infections requiring frequent admissions to the intensive care unit for mechanical ventilation; eventually he required nocturnal BiPAP. He continues on biweekly ERT.

Case 5: This UAE female is the sibling of case 4. Her diagnosis was suspected by prenatal obstetrical ultrasound demonstrating cardiac hypertrophy. She was started on ERT at 24 h of age based on clinical suspicion (hypertrophic cardiomyopathy) and family history (an affected sibling and confirmed carrier status in both parents). Dried blood spot testing confirmed her diagnosis within 3 days of sending her specimen (α-glucosidase at pH 3.8 was 0.90 nmol/spot*21 h, reference range 1.5–10 nmol/spot*21 h). Genetic testing in a lab abroad later confirmed homozygous mutation (exact mutation not available in our records). Hypertrophic cardiomyopathy resolved completely by 3 months of age. At 21 months of age she is bearing weight and taking steps with support, but having mild pooling of saliva. Speech however is not well developed. She continues on biweekly ERT.

Discussion

Our case series demonstrates several important points in the evolving picture of infantile Pompe disease. First, given the rates of consanguinity in the UAE, reported in one study to be as high as 40% to 54.2% in two sampled cities in the UAE (Al-Gazali et al. 1997), one would expect that cases are underdiagnosed; increased awareness becomes critical in early recognition and referral of such cases. This is indeed the case in our patient series, were the trends of referral have changed from more vague complaints of delayed development, as in cases 1 and 2, to a more focused referral to exclude infantile Pompe disease per se as in cases 3 and 4. This in our review represents increased awareness of what is now a treatable orphan disease.

Second, all of our patients, except case 3 which was in severe decompensated cardiac failure and did not survive beyond her first dose of ERT, had hypertrophic cardiomyopathy that responded to ERT including complete resolution in cases 4 and 5. However, the skeletal muscle response to ERT was less robust except for case 5 which

was started on ERT for the sole indication of mild hypertrophic cardiomyopathy prior to development of weakness and hypotonia. These observations may in part explain the contribution of ERT to a shift of disease burden from mortality to morbidity; infants who would have died of progressive cardiac involvement are now living longer only to manifest a more protracted course of muscle weakness and risk of respiratory complications, i.e., unless started on ERT before development of clinically noticeable motor weakness. The attenuated skeletal muscle response to ERT has been attributed to many factors such as the degree of pre-ERT muscle damage, humoral immunity, and paucity of mannose 6 phosphate receptors (M-6-P), the latter was the focus of Koeberl et al.'s recent publication in which they demonstrated the positive role of increasing M-6-P expression in mice, using the β2-agonist therapy with clenbuterol, on enhancing the efficacy of ERT in Pompe disease (Koeberl et al. 2011). Until such strategies are clinically proven, the best chance for these patients remains in initiating ERT before onset of weakness. The third observation is the early recognition of infantile Pompe cases through targeted prenatal ultrasound in high-risk families, defined as parents with previously affected children or a known carrier state such as the situation in case 5. This approach was previously reported in another city in the UAE, and resulted in early successful therapy with ERT (Hamdan et al. 2008), and later expanded to a full fetal echocardiography screening program as demonstrated by the same group (Hamdan et al. 2010). The advantages of prenatal ultrasonography approach is that it picks up affected patients in whom the indication for ERT is unequivocal; it bypasses the dilemma of what to do with neonates with biochemical diagnosis of Pompe with no symptoms. The other advantage is that the practice of prenatal ultrasonogrophy is already in place, it only requires increased awareness for early postnatal evaluation of newborns in which hypertrophic cardiomyopathy has been identified.

The fourth observation in our patient cohort is the high prevalence (80%) of feeding problems compared to a reported 44–55% in non-Dutch and Dutch groups (Van den Hout et al. 2003). Cases 1, 2, and 4 all had swallow studies confirming significant dysphagia and aspiration risk requiring placement of gastrostomy tubes in all three patients. Despite that, they continued to have difficulty handling their secretions. Feeding problems are a significant morbidity in infantile Pompe, and early intervention is essential in these cases. Case 5 demonstrates that this symptom too is potentially preventable with early ERT.

The fifth alarming observation is represented in the sudden unexpected death of case 2. Unfortunately, autopsies are not routinely performed in UAE, and hence we were not able to confirm the cause of death in this infant who was responding very well to ERT having normalized cardiac function and acquiring new motor skills. Interestingly, Dr. Pompe's first report of the disease was that of a 7-month-old girl with sudden death from "idiopathic myocardial hypertrophy" (Pompe 1932). Metzl et al reported a case of sudden death in an infant with severe hypertrophic cardiomyopathy due to Pompe disease (Metzl et al. 1999); the mechanism attributed to the effect of glycogen on the conduction system of the heart. Whether ERT contributes to the risk of conduction system abnormalities during the phase in which the glycogen overload is mobilized by the effect of ERT is a theoretical but important question. The involvement of the cardiac conduction system may be supported by the association of the general anesthesia with increased risk of cardiac arrhythmias in infantile Pompe disease (Wang et al. 2007). Our case died unexpectedly at home, raising concerns of other contributing factors to mortality in infantile Pompe disease despite successful ERT.

Finally, our 2 sibships (cases 4 and 5) demonstrate the clear impact of early ERT, especially with the known fact of phenotype concordance among infantile Pompe sibships (Smith et al. 2007). They also demonstrate the effectiveness of targeted neonatal screening as outlined earlier, and show how the face of infantile Pompe disease is changing even within an individual family.

Conclusion

The diagnosis of infantile Pompe disease is no longer just a matter of academic curiosity and family counseling. It has become an important commitment of the medical community caring for children with orphan diseases. Increasing awareness of the manifestations of the disease and targeted screening in high-risk families are readily available approaches that unequivocally contribute to better outcomes. The concern over shift of burden of disease from mortality to morbidity is legitimate, but should only lead to further improving our skills and wisely directing our resources to provide our patients with early access to effective therapies, while curative treatments continue to be sought.

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RESEARCH REPORT

A Pilot Study of the Effect of (E, E)-2, 4-Undecadienal on the Offensive Odour of Trimethylamine

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Abstract *Introduction*: Trimethylaminuria is a malodour syndrome caused by a functional defect of flavin-containing monoxygenase 3 (FMO3), resulting in accumulation of trimethylamine in body secretions. Recently, (E, E)-2, 4-undecadienal has been shown to deodorize the offensive odour of cooked porcine intestines (chitlins). We tested the deodorizing effect of commercially available (E, E)-2, 4-undecadienal on the odour of trimethylamine (TMA) in solution.

Study Participants: Eleven volunteers among staff of the Children's Hospital at Westmead, Sydney, Australia.

Methods: This was a study in three stages. In the first stage,12 volunteers sniffed and graded a commercially available trimethylamine at variable concentrations (12.5–10,000 μmol/L). Those who could smell trimethylamine scored the odour of mixtures of (E, E)-2, 4-undecadienal and trimethylamine. Finally, the odour of trimethylamine was graded with increasing concentrations of (E, E)-2, 4-undecadienal (0.1–100 ppm).

Results: All except one could detect the characteristic trimethylamine odour at varying concentrations (12.5–10,000 μmol/L) and reported the odour as offensive and fish like. There was a dose response effect of the ability of (E, E)-2, 4-undecadienal to deodorize the odour of trimethylamine. (E, E)-2, 4-undecadienal at 10 ppm appeared to deodorize the odour of trimethylamine at 1,000 μmol/L without making the former's odour obvious.

Conclusions: We have demonstrated that (E, E)-2, 4-undecadienal has a deodorizing effect on the offensive odour of trimethylamine in solution. The mechanism of action for this effect and potential for treatment of affected individuals needs further research.

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Introduction

Trimethylaminuria (TMAU) (OMIM # 602079) is a metabolic disorder characterized by decreased ability to oxidize and convert dietary-derived trimethylamine (TMA), an aliphatic tertiary amine, to odourless trimethylamine N-Oxide (TMAO) (Humbert et al. 1970; Chalmers et al. 2006). This disorder results in affected patients secreting volatile and malodorous (like rotting fish) TMA in their breath, sweat, urine and other body secretions. This compound can be detected by humans

at very low concentrations (<1 ppm) (Willey 1985). Fortunately, accumulation of TMA has no deleterious physical effect but may cause devastating social debilitation and long-term psychiatric consequences (Christodoulou 2012; Mountain et al. 2008). The few therapeutic options for management include dietary restriction of choline- and lecithin-containing foods, avoidance of drugs like carnitine and betaine, copper chlorophyll and/or activated charcoal as binding agents to reduce systemic absorption, probiotics and intermittent antibiotics to balance and/or reduce gut bacterial load (Chalmers et al. 2006; Buby et al. 2004; Treacy et al. 1995).

There is a need to expand the available therapeutic armamentarium. The mechanism of action of chlorophyll in deodorizing TMA odour by forming complexes opens avenues for exploring other compounds (Dashwood et al. 1996). This is intriguing in the context that recently (E, E)-2, 4-undecadienal, a naturally derived compound isolated from coriander leaves, was demonstrated to have a deodorant effect against the offensive odour of cooked porcine large intestine (chitlins) (Ikeura et al. 2010; Kohara et al. 2006).

We decided to test the hypothesis that commercially available (E, E)-2, 4-undecadienal may have a neutralizing effect on the malodour of TMA in solution.

Methods

The study protocol was approved by the Human Research Ethics Committee (HREC), the Children's Hospital at Westmead, Sydney, Australia.

One hundred and twenty staff members of the Western Sydney Genetics Program were invited in writing to participate in this study. Twelve volunteers (age range 23–57 years, eight females and four males) responded and provided written consent to participate in the study. Five volunteers reported some kind of nasal allergies.

The study was conducted in three stages. In each stage a five-point odour detection scale (given below) was used for volunteers to grade the odour of the TMA alone (stage one) or mixture of TMA and (E, E)-2, 4-undecadienal (stages two and three).

1. Barely detectable
2. Mild odour (faint)
3. Moderately strong odour (obvious)
4. Strong odour
5. Very strong repulsive odour

The samples were presented in 60 mL open specimen containers, starting with a distance of about a forearm length from the nose. Subsequently, the container was slowly brought closer to the nose until the volunteer could clearly smell the odour. In order to avoid habituation of odour stimuli, each presentation was followed by a rest period of about 30 s. This coincided with the time taken for opening of jars and communicating the next step to the volunteer. As a rule, each sample was presented only once except in some cases where a volunteer requested to smell again to be sure to quantify the difference in odour intensity. In such a situation, a rest period of about 30 s was given.

In stage one, 12 volunteers were asked to rate increasing concentrations of TMA (12.5−10,000 μmol/L). One volunteer was excluded from entering into the next stage of the study as she could not detect the odour of TMA at any concentration.

In stage two, 11 volunteers rated three concentrations of TMA (1,000, 5,000, and 10,000 μmol/L) as controls, followed by ten mixtures of variable concentrations of TMA and (E, E)-2, 4-undecadienal (Table 1). Volunteers were asked to compare the odour of TMA on its own with the odour of the mixtures, comment on the type of odour (whether it is TMA or some other odour), and report whether it was offensive or non-offensive.

In stage three, 11 volunteers rated the odour of a mixture with 1,000 μmol/L of TMA and increasing concentrations of (E, E)-2, 4-undecadienal (0, 0.1, 10, 20, 25, 50, and 100 ppm).

Preparation of the Study Reagents

Trimethylamine hydrochloride was obtained from Sigma-Aldrich, Castle Hill, NSW, Australia. Stock standards were prepared by dissolving in ultra-pure water and stored in sealed containers at room temperature.

(E, E)-2, 4-undecadienal was obtained from Alfa Aesar, Bioscientific, Gymea, NSW, Australia. The compound is an oily liquid at room temperature and varying concentrations were produced from a stock emulsion of 1,000 ppm in ultra-pure water and stored at 4 °C. There was no evidence of the compound coming out of emulsion on storage at the concentrations used.

Mixtures of (TMA and (E, E)-2, 4-undecadienal) in water were prepared at various concentrations from the stocks and stored in sealed containers at room temperature.

Statistical Analysis

The relationship between odour scores and concentrations of TMA and (E, E)-2, 4-undecadienal was examined using Generalized Estimating Equations (GEE) regression. This regression analysis accounts for the potential correlation between odour scores of the same patient (Hanley et al. 2003).

Table 1 Proportion of volunteers who could smell TMA in mixtures

Stage	**TMA mixtures[a] (Stage II)**										
Mixture *n* (%)		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
II	Yes	4 (36.4)	4 (36.4)	1 (9.1)	5 (45.5)	4 (36.4)	3 (27.3)	7 (63.6)	4 (36.4)	3 (27.3)	11 (100.0)
	No	7 (63.6)	7 (63.6)	10 (90.9)	6 (54.5)	7 (63.6)	8 (72.7)	4 (36.4)	7 (63.6)	8 (72.7)	0
III	**Fixed TMA and variable (E, E)-2, 4-undecadienal (Stage III)**										
Concentration *n* (%)	0	0.1	1	10	25	50	100				
	Yes	11 (100.0)	10 (90.9)	9 (81.8)	4 (36.4)	0	1 (9.1)	1 (9.1)			
	No	0	1 (9.1)	2 (18.2)	7 (63.6)	11 (100.0)	10 (90.9)	10 (90.9)			

[a] M1–M3 mixtures of 1,000 μmol/L TMA and 1, 10, and 100 ppm (E, E)-2, 4-undecadienal respectively; M4–M6 (5,000 μmol/L TMA and 1, 10, and 100 ppm (E, E)-2, 4-undecadienal, respectively); M7–M9 (10,000 μmol/L TMA and 1, 10, and 100 ppm (E, E)-2, 4-undecadienal, respectively); M10 (10,000 μmol/L TMA and 0.1 ppm (E, E)-2, 4-undecadienal)

The relationship between binary outcomes (proportion detecting TMA or not) and the concentration levels was examined using GEE logistic regression. Compound symmetry covariance matrix was applied in the models to treat repeated measurements as independent in time, but constantly correlated for the odour scores for the same subjects (Little et al. 2000). All analyses were performed using SAS version 9.2 (SAS Institute. SAS: statistical software. 9.1 ed. Cary, North Carolina. SAS Institute, 2003).

Results

In stage one, all except one test subject could smell TMA at varying concentrations (12.5–10,000 μmol/L). There was a wide variation of odour scores with increasing concentration of TMA indicating inter-observer variation. There was a positive relationship between odour scale and TMA, which plateaued at around a concentration of 1,000 μmol/L (Fig. 1).

In the second stage of the study, the proportion of subjects who could smell TMA was significantly less in mixtures (M3, M6, M9) with high concentration of (E,E)-2,4-undecadienal (Table 1). There were significantly increased odds of smelling TMA with increasing concentration of TMA, while increasing the concentration of (E, E)-2, 4-undecadienal decreased the odds of smelling TMA in mixtures (Table 2). For an increase of 10 μmol/L TMA, there was a 0.4% increased odds of smelling TMA.

In stage three of the study, keeping the concentration of TMA fixed, the proportion of subjects smelling TMA decreased with increasing concentration of (E, E)-2, 4-undecadienal (Table 1). There was a dose response effect of the ability of (E, E)-2, 4-undecadienal to deodorize the odour of TMA (Fig. 2). Every 10 ppm of (E, E)-2, 4-undecadienal decreased the odour scale of TMA by 0.2 units (95 % CI −0.3, −0.07, $p = 0.005$). (E, E)-2, 4-undecadienal at a concentration of 10 ppm deodorized the odour of TMA at 1,000 μmol/L without the odour of the (E, E)-2, 4-undecadienal becoming obvious.

Discussion

We have demonstrated that (E, E)-2, 4-undecadienal has the ability to deodorize the odour of TMA in solution. This is a first such study where this chemical compound has been demonstrated to deodorize the offensive odour of TMA. Previously, the deodorizing ability of (E, E)-2, 4-undecadienal on the malodour associated with cooking porcine intestine (chitlins) has been demonstrated (Kohara et al. 2006; Ikeura et al. 2010). However, the malodour of chitlins does not come from TMA (Kohara et al. 2006). Possible mechanisms for the deodorizing effect of (E, E)-2, 4-undecadienal on TMA include chemical, physical, biological and sensory actions (Ikemoto 1996). Kohara et al. has speculated that the most likely mechanism of action of (E, E)-2, 4-undecadienal (active ingredient in coriander) on the offensive odour of chitlins was a masking effect, that is, hiding the offensive odour with another strong odour, and/or a modification effect, modulating the compound with an offensive odour to a different compound that no longer has the offending odour. They found that the treatment with coriander did not decompose or reduce the main offensive odours. On the other hand, Ikeura et al. has expressed doubts about the masking agent hypothesis, as they used much lower concentrations of (E, E)-2, 4-undecadienal (0.1–100 ppb) at which this compound is odourless.

We used a much higher concentration of (E, E)-2, 4-undecadienal (0.1–100 ppm) at which its specific odour was obvious. In the current study, at higher concentrations of (E, E)-2, 4-undecadienal, the odour of (E, E)-2, 4-undecadienal became obvious (Table 1). Fortunately the odour of (E,E)-2, 4-undecadienal was mostly reported as much less offensive

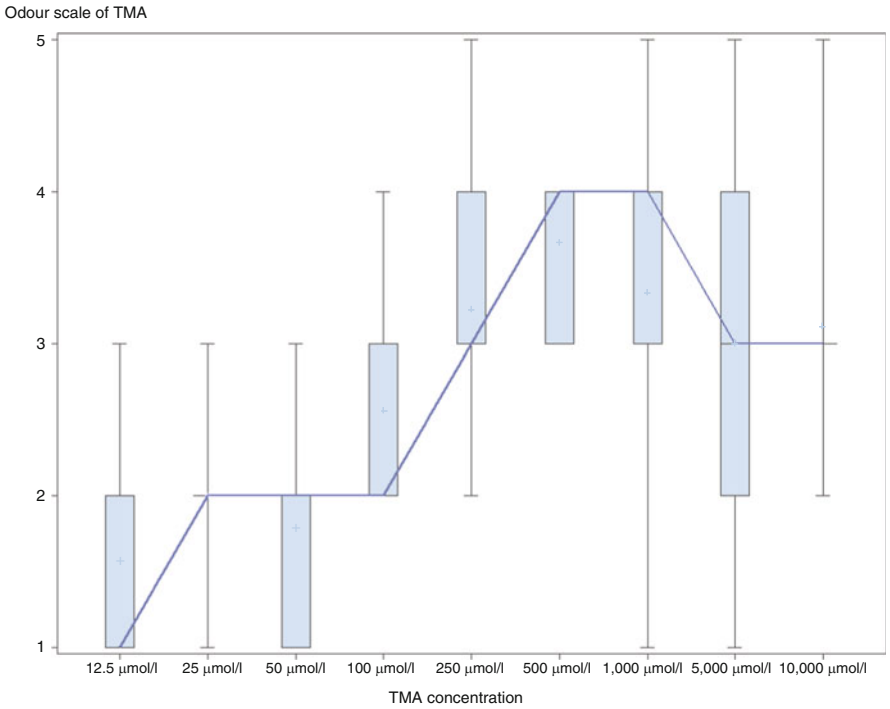

Fig. 1 Boxplot of odour scores for increasing concentration of TMA (Stage I). **Bold line* represents median and '+' represents mean

Table 2 The relationship between the odds of smelling TMA with variable concentration of TMA and (E, E)-2, 4-undecadienal (Stage II)

	OR[a]	95 % CI	*p*-value
TMA	1.004	(1.00–1.01)	0.003
Undecadienal	0.79	(0.66, 0.95)	0.014

[a] Odds ratios for every 10 unit increase

and described differently as "coriander-like", "oily", "vegetable oil", "rancid fat", "some plant odour" and "paint-like". This is in contrast to the odour of TMA alone, which was universally reported as offensive and fish-like. The possibility of a chemical reaction between (E,E)-2, 4-undecadienal and TMA warrants further research using chromatographic and other extraction methods.

The plateau effect of intensity of odour of TMA with increasing concentrations is an interesting finding. This could have two possible explanations, saturation of the head space gas concentrations or saturation of the nasal receptors leading to no increase in reported odour strength. Head space concentrations relate to the fact that the concentration of volatile molecules in the air space above a liquid will be determined by TMA's Henry law constant that is the ratio of concentration in air/concentration in water. The constant for TMA is 1.96×10^2, that is, it much prefers to be in air and the ratio holds until the sample reaches saturation in either phase. As the volume of liquid in our containers was 5 mL and the air volume was 55 mL it is possible that the TMA reached maximum concentration in the air even at sub-saturated aqueous concentrations. Greenman et al. have suggested two-site binding for TMA, which produces high affinity at low concentrations and low affinity binding at higher concentrations (Greenman et al. 2004). This could have given an apparent plateau effect in our study.

A limitation of our study was the inherent variability due to the subjective nature of detecting the TMA odour. Also, this was a pilot study on a small sample of volunteers which might have contributed to wide variation in the odour scale. However, we could clearly demonstrate a robust dose-response relationship of the ability of (E, E)-2, 4-undecadienal to deodorize the odour of TMA.

The results of study have implications for further research into the use of (E, E)-2, 4-undecadienal for treatment of malodour in patients with TMAU. We are not aware of any studies which have demonstrated whether

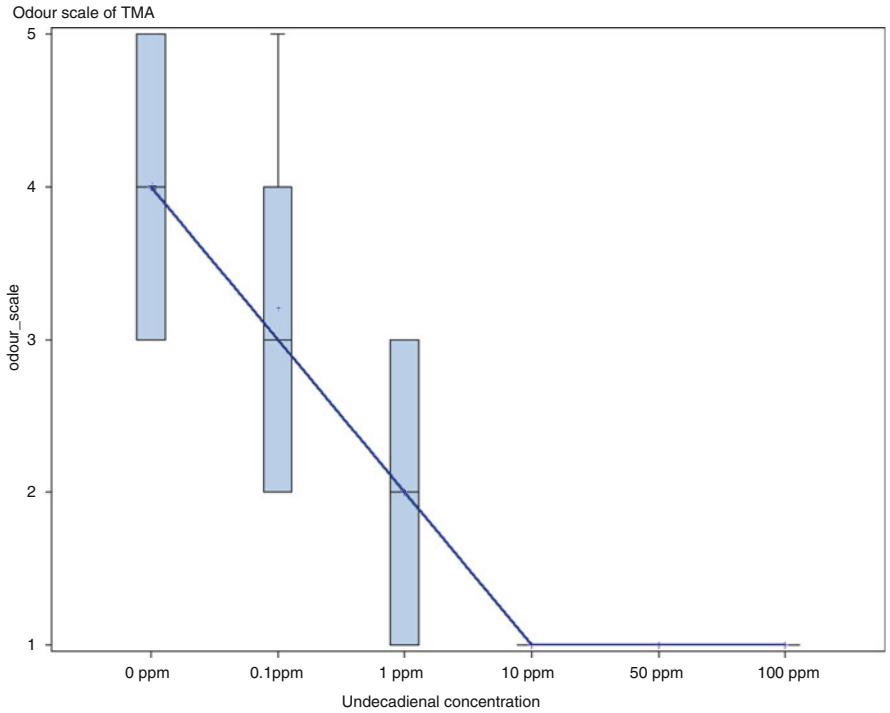

Fig. 2 Odour scale of fixed concentration of TMA (1,000 μmol/L) with increasing concentration of (E, E)-2, 4-undecadienal (Stage III). **Bold line* represents median and '+' represents mean

topical use of (E, E)-2, 4-undecadienal cause any local skin irritation. As this is a naturally occurring compound isolated from coriander, it could potentially be tested both as a topical as well as an oral preparation.

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CASE REPORT

ALG6-CDG in South Africa: Genotype-Phenotype Description of Five Novel Patients

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Abstract ALG6-CDG (formerly named CDG-Ic) (phenotype OMIM 603147, genotype OMIM 604566), is caused by defective endoplasmic reticulum α-1,3-glucosyltransferase (E.C 2.4.1.267) in the N-glycan assembly pathway (Grünewald et al. 2000). It is the second most frequent N-glycosylation disorder after PMM2-CDG; some 37 patients have been reported with 21 different ALG6 gene mutations (Haeuptle & Hennet 2009; Al-Owain 2010). We report on the clinical and biochemical findings of five novel Caucasian South African patients. The first patient had a severe neuro-gastrointestinal presentation. He was compound heterozygous for the known c.998C>T (p.A333V) mutation and the novel c.1338dupA (p.V447SfsX44) mutation. Four more patients, presenting with classical neurological involvement were identified and were compound heterozygous for the known c.257 + 5G>A splice mutation and the c.680G>A (p.G227E) missense mutation. The patients belong to a semi-isolated Caucasian community that may have originated from European pioneers who colonized South Africa in the seventeenth/eighteenth centuries.

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Introduction

ALG6-CG (phenotype OMIM 603147, genotype OMIM 604566) is a genetic disorder in the assembly of N-glycans due to ER alpha-1,3-glucosyltransferase deficiency (E.C 2.4.1.267). It was first reported in 1998 (Burda et al. 1998; Körner et al. 1998) and about 37 patients have been described (Imbach et al. 1999, 2000; Grünewald et al. 2000; Hanefeld et al. 2000; Westphal et al. 2000a, b, 2003; de Lonlay et al. 2001; Schollen et al. 2002; Newell et al. 2003; Sun et al. 2005; Vuillaumier-Barrot 2005; Eklund et al. 2006; Haeuptle and Hennet 2009; Al-Owain et al. 2010). The clinical presentation is mainly characterized by feeding problems and a moderately pronounced neurological disorder with psychomotor retardation, hypotonia, epilepsy, and internal strabismus (Grünewald et al. 2000; Newell et al. 2003). A minority of patients show other symptoms, particularly intestinal (such as protein-losing enteropathy (PLE)) and liver involvement (Damen et al. 2004). Striking biochemical features are unusually low serum cholesterol; blood coagulation factor XI and anticoagulation factors antithrombin, protein C, and protein S; as well as variable hypoalbuminemia; low serum LDL-cholesterol; and endocrinological abnormalities (Körner et al. 1998; Grünewald

et al. 2000; Hanefield et al. 2000; Newell et al. 2003; Sun et al. 2005; Westphal et al. 2000a, b). Table 1 summarizes clinical and molecular findings in ten ALG6-CDG patients reported since 1998.

Twenty-one different mutations have been described but the majority of patients have the c.998C>T (p.A333V) mutation, either in a compound heterozygous or homozygous state (Imbach et al. 1999, 2000; Grünewald et al. 2000; Hanefeld et al. 2000; Westphal et al. 2000a, b, 2003; de Lonlay et al. 2001; Schollen et al. 2002; Newell et al. 2003; Sun et al. 2005; Vuillaumier-Barrot 2005; Eklund et al. 2006; Haeuptle and Hennet 2009; Al-Owain et al. 2010). Haeuptle and Hennet (2009) reported that most of the mutations affect amino acids positioned within the 11 TM domains, which compromise the integrity of the protein structure and alter the properties responsible for the binding of dolichol-linked glycans.

Serum transferrin IEF profiles of ALG6-CDG patients show a type 1 CDG pattern. LLO (lipid-linked oligosaccharides) analysis of fibroblasts shows an accumulation of Man9GlcNAc2 (Imbach et al. 1999; Burda et al. 1998; Marklova and Albahri 2007). A few isolated CDG-ALG6 patients (e.g., patients with the homozygous c.391TT>C, p.Y131H mutation) presented with a normal transferrin and LLO profile (Westphal et al. 2003; Miller et al. 2011). The Laboratory for Inborn Errors of Metabolism started screening for CDG in 1998 and Du Plessis et al. (2001) reported on the first South African CDG patient. We report on five novel Caucasian patients from South Africa with previously described mutations and one novel mutation.

Patients and Methods

Patients

The ALG6 patients in this study formed part of the semi-isolated Caucasian society, which can be defined as a population which is not found in a particular area in South Africa, but share the same historical past of forced segregation. These families may have originated from European pioneers who colonized South Africa in the seventeenth/eighteenth centuries, but further genealogy studies are needed in the future.

Patient 1 was referred to the Potchefstroom Newborn Screening (NBS) Laboratory in South Africa. A follow-up metabolic screen and CDG assays were suggested. Samples of patient 2, 3, 4, and 5 (Table 2) were initially referred to the Laboratory for Inborn Errors of Metabolism in Potchefstroom, South Africa, for a full metabolic investigation. The parents of the patients were all non-consanguineous and the patients were all Caucasian South Africans. Patients 1, 2, and 3 had no family ties, but patients 4 and 5 were first cousins. The latter two patients were not related to patients 1, 2, and 3. Informed consent was given by the families for the CDG genotype studies and the group formed part of the EUROGLYCANET project.

Blood/Serum Transferrin IEF

Transferrin IEF analysis was performed on blood cards, of all five patients in this study at the Laboratory for Inborn Errors of Metabolism in Potchefstroom, South Africa, according to the methodology of Du Plessis et al. (2001). The validated blood card approach ensured a suitable sample for the transferrin analysis in a country, such as South Africa, in which transport and viability of samples could be compromised. Additional serum was collected from the five patients, who had a type 1 transferrin pattern on the blood card IEF analysis, and referred to the Academic Medical Centre (AMC), Amsterdam for confirmation.

ALG6 Mutation Analysis

Genomic DNA was isolated from EDTA blood of the patients, using the "NucleoSpin blood genomic DNA purification kit" (Macherey-Nagel, Germany, Düren). The ALG6 mutation analysis was performed by direct gene sequencing on DNA specimens, at the Department of Human Genetics of the University of Leuven (Imbach et al. 2000; Schollen et al. 2002) (The GenBank accession number of the ALG6 gene used in our study was NM_013339.3). An SNP database comparison and multiple alignment of the ALG6 protein were done for the novel mutation in this study.

Results

Clinical and Biochemical Description of Patients

Clinical data are summarized in Table 2. Patient 1 had severe PLE and neurological involvement. The newborn screening (NBS) result of patient 1 was normal except for an abnormal IRT (immunoreactive trypsinogen) result ([IRT = 145 μg/L, Ref value for 2–7 days < 75 μg/L]). Cystic fibrosis was excluded and no other metabolic abnormalities were observed. A metabolic screening and CDG analysis were requested, after the patient presented with clinical deterioration.

Patients 2 to 5 did not undergo NBS. Specimens of patient 2, 3, and 5 were sent to the metabolic unit for full metabolic screening and a transferrin IEF CDG analysis was specifically requested from clinicians. A CDG analysis was performed on

Table 1 Genetic and clinical description of ALG6-CDG patients published from 1998 to 2011

Reported ALG6-CDG patients	1	2	3	4	5	6	7	8	9	10
ALG6 gene mutation	c.998C>T (Homozygous and heterozygous)	Paternal: c.924C>A and c.391T>C, Maternal: c.998C>T (Heterozygous)	c.895-897 delATA and c.IVS3 + G>A (Heterozygous)	IVS 3 + 5G>A and c.998C>T or c.680G>A (Heterozygous)	c.509G>T and 1330_1332delCTT (Heterozygous)	c.391T>C and c.998C>T (Heterozygous) or c.391TT>C (Homozygous)	IVS3 + 3_3insT and c.998C>T (Heterozygous)	IVS7 + 2T>G and 897-897-899delAAT (Heterozygous)	c.338G>A and 10–12 Mb del(1)(p31.2p32.3) (Heterozygous)	c.482A>G (Homozygous)
Amino acid change	p.A333V(or combined with other mutation)	p.S308A, p.Y131H and p.A333V	In-frame deletion (dell299) and in-frame skipping	Aberrant splicing and p.A333V or p.G227E	p.S170I and in-frame deletion (ΔL444)	p.Y131H and p.A333V or p.Y131H alone	In-frame skipping and p.A333V	Aberrant splicing and in-frame deletion (dell299)	p.R113H and a deletion described on gDNA level	p.Y161C
Neurological abnormalities										
Microcephaly/ macrocephaly	Not reported	–	–	+ (microcephaly)	Not reported	–	–	–	+ (macrocephaly)	–
Hypotonia	+	+	+	+	+	+	+	+	+	+
Strabismus (internal or external if mentioned)	+ (internal)	Not reported	+	+	–	+	+	–	+	–
Psychomotor retardation (including speech delay)	+	+	+	+	+ (normal speech development)	+	+	+	+	+
Epilepsy	+	+	–	+	+	+	+	+	Not reported	+
Hypokinesia/ tremors	−/+	–	–	–	–	–	+ (tremors)	+ (tremors)	–	+ (hypokinesia)
EEG	Mostly normal	Normal	Abnormal	Not reported	Not reported	Normal	Not reported	Not reported	Normal	Not reported
Brain imaging	Cortical atrophy	Delayed myelination, slight cortical atrophy	Thin corpus callosum, mild cerebellar atrophy , mild widening of CSF spaces and ventricular system	Minimal cortical atrophy	–	Agenesis of corpus callosum (Westphal et al. 2003), cerebellar dysfunction (Miller et al. 2011)	Delayed myelination, normal cerebellum	Papilledema, but MRI normalized with age	Hypoplastic corpus callosum and absent septum pellucidum, white matter abnormality	Widening of CSF spaces and ventricular system, normal cerebellum
Optic dysfunction and/or -atrophy	−/+	+	+	+	+	+ (Miller et al. 2011)	Not reported	+	–	+
Dysmorphism	Broad nasal bridge, prominent forehead and large ears	–	–	Anormal long and narrow distal limbs, inverted nipples (noted at 6.5 months)	–	Broad nasal bridge	–	Abnormal fingers and toes (distal phalangeal hypoplasia with shortened toes and fingers)	Facial and limb abnormalities, deep set eyes, broad nasal bridge, loose skin, unusual fat distribution and other dysmorphic features	Severe body and facial abnormalities, inverted nipples and broad nasal bridge

(continued)

Table 1 (continued)

Reported ALG6-CDG patients	1	2	3	4	5	6	7	8	9	10
Gastrointestinal symptoms	–	Severe protein-losing enteropathy, gastroenteritis and diarrhea	–	Protein-losing enteropathy	–	Gastrointestinal disturbances	Peripheral edema and diarrhea, abdominal distention, protein-losing enteropathy	–	Mild hepatomegaly	–
Other symptoms	Poor feeding, recurrent infections	Hormonal abnormalities, e.g., cortisol deficiency	–	Poor feeding, apnea at the age of 4.5 months	–	Abnormal endocrinological function	Poor feeding (anorexia), vomiting, cardiac abnormalities, recurrent infection	Apnea, cyanosis, abnormal hair growth and distribution, ovaria and hormonal abnormalities	Poor feeding, cardiac abnormality: biventricular hypertrophy	Poor feeding, cardiomyopathy, brachycephaly, bilateral cryptorchidism, bilateral esotropia
Reference	Imbach et al. 1999 (Genotype) and Grünewald et al. 2000 (Phenotype), Körner et al. 1998	Westpal et al. 2000 (Dec)	Hanefeld et al. 2000 (Phenotype) and Westphal et al. 2000a (Genotype)	Imbach et al. 2000 (genotype), Du Plessis et al. 2001 (phenotype) and Schollen et al. 2002	De Lonlay et al. 2001	Westphal et al. 2003 and Miller et al. 2011	Newell et al. 2003	Sun et al. 2005	Eklund et al. 2006	Al-Qwaine et al. 2010

– : Not present

+ : Present (severity not indicated)

Table 2 Clinical presentation of South African patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Gender and age	Male, died at 5 months	Male, died at 3.1 years	Male, 3.1 years	Female, 6.9 years	Male, 3.9 years
Neurological abnormalities					
Microcephaly	–	+	–	+	+
Hypotonia	++	++	++	++	+++
Strabismus (internal or external)	–	+ (internal)	–	+ (internal)	+ (internal)
Psychomotor retardation (including speech delay)	–	+++	+++	+++	+++
Epilepsy	–	+++	+++	+++	+++
Hypokinesia/Tremors	–	–	Hypokinesia	Hypokinesia	Hypokinesia
EEG	Not performed	Abnormal	Abnormal	Abnormal	Abnormal
Brain imaging	Thin corpus collosum	Mild cerebral atrophy	Mild cerebral atrophy	Mild cerebral atrophy	Severe atrophy, small cerebellum
Optic dysfunction/atrophy	–	+	+	++	+++
Dysmorphisms	Enlarged eyes, micrognathia, broad-nasal bridge; camptodactyly (observed in fingers)	Broad nasal bridge	Broad nasal bridge	Broad nasal bridge; inverted nipples	Broad nasal bridge; small feet and fat pads on finger tips
Gastrointestinal symptoms	Protein-losing enteropathy with severe edema and ascites	–	Neonatal constipation and hepatomegaly	–	–
Other symptoms	Poor feeding	Recurrent infections	Poor feeding; recurrent infections	Recurrent infections	Poor feeding; recurrent infections

Patients 1 to 5 represent CDG cases in five separate families

– : Absent

+ : Mild

++: Moderate

+++: Severe

a blood card sample of patient 4, after the exclusion of other metabolic abnormalities. Biochemical information on patients 2, 3, 4, and 5 was limited. Patient 2 had normal liver and thyroid function. No biochemical parameters were available for patient 3. Patients 4 and 5 had recurrent episodes of severe infections. No chromosomal abnormalities were documented. Additional blood parameters, which might have been of interest (e.g., lipidogram, coagulation and endocrinological tests), were not requested for any of the five patients in this investigation.

Blood/Serum Transferrin IEF

All five patients showed a type 1 serum transferrin IEF profile, that was most pronounced in patient 1 (almost no tetrasialotransferrin) (Fig. 1).

ALG6 Mutational Analysis

Patient 1, with an intestinal-neurological phenotype, was found to be compound heterozygous for the c.998C>T (p.A333V) and c.1339dupA (p.V447SfsX44) mutations. Patient 2, 3, 4, and 5, with a predominantly neurological phenotype, were all compound heterozygous for the c.257 + 5G>A (originally described by Imbach et al. 2000 as the IVS3 + 5G→A mutation) and c.680G>A (p.G227E) mutations. The polymorphism, c.911C>T (p.S304F) was present in heterozygous state in patients 2, 3, 4, and 5. The carrier (father of patient 1) of the c.1339dupA (p.V447SfsX44) did not portray CDG-related symptoms.

The novel frameshift mutation, c.1339dupA (p.V447SfsX44), which we identified in this study, is not

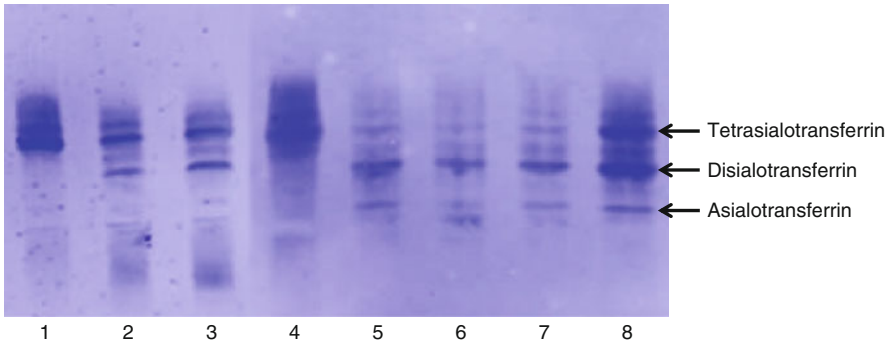

Fig. 1 Lanes 1 and 4 represent control IEF transferrin patterns (the presence of tetratransferrin indicates a normal profile). Lane 2 indicates the IEF profile of patient 2 and lane 3 indicates the IEF profile of patient 4. Lanes 5, 6, and 7 represent the IEF profiles of patient 1. The IEF profiles were consecutively performed at 2 , 3 , and 4 months of age. Lane 8 represents the IEF profile of patient 3

listed in the SNP database and affects a well-conserved amino acid in the ALG6 protein. Alignment studies were performed, using the computer software Alamut (version 1.5 rev. 32) of Interactive Biosoftware. The multiple alignment study of the ALG6 proteins of *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Rattus rattus* (rat), *Mus musculus* (mouse), *Canis familiaris* (dog), *Gallus gallus domesticus* (chicken), and *Tetraodon nigroviridis* (pufferfish), indicated that the valine on position 447 is highly conserved. The frameshift mutation also results in the premature termination of the ALG6 protein (44 amino acids after the amino acid change) and therefore the formation of a truncated protein.

Discussion

The described patients showed two distinct phenotypes of ALG6-CDG deficiency. Patient 1 showed a severe, early-onset, neuro-gastrointestinal presentation comparable with previously described case studies (Westphal et al. 2000b; Damen et al. 2004; Newell et al. 2003). The patient was compound heterozygous for the prevalent c.998C>T (p.A333V) missense mutation (originally described by Imbach et al. 1999) and a novel c.1338dupA (p.V447SfsX44) frameshift mutation in the ALG6 gene. The p.A333V mutation is associated with a mild to moderate phenotype which indicates that the novel frameshift mutation (c.1338dupA), which results in the formation of a truncated ALG6 protein, probably explains the severe phenotype and early death of the patient as well as the unusually low serum tetrasialotransferrin. The phenotypic variation from the typical ALG6 deficiency concurs with previous literature of rare ALG6-CDG cases with multiple organ involvement and intestinal abnormality, e.g., PLE (Westphal et al. 2000b; Newell et al. 2003; Al-Owain et al. 2010).

Patients 2, 3, 4, and 5 presented with the classical ALG6-CDG phenotype comprising of a variable degree of neurological involvement, including hypotonia and resistant epilepsy. Other features in these patients were feeding problems, recurrent infections, and mild facial dysmorphism (Grünewald et al. 2000, Du Plessis et al. 2001). The c.257 + 5G>A splice site mutation (originally described by Imbach et al. 2000) in combination with the c.680G>A (p.G227E) missense mutation (originally described by Schollen et al. 2002) was identified in all four patients. The same combination of mutations in four patients may suggest that they have a common (most probably European) ancestor.

In summary, beside the patients with the classical neurological ALG6-CDG phenotype described in this study, we also identified an ALG6-CDG patient with an early onset of severe neurological and intestinal involvement associated with a novel frameshift mutation. This variation in ALG6 phenotype is in agreement with the literature summarized in Table 1 and suggests a genotype-phenotype correlation.

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Competing Interests

All parties confirmed that they have no competing interest for declaration.

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CASE REPORT

Unexplained Hypoglycemia During Continuous Nocturnal Gastric Drip-Feeding in a Patient with Glycogen Storage Disease Type Ia: Is It a Dumping-Like Syndrome?

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Abstract A 5 years old boy affected with Glycogen Storage Disease type Ia (GSD-Ia) with previous optimal metabolic control developed severe erratic hypoglycemic episodes during continuous nocturnal gastric drip-feeding (CNGDF) administered by nasogastric tube. The episodes of hypoglycemia were not related to pump failure or human errors or wrong position of the tube in the gastrointestinal tract. Hyperinsulinism was also considered in this patient but it was excluded mainly because hypoglycemia was only nocturnal. Moreover, hypoglycemic episodes disappeared when CNGDF was stopped and he was fed with normal meals. The fact that hypoglycemia resolved after stopping CNGDF when nocturnal meals were introduced led us to hypothesize that CNGDF rich with simple carbohydrates might have been the cause of a sort of dumping-like syndrome. Dumping syndrome (DS) develops when a large amount of carbohydrate reaches the small intestine due to rapid gastric emptying (Tack et al. 2009; Hejazi et al. 2010). We suppose that CNGDF induced a disturbance of gastric motility with a gastric accumulation of fluids at a certain time of the night followed by a rapid voiding of the stomach leading to DS.

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Abbreviations

CNGDF	Continuous nocturnal gastric drip-feeding
DS	Dumping syndrome
G6PC	Glucose-6 phosphatase gene.
GET	Gastric emptying test
GSD-Ia	Glycogen storage disease type Ia
GSDs	Glycogen storage diseases

Introduction

Glycogen Storage Diseases (GSDs) are a group of inherited disorders characterized by enzyme defects that affect the glycogen metabolism. The deficiency of the glucose-6 phosphatase (EC 3.1.3.9) activity causes Glycogen Storage Disease type Ia (GSD-Ia, MIM 232200) that produces fasting hypoglycemia, hyperlacticacidemia, hyperlipidemia and hyperuricemia, growth retardation, enlargement of kidneys and progressive renal involvement, and risk of development of hepatocellular adenoma and carcinoma.

The current treatment of GSD-Ia is frequent diurnal small meals followed by orally administered uncooked cornstarch, a complex carbohydrate composed of highly branched glucose chains and slowly hydrolyzed in the small intestine, and continuous nocturnal gastric drip-feeding (CNGDF) through nasogastric tube or through gastrostomy with maltodextrins alone or free-lactose milk and maltodextrins. Diet composition is recommended to be hypolipidic, with normal protein and elevated carbohydrates intake. Carbohydrates are all complex during the day and maltodextrins are given continuously through the night. These measures are effective in maintaining the metabolic control (Rake et al. 2002) although growth retardation and liver, renal, and other complications may still occur in the long-term

follow-up even in well-controlled patients (Chen 2001; Heller et al. 2008; Melis et al. 2010).

Suggested rates of CNGDF are different at various ages on the basis of different energy needs: a rate of 7–9 mg/kg/min of carbohydrates is recommended in infants below 1 year of age, 6–8 mg/kg/min in children younger than 6 years, 5–6 mg/kg/min in school children and adolescents, and 3–4 mg/kg/min in adults (Rake et al. 2002).

Dumping syndrome (DS) consists of early (abdominal and vasomotor) and late (hypoglycemia) symptoms (Tack et al. 2009) and results from rapid delivery of simple carbohydrate-rich osmotic fluids to the small intestine. In normal conditions, the presence of glucose in the jejunum is a powerful stimulus for insulin secretion. Hyperosmolar significant amount of glucose in the duodenum substantially produces two effects: one is shift of fluids from the intravascular component to the duodenum leading to release of vasoactive agents and probably responsible of early symptoms of DS, such as tachycardia, nausea, abdominal pain, and hypotension; the other is a hyperinsulinemic response inducing hypoglycemia, the late effect of DS (Tack et al. 2009; Hejazi et al. 2010).

DS has mainly been described in children after surgical procedures for gastroesophageal reflux, like Nissen fundoplication, or in rare cases of microgastria, partial or total gastrectomy, accidental intraduodenal or jejunal administration of bolus feeding, or inadequate meals with high osmolarity, as well as rare cases of generalized autonomic dysfunction in two children operated for esophageal atresia without gastroesophageal reflux (Michaud et al. 2010), these last indicating that only esophageal dysmotility may be the trigger point. DS is also diagnosed in patients with type 2diabetes mellitus and in many patients previously diagnosed as cyclic vomiting syndrome (Hejazi et al. 2010).

We here report the case of a patient with GSD-Ia who had several unexplained episodes of hypoglycemia during CNGDF administered by nasogastric tube. Hypoglycemic episodes disappeared when CNGDF was stopped and he was fed with normal meals. By exclusion of other causes of hypogycemia, we hypothesize that this patient had a form of dumping-like syndrome caused by a gastric motility disorder associated to CNGDF.

Case Report

The patient is the first male child of nonconsanguineous Italian parents. He was born via a spontaneous vaginal delivery at 39 weeks gestational age with birth weight of 3.150 kg. His perinatal period was normal. At 4 months of life, he was hospitalized for frequent regurgitation, failure to thrive, and severe hepatomegaly (at the umbilical plane). On the basis of the clinical picture, the finding of fasting hypoglycemia, hypertriglyceridemia, and hypercholesterolemia and hyperechogenic liver tissue at ultrasound, a GSDs was suspected and later GSD-Ia was confirmed through the molecular analysis of glucose-6 phosphatase gene (G6PC) which showed that the patient carried the mutations p.Y172X/p.W86C.

The boy was regularly followed and maintained optimal growth and metabolic control of the disease with frequent meals, followed by cornstarch and CNGDF. At the age of 4 years and 6 months, he had mild hepatomegaly (1 cm from rib cage) and normal psychomotor development, the weight was 16.0 kg (25th centile) and the height was 103 cm (25th–50th centile).

His diet consisted of five diurnal meals every 3–4 h with cornstarch 35–40 g/day and CNGDF prepared with 15 g of lactose-free milk AL 110 (Nestlè, Milan, Italy), 70 g of maltodextrins powder Fantomalt (Nutricia, Milan, Italy) in 420 mL of water at rate 42 mL/h (8.31 mg/kg/min). Intake of glucose/kg/min with CNGDF (8–10 mg/kg/min) was elevated for patient's age because he has always been a lean, very active child with augmented energy needs.

He had normal pre-prandial glucose levels (70–90 mg/dL), no post-prandial hyperglycemia (90–100 mg/dL), normallactate (2.6-3.3 mmol/L, normal range 0.44-2.22 mmol/L), triglycerides (71–159 mg/dL, normal range 50–200 mg/dL), cholesterol (93–136 mg/dL, normal range 130–200 mg/dL), and uric acid (3.8-5.2 mg/dL, normal range 3–7 mg/dL).

At the age of 5 years, he had a generalized seizure with hypoglycemia (23 mg/dL measured with finger-stick glucose at home) at 4 am while he was fed with CNGDF; he had a rapid resolution after the administration of 30 mL of 33 % glucose solution via nasogastric tube. The child was then admitted to the local hospital. Feeding-pump functioning was verified and the nasogastric tube was repositioned. Rate of CNGDF was temporarily increased to 110 mL/h and then modified to maintain blood glucose in the normal range but he had another generalized seizure at 8.30 am caused by hypoglycemia (34 mg/dL) during the CNGDF (rate 45 mL/h).

After the resolution of the symptoms and the improvement of blood glucose, the patient was discharged that day and the parents were instructed in measuring glycemia with finger-stick glucose monitoring every hour during the night. The night after, the patient had another asymptomatic hypoglycemia at home (39 mg/dL at 4 am) during CNGDF (rate 50 mL/h). He was then accompanied to our Department. In his first night in our hospital, he had an important asymptomatic hypoglycemia (26 mg/dL at 3.30 am) during CNGDF prepared with 34 g of lactose-free milk AL 110, 96 g of maltodextrins powder Polycose in 600 mL of water at a rate of 55 mL/h (10.8 mg/kg/min). The day after, he had another asymptomatic hypoglycemia (39 mg/dL) at 4.15 am and in this occasion growth hormone (0.11 ng/mL, normal range 0–10 ng/mL), insulin (1.0 μUI/mL, normalrange

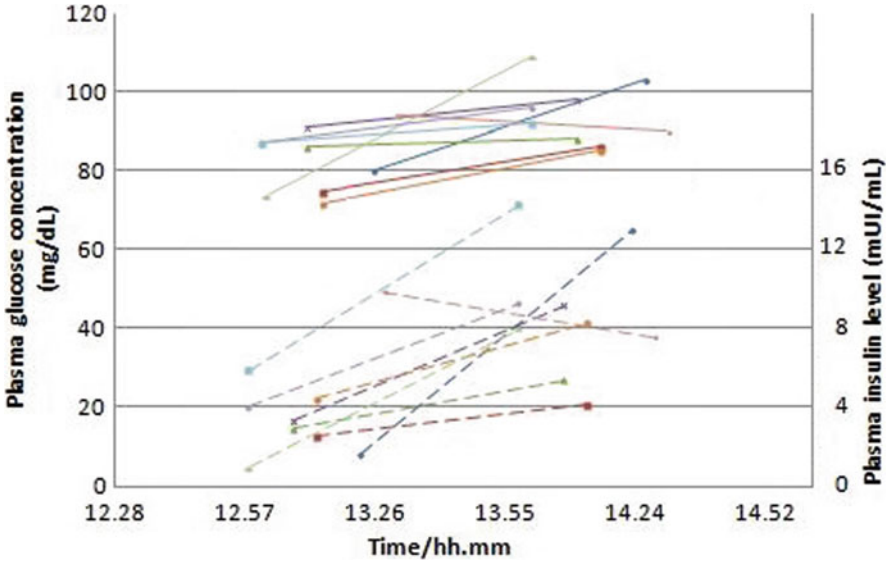

Fig. 1 Monitoring of blood glucose (*solid line*) and insulin (*broken line*) during the day, immediately pre-prandial and 1 h later (9 meals). We observed optimal values for a GSD-Ia patient metabolic profile

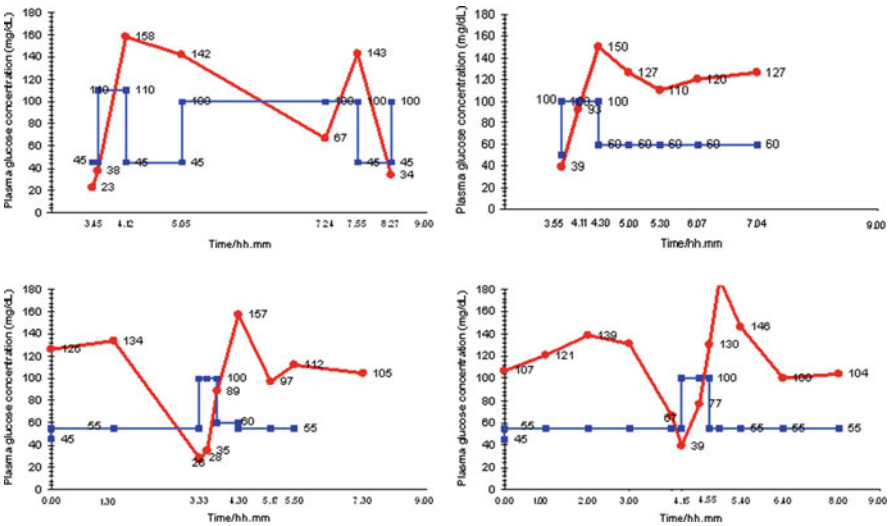

Fig. 2 Nocturnal blood glucose concentration with finger-stick glucose monitoring (●) and the rate of CNGDF (■). We report five episodes of nocturnal hypoglycemias during 4 nights of CNGDF

2.6–24.9 μUI/mL), C-peptid (1.1 ng/mL, normal range 0.8–4.2 ng/mL), and cortisol (99.6 ng/mL, normal range 62–194 ng/mL) were analyzed and resulted normal. We checked with X-rays that the tube was correctly positioned in the stomach. Monitoring of blood glucose and insulin during the day, immediately pre-prandial, and 1 h later did show an optimal metabolic profile for a GSD-Ia patient (Fig. 1). Munchausen syndrome was excluded first for the fact that different relatives were staying in hospital with the child (father, mother and grandmother) in the nights when he had hypoglycemias and furthermore because the psychologist excluded this possibility and defined this family as very well structured and optimally self- supporting.

At this point the composition and the rate of CNGDF were changed (35 g of lactose-free milk AL 110, 100 g of maltodextrins powder Polycose in 625 mL of water at 40 mL/h from 9.30 pm to 00.00 and at increased rate to 60 mL/h from 00.00 to 7.30 am): an improvement of nocturnal blood glucose was observed for 4 days and the patient was discharged.

At home, 18 days after the discharge, he experienced three other hypoglycemias during CNGDF (rate 55–60 mL/h), one with generalized seizure (35 mg/dL) and the other two

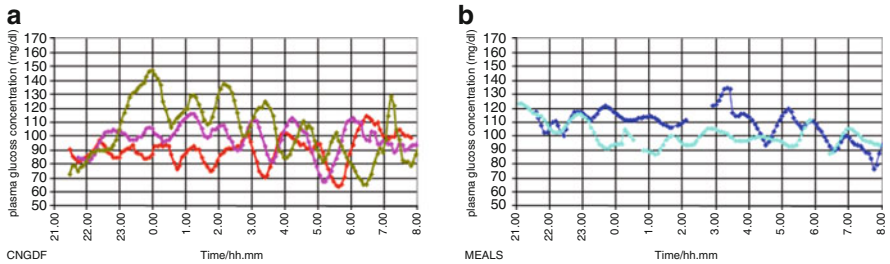

Fig. 3 Continuous monitoring of nocturnal blood glucose with Glycemic Holter: we report in Fig. 3a blood glucose of 3 nights during CNGDF and in Fig. 3b blood glucose of 2 nights during nocturnal meals. During CNGDF, glucose values in this patient show important variations with significant up and down even when he does not reach hypoglycemic values. Glucose blood variations are less relevant with nocturnal meals

asymptomatic (39 mg/dL, 46 mg/dL). The episodes of hypoglycemia in hospital and at home monitored with finger-stick glucose are reported in Fig. 2. Figure 3a shows results of continuous glucose monitoring (Guardian REAL-Time CGM System, Medtronic, Northridge, USA) at home during CNGDF. In these nights no hypoglycemia was detected but blood glucose values were fluctuating much more than we would expect with a CNGDF.

Then we stopped CNGDF and introduced nocturnal meals with 7 g of lactose-free milk AL 110, 8 g of multicereal cream and 40 g of uncooked cornstarch maizena at 10.30 pm and 3.30 am and 15 g of lactose-free AL110 and 25 g of uncooked cornstarch maizena at 7.30 am. The patient did not have any other nocturnal hypoglycemia. The continuous glucose monitoring with meals at home is reported in Fig. 3b. Surprisingly it was less fluctuating than the profile obtained with CNGDF.

Nowadays, at the age of 12 years, the boy is still having frequent meals during the night and has an optimal metabolic balance (last triglycerides 78 mg/dL, cholesterol 163 mg/dL, uric acid 3.6 mg/dL and lactate 2.1 mmol/L) and his dietary treatment consists of seven meals at 7.15 am, 10.45 am, 1.15 pm, 5.15 pm, 7.00 pm, 10.30 pm, and 3.15 am with a glucose intake of 7 mg/kg/min.

From the age of 6 years and 6 months, the patient presented glomerular hyperfiltration without microalbuminuria and proteinuria and we started at the age of 10 years the treatment with Ramipril 1.25 mg/day, incremented to 2.5 mg/day for persistent hyperfiltration without hypotension.

Discussion

Our patient had severe repeated episodes of symptomatic hypoglycemia while he was fed through CNGDF prepared with a high amount of simple carbohydrates. The rationale of giving simple carbohydrates continuously during the night relies on the assumption that CNGDF is associated with regular continuous absorption of nutrients.

We first excluded human errors, misfunctioning of the feeding pump, tube entanglement or blockages, faulty equipment, and child tampering with pumps and feeding equipment. Our second hypothesis was that of a wrong position of the tube in the gastrointestinal tract but, as the tube was replaced every night and taken away in the morning, we should assume that every night, at a certain time, the tip of the tube went down to the duodenum and this is not easily happening. When we checked with X-rays, the tube was correctly positioned in the stomach.

Hyperinsulinism was also considered: in fact sometimes GSD-Ia patients who are excessively fed can manifest hypoglycemia around 2–2.5 h after the meal but it does not happen during CNGDF. However, this child was never hypoglycemic during the day but only during CNGDF. Even though he had quite an elevated intake of glucose/kg/min for his age, he was really thin and much physically active every day; he was also optimally controlled from the metabolic point of view, with normal blood lactate, triglycerides, and uric acid. For all these reasons we had to exclude that this patient was affected by hyperinsulinism. We also excluded a Munchausen syndrome: the patient was followed by different relatives when had hypoglycemias in the hospital and the family was well structured from a psychological point of view.

We first tried to modify composition and rate of delivery of CNGDF in this child without success. Only after all these unsuccessful trials, considering that hypoglycemia occurred only during CNGDF, we decided *ex juvantibus* to stop the CNGDF and substitute it with meals. The fact that hypoglycemia resolved after the introduction of nocturnal meals led us to suppose that CNGDF rich with simple carbohydrates might have been the cause of a sort of dumping-like syndrome. Our interpretation is that it may be possible that this kind of artificial feeding during the night interfered with gastric motility and induced at a certain time

of the night an accumulation of fluids, followed by a rapid voiding of the stomach leading to DS.

DS is a constellation of gastrointestinal and systemic symptoms secondary to volume shifts and excessive release of insulin and various vasoactive peptides due to abnormal emptying of gastric content (Tack et al. 2009).

The physiopathology of DS is still controversial and probably multifactorial. The rapid passage of gastric content, especially if it is rich in small molecules, into the small bowel results in a shift of fluids and electrolytes into the intestinal lumen, loss of intravascular volume, and small bowel distension leading to the release of gastrointestinal hormones (Caulfield et al. 1987; Samuk et al. 1996). The rush of fluid can cause bowel stretching and cramps, nausea and vomiting, bloating, and diarrhea (early dumping syndrome). Late DS occurs 1–2 h after the meal and is characterized by systemic vascular symptoms including flushing, dizziness, weakness, sweating, fainting and palpitation, fall in blood pressure, and increase of heart rate (Hejazi et al. 2010). The rapid absorption of glucose is countered by excessive release of insulin responsible for the subsequent reactive hypoglycemia (Hejazi et al. 2010).

DS is suspected according to symptoms and medical history and confirmed through measurement of gastric emptying by scintigraphy. A 4 h Gastric Emptying Test (GET) using a low fat meal is the gold standard to investigate DS (Hejazi et al. 2010). However, this diagnostic method is rarely used in pediatric population and we lacked normal range values for this age. Second, this patient had symptoms only during the CNGDF and it was clear that he did not have problems after a normal meal. Third, the standard meal that is suggested for this test is not appropriate to a GSD-Ia patient. Another test which is suggested for DS diagnosis is dumping provocation by the oral glucose challenge (50 g after 10-h fasting): higher plasma levels of glucose during the first 60 min after provocation and reduced plasma glucose levels 60–180 min later have been used as diagnostic criteria for DS (Ukleja 2005).

The treatment of DS primarily consists of dietary manipulations as limiting fluid intake while eating solid small meals, high in protein and fat and low in carbohydrates, increasing meal viscosity and assuming a recumbent position following a meal (Bouras and Scolapio 2004). Pectins, glucomannan, and guar gum have been tested with good results, especially in the pediatric population (Tack et al. 2009). Uncooked cornstarch after the meals has been shown to be effective in a small number of children (Gitzelmann and Hirsig 1986; Borovoy et al. 1998).

We decided not to perform diagnostic conclusive tests in this child because they were too demanding for him and his family and because we had already obtained the resolution of symptoms. DS has mainly been described in children after surgical procedures on the gastrointestinal tract, and in few rare cases of generalized autonomic dysfunction in two children operated for esophageal atresia without gastroesophageal reflux (Michaud et al. 2010). In analogy with these few cases we hypothesized a posteriori that CNGDF could have been the cause of gastro-esophageal dysmotility in this child. It appeared to be confirmed by the trace of continuous nocturnal glucose monitoring which showed more relevant ups and downs during CNGDF than when the patient was fed with nocturnal meals (Fig. 3). This finding is enough to say that, despite continuous feeding with simple carbohydrates, the absorption of glucose during the night was not continuous. The explanation might only be a disturbance of gastrointestinal motility.

In conclusion, we present a 5-year-old GSD-Ia patient with previously optimal metabolic control who developed severe erratic hypoglycemic episodes during CNGDF. By exclusion, we hypothesized a disturbance of gastric motility during CNGDF.

We think that the more reasonable explanation of these findings is that nasogastric drip feeding induced a disturbance of gastric motility. It would be interesting to know if other cases exist with these symptoms and whether they are related to the use of nasogastric tube instead of gastrostomy.

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RESEARCH REPORT

A Dysmorphometric Analysis to Investigate Facial Phenotypic Signatures as a Foundation for Non-invasive Monitoring of Lysosomal Storage Disorders

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Abstract *Background:* Some lysosomal storage disorders (LSDs), including Muccopolysaccharidosis type 1 (MPSI), are associated with characteristic facies. Methods such as three-dimensional (3D) facial scanning and geometric morphometric techniques can potentially generate detailed objective descriptions of these facial phenotypes. This approach can facilitate discriminating the inherent overlap in facial phenotypes within these disease spectra, and the non-invasive monitoring of disease progression and treatment.

Methods: 3D facial images of three MPS I-affected individuals and 400 reference subjects (aged 5–25 years) were obtained using a 3dMD camera (Atlanta, Georgia). Images were fitted with an anthropometric mask, comprising a set of spatially dense quasi-landmarks. A statistical face-space was constructed from the reference image set and the MPS I-affected individuals were compared to this face-space utilising an emerging methodology known as dysmorphometrics. This facilitated simultaneous identification of harmonic and discordant facial regions. A relative significant discordance (RSD) score quantified proportional facial discordance for a given individual, whilst a root-mean-squared-error (RMSE) score measured the degree of facial discordance providing a severity measure.

Results: A consistent facial pattern, with differential severities, primarily affecting the frontal, nasal, infraorbital and cheek regions, was detected in all three individuals. As expected, there was greater discordance (RMSE, RSD) with clinically severe MPS I when compared to attenuated disease.

Conclusions: Objective detection and localisation of MPS I facial characteristics was achieved, and severity scores were attributed. This spatially dense dysmorphometric facial phenotyping technique has the potential to be used for non-invasive treatment monitoring and as a discriminatory tool.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AM	Anthropometric mask
BMT	Bone marrow transplantation
EMA	European medicines agency
ERT	Enzyme replacement therapy
IDUA	α-L-iduronidase
LSDs	Lysosomal storage disorders
MPS I	Muccopolysaccharidosis type I
MPS IH	Muccopolysaccharidosis type I, Hurler Syndrome
MPS IHS	Muccopolysaccharidosis type I, Hurler-Scheie Syndrome
MPS IS	Muccopolysaccharidosis type I, Scheie Syndrome

NE	Normal equivalent
PCA	Principal component analysis
PCs	Principal components
RMSE	Root-mean-squared-error
RSD	Relative significant discordance
SD	Standard deviation
SMAS	Submuscular aponeurotic system

Background

Lysosomal storage disorders (LSDs) are a group of inborn metabolic disorders, associated with disrupted lysosomal function that causes widespread lysosomal accumulation of undegraded macromolecules (Aldenhoven et al. 2009). Untreated, they inevitably result in progressive multisystem disease. With the advent of disease-modifying treatments, timely diagnosis and monitoring is increasingly pivotal to improving prognosis of affected individuals including, but not limited to, some muccopolysaccharidoses (e.g. MPS I) and Fabry disease. MPS-affected individuals are described as having characteristic 'coarse' facies. However, the detection of LSDs based on facial features can be challenging owing to overlapping facial phenotypes (Clarke 1997), clinical inexperience and attenuated disease. Supplementary approaches to discriminate between and within disorders and monitor treatment in a definitive manner are, therefore, required.

There have been promising, but limited, attempts towards objective definition of LSD-associated facial phenotypes, and advances in imaging technology are facilitating extension of these preliminary investigations. A two-dimensional (2D) photogrammetric study by Boehringer et al. (2006) discriminated MPS III from a number of non-LSD syndromic conditions, and a proof-of-principle study by Cox-Brinkman et al. (2007) utilised three-dimensional (3D) face shape analysis to characterise the face of Fabry disease. The latter study used spatially dense 3D surface modelling and morphometric analysis to quantify differences between male and female Fabry individuals, as well as in comparison to healthy controls, with classification specificities of 85 % and 67 %, respectively (Cox-Brinkman et al. 2007).

Quantitative morphometric techniques have demonstrated great potential in detecting facial dysmorphology and variation. Hammond et al. (2007) established facial archetypes for a small number of syndromic conditions. However, this methodology did not discriminate the impact of the condition from normal facial variation, and only visualised the facial dysmorphology expressed as net population difference. It was also based on a closed classification setup, with the individual always attributed to either one of the defined populations, even when they did not belong to any of them. Statistically, this setup is equivalent to an unpaired analysis, which requires the appropriate sample number to obtain sufficient statistical power. These limitations have, in part, been recognised by Hammond et al. (2012) and they have similarly been addressed by Claes et al. (2012a) with, an alternate dysmorphometrics strategy that makes comparisons to population-based 'averages'. This was previously applied to measure changes in facial shape as a result of surgical intervention (Claes et al. 2012b) and asymmetric abnormalities (Claes et al. 2011).

This chapter explores the use of dysmorphometrics (Claes et al. 2010, 2012a) to discriminate subtle facial characteristics of MPS I. Normal variation can be learned from a reference dataset of individuals without pathology and obtained with relative ease. However, the collection of facial data from individuals with rare diseases is more challenging. A dysmorphometric approach does not require comprehensive datasets of patient groups to generate a facial anomaly map. This facilitates investigations into facial phenotypic signatures in rare conditions. In difference to archetype analysis, an open-classification is performed, using only the normal variation from an appropriate reference dataset, which is encoded into a statistical face-space (Aeria et al. 2010; Wei et al. 2011; Claes et al. 2012a). This facilitates an individual-specific assessment and visualisation of a problem or hypothesis, through the construction of a normal-equivalent or case-specific control (Claes et al. 2010, 2012a).

This approach facilitates investigations into syndromic phenotypic signatures of rare conditions and a process to address the inherent overlap in facial phenotypes within disease spectra. It can also be applied to the non-invasive monitoring of disease progression and evaluation of therapeutic effects. From this exploratory study of three MPS I–affected individuals, we describe regional discrepancy scores that can be employed to establish discordance signatures in combination with a continuous severity index, to investigate aspects of metabolic and other rare diseases.

Methods

Ethics Approvals

Ethics approvals (PMHHEC: 1801/EP, 1443/EP and 1488/EP) were granted by the Princess Margaret Hospital for Children Ethics Committee in Perth, Australia.

Participants

The normative reference cohort (Perth face-space project) consisted of 400 healthy individuals aged 5–25 years. Subjects completed a questionnaire on relevant medical history

and informed consent. Subjects with prior craniofacial surgery or a suspected syndromic condition with craniofacial manifestations were excluded.

Three participants clinically diagnosed with MPS I (severe or attenuated), and of Western European ancestry, were recruited at the 11th International Symposium on MPS and related diseases (Adelaide, SA, Australia; July 2010). Individual I was a 21-year-old female diagnosed with Hurler-Scheie syndrome (MPS IHS, OMIM #607015); Individual II was a 23-year-old female diagnosed with Scheie syndrome (MPS IS, OMIM #607016); and Individual III was a 14-year-old male diagnosed with Hurler syndrome (MPS IH, OMIM #607014).

3D Image Acquisition

3D images were captured using a 3dMDFacial™ stereo-photogrammetric system (3dMD Inc., Atlanta, Georgia, USA) with sub-millimetre precision (Aldridge et al. 2005). Facial shape was represented as a point cloud consisting of approximately 200,000 points defined in a 3D coordinate space.

Anthropometric Masks and Facial Mapping

An anthropometric mask (AM) (Claes et al. 2012b) consisting of a spatially dense ~10,000 quasi-landmark configuration, was non-rigidly mapped onto 3D facial scans of affected and normative reference individuals (403 3D images). This mapping process is equivalent to the indication of traditional anthropometric landmarks (Claes et al. 2011, 2012b) and establishes homology among the 3D faces, thus allowing image data from different individuals to be standardised and analysed in a spatially dense manner.

Statistical Face-Space

A statistical face-space, constructed from normative reference individuals, describes facial variations and covariances (harmonic interrelationships) present within the general population (Aeria et al. 2010; Wei et al. 2011; Claes et al. 2012a). A generalised Procrustes fit (Rohlf and Slice 1990) rotates, translates and scales the quasi-landmark configurations into the same coordinate space. Shape variation is then described by Procrustes distance residuals. Subsequently, the extent and modes of facial variation are calculated using Principal Component Analysis (PCA) to elucidate complex harmonic interrelationships found in facial form variations. This 'normative' statistical face-space was created to define the boundaries or statistical limits of typical facial variation found in the reference population (Claes et al. 2012a).

Dysmorphometrics and Normal Equivalents

Dysmorphometrics identifies abnormal facial morphology, as outliers in comparison to a given normative reference (Claes et al. 2012a). In this scenario, normative references are encoded within the face-space and the outliers reflect discordancy in facial form. Dysmorphometrics involves a robust superimposition of the reference face-space onto the patient's facial scan, where each of the 10,000 quasi-landmarks is assigned a confidence value. This reflects the confidence of such a point being harmonic (value closer to 1) or discordant (= outlier, value closer to 0) against a p-value of 0.05.

This superimposition establishes the best description of a given face only in terms of harmonious facial variation, known as the 'normal equivalent' (NE). It eliminates confounding variables like position and orientation differences, and considers typical within-population differences (e.g. variances induced by gender, age, BMI, ethnicity, etc.) as confounders as well. This allows for construction of case-matched controls, more specifically, generation of a *patient-specific* and *population-based* matched reference (see Fig. 1 for comparison to averaged age-gender-matched control), as long as the variation is described by the face-space. Thus, providing a more sensitive and specific analysis on the syndromic facial phenotype. We used a face-space comprised predominantly of Western European ethnic variance. Technical details can be found in (Claes et al. 2012a).

Scoring, Analysis and Visualisation of Facial Variants

When the NE is superimposed on to the patient scan, differences between corresponding landmarks of the two configurations provides the means to measure magnitude (distance) and directions (vector) of facial discordances. Distance maps were summarised by a root-mean-squared-error (RMSE) score, which takes into account both variance and possible bias, as an error in millimetres (mm). This RMSE score was applied to measure severity for the observed discordancy. Confidence maps were summarised by relative significant discordance (RSD) percentages. This localised the discrepancy and provided an overall proportion of dysmorphology for a given individual. Finally, vector maps provide directional information on the observed facial discordance. The distance, outlier and vector maps collectively provide a facial discordance signature specific to the MPS I–affected individual.

Normative Population Reference Statistics

Some discordance is to be expected in the 'normative' reference range as a consequence of scan/mapping artefacts and/or the reduction of total variance modelled to 98 %.

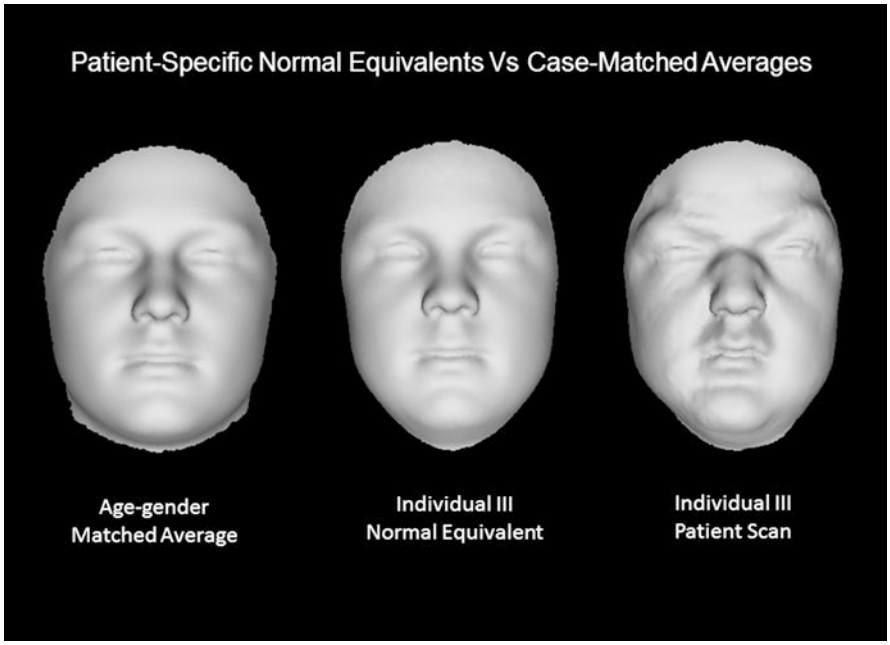

Fig. 1 Comparison of the normal equivalent (NE) versus an average case-matched control. The NE of Individual III (*middle*), synthetically matched for variables (e.g. age, gender and ethnicity) according to the dysmorphometric approach, is visually a closer matched reference to the original patient scan (*right*), in comparison to an average 14-year-old male scan (*left*)

Typical facial values in the reference population were established using a leave-one-out approach to compute discordance scores for the 400 healthy individuals. As a means of reference, distributions of overall RMSE and RSD scores for the 'normative' cohort were used to express patient values as Z-scores.

Results

Reference cohort distributions were characterised by a mean of 10.6 % RSD (1.8 % SD) and 0.91 mm RMSE (0.22 mm SD), summary statistics are provided in Table 1. These summary statistics provided the data for the calculation of Z-scores for the MPS I subjects.

Discordance signatures (i.e. outlier and distance maps) for the three MPS I individuals are depicted in Fig. 2. A consistent facial discordance with varying severity was observed for all three MPS I individuals (Fig. 2; Table 1). Individual I exhibited facial variants in the infraorbital region, nasal tip and nasio/mento-labial sulci (Fig. 2a, d). A similar discordance pattern was observed in Individual II with an additional variant in the frontal region (Fig. 2b, e). All facial variants detected in Individuals I and II were also observed in Individual III, with the exception of the nasal tip (Fig. 2c, f). Among all discordant facial regions observed in Individuals I, II and III, two individuals were found to be discordant at the naso/mentolabial sulci, as well as the frontal, nasal and infraorbital regions.

Sorting the cases on RMSE and RSD values provides a severity differential, with Individual I presenting discordance scores of 0.95 mm RMSE and 9.08 % RSD, these were elevated in Individual II (RMSE 1.19 mm; RSD 10.31 %), Individual III had the most severe discordance scores of 1.46 mm RMSE and 12.84 % RSD (Table 1). The facial dysmorphology for both Individual I and II was subtle with discordance scores that were within the reference range (Z-RSD: –0.84 % and Z-RSD: –0.16 % respectively). The severity of the observed facial discordance was likely to be mediated, as all patients were treated (e.g. ERT/BMT), indicating the sensitivity of the technique to detect subtle differences.

The vector map provided information on magnitude and direction of the facial discordance. In Individual III, the vector map revealed an overall 'ballooning effect' (Sandubray et al. 2012) of the facial tissues; a close up of this in profile view showed specific vector displacements at the brow ridges, glabella/frontal region, infraorbital region, upper lip and both nasiolabial and mentolabial sulci (Fig. 3). Expansive displacements were observed at the brow ridges and upper lip, different to the glabella, nasolabial and mentolabial sulci, and infraorbital regions that were depressed.

Table 1 Overall NE assessment and Z-scores for the three MPS I individuals in relation to summary reference statistics

	Dysmorphology (RSD in %)	Relative severity (RMSE in mm)	Z-RSD (%)	Z-RMSE (mm)
Reference (mean)	10.6	0.91	-	-
Reference (SD)	1.8	0.22	-	-
Individual I (MPS IHS)	9.08	0.95	−0.84	+0.18
Individual II (MPS IS)	10.31	1.19	−0.16	+1.27
Individual III (MPS IH)	12.84	1.46	+1.24	+2.50

% Dysmorphology scores were based on RSD values of the outlier map, which quantified the extent of the discordance and depicted the 'affected area'. The RMSE (mean + SD) score quantified the overall degree of discordance in millimetre and provided a measure of severity of the 'affected area'. Z-scores described the extent of difference to reference distributions (reference range). MPS IHS (Hurler-Scheie Syndrome); MPS IS (Scheie Syndrome); and MPS IH (Hurler Syndrome)

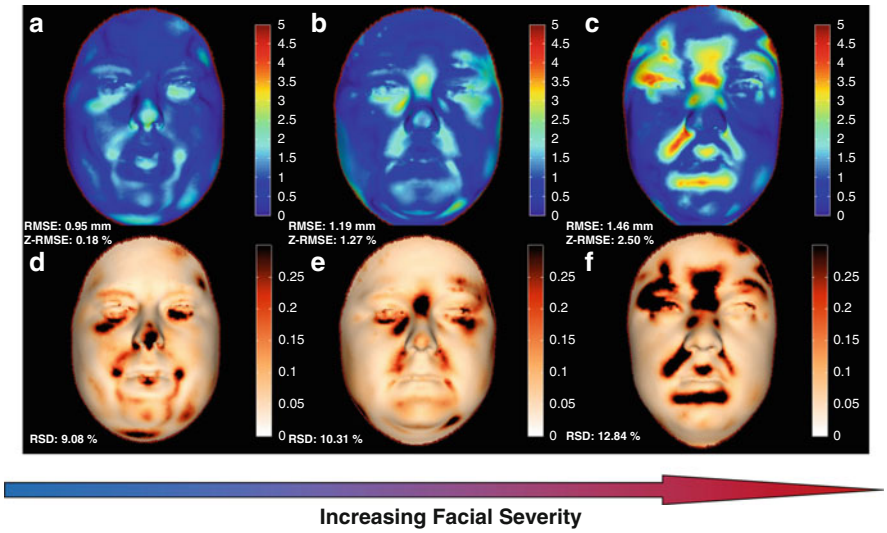

Fig. 2 Discordance severity in MPS I clinical subtypes. Dysmorphometric facial assessment depicting the distance and outlier map components of each MPS I–affected individual's discordance signature. The distance maps (*top row*) of Individuals I, II and III, respectively (**a**, **b** and **c**), illustrates in millimetre the magnitude of the facial discordance observed where regions of discordance above a threshold of 3 mm (0 on the scale) are visualised using a colour bar. The corresponding images **d**, **e** and **f** (*bottom row*) are outlier maps that depict statistically significant areas of facial discordance. Distance map and scale bar in 0–5 mm (0 *blue*, 5 mm *red*), while outlier map and scale bar in 0–0.3 (0 *white*, 0.3 *black*)

Discussion

Lysosomal Storage Disorders (LSDs) can manifest a broad phenotypic spectrum. MPS I–affected individuals have been classified to have Hurler, Hurler-Scheie or Scheie syndrome. Previously this classification was thought to reflect separate entities; however, they are now recognised as part of a continuum and affected individuals can be classed on a spectrum of severe or attenuated MPS I (Muenzer 2004). We objectively quantified a facial phenotype of three individuals affected with MPS I and sorted them on a severity scale using summary discordance values (Fig. 2). This severity scale reflected established criteria (severe - attenuated) and found a consistent pattern of facial discordance indicative of a specific signature.

This signature was detectable in Individuals I and II, with attenuated forms, despite discordance scores that fell within the limits of the normative reference. It is not unexpected that phenotypic facial signatures, particularly of attenuated MPS I, can exist within normative ranges. However, a consistent pattern of facial discordance was found, indicating that detection of subtle phenotypes was achievable. This signature was similarly seen in Individual III who was diagnosed with a more severe form of MPS I. He presented with the greatest facial variation, as reflected in the dysmorphometry scores that were outside the reference range (Z-RSD; 1.24; and Z-RSME; 2.50).

A pattern of accumulation of extracellular substrates in facial compartments may be delineated by tethering of the submuscular aponeurotic system (SMAS) (Thaller et al. 1990).

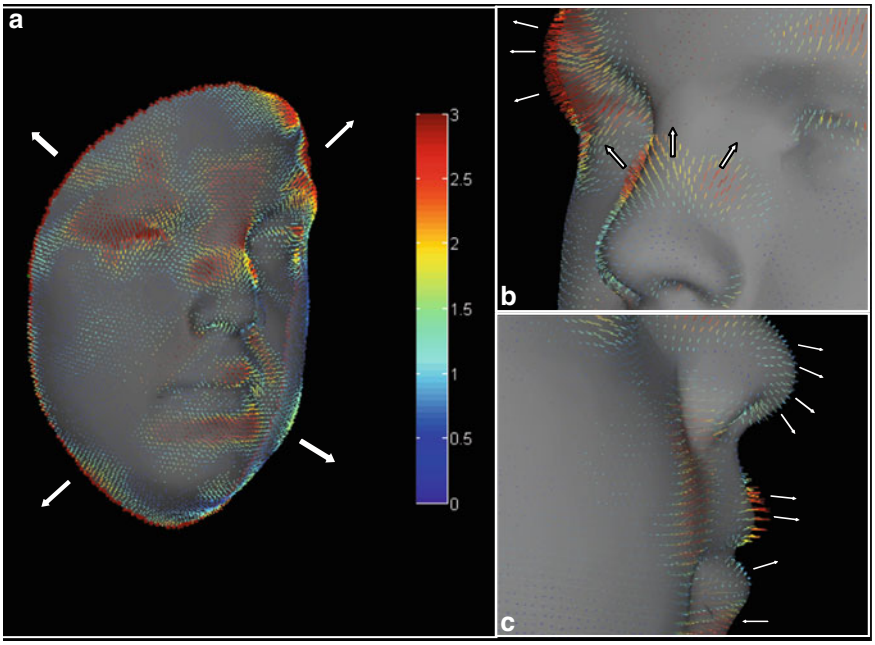

Fig. 3 Individual III MPS I–affected vector map. Dysmorphometric vector map of Individual III depicting vectors associated with substrate accumulation resulting in a 'ballooning' effect (**a**) of facial features and tethering in the nasiolabial folds. A close up view (**b**) and a profile view (**c**) are also shown, illustrating specific vector movements at the brow ridges, nasal bridge/tip, upper lip and infraorbital, nasiolabial and mentolabial regions

The pattern of facial variation was consistent with this proposition, manifesting a flattened frontal region in relation to prominently raised brow ridges, depressed infraorbital regions, and a prominent nasal tip in relation to a flattened nasal bridge and expansive alae nasi. The hypothesised tethering was, in particular, evident in the depressed nasolabial and mentolabial sulci; this is in accordance with the overall facial ballooning (Figure 3A), or 'puffiness', characteristic of this condition (Sandubray et al. 2012).

The potential of dysmorphometrics to provide an objective facial discordance signature and attribute severity on a continuous scale was demonstrated in a small cohort of MPS I individuals. Facial histograms provide data of the overall pattern of dysmorphology as well as patterns most frequently encountered, facilitating detection of subtle presentations. This is made possible by using NE controls that effectively remove confounding normal facial variations associated with gender, age and population affinity (Claes et al. 2012a). This approach, when combined with a large normative dataset, can potentially address sample size limitations inherent in the study of rare disorders. Additionally, in contrast to other morphometric analyses (Hammond et al. 2005; Shaweesh et al. 2006), it allows for open classifications and individual-specific assessments, which facilitates a progression from descriptive dysmorphology to quantitative dysmorphometrics. Our findings supported the possibility that using these techniques on an expanded cohort of individuals, a consistent pattern of facial variation may be revealed in both severe and attenuated MPS I. Given the rarity of these conditions, further investigations will optimally be approached by multicenter collaborations.

In theory, dysmorphometrics allows the use of a mixed control group (e.g. in gender, age and ethnicity). The face-space is a global shape model of covariance requiring all facial areas and features to be consistent, and in harmony, with each other for construction of the NE. It is therefore impossible for the face-space to randomly combine facial features from different subgroups that do not match. The reference face-space is *inclusive* in nature, enabling the same face-space to generate different age-, sex- and ethnicity-matched controls for answering distinct biological questions, as long as the variation is within the face-space. If an affected individual has facial features that show some degree of overlap with a subgroup of individuals of another gender or ethnicity, it is possible to make comparisons to a subspace constructed from subjects of the overlapping gender/ethnicity. However, this may only be required when the overlap extends to the majority of the face (e.g. > 40 % of the facial area). Limits of the applicability of subspace reference will require further testing.

There are considerable difficulties in objectively monitoring LSD treatment and establishing optimal dosages, particularly for presymptomatic patients in the absence of overt clinical features or suitable biomarkers (Fuller et al. 2004; Langford-Smith et al. 2010). Given the finding of

apparent gradation of facial phenotype, we propose further study on the potential utility of 3D facial analysis for non-invasive disease monitoring and assessment of treatment response.

The development of new therapeutics (Wraith et al. 2005; Sifuentes et al. 2007; Munoz-Rojas et al. 2008; Bijarnia et al. 2009; Clarke et al. 2009; Cox-Brinkman et al. 2010; Lachmann 2010; Langford-Smith et al. 2010; Schiffmann 2010) provides an impetus for timely LSD diagnosis, as the efficacies of these treatments are dependent on initiating therapy prior to the development of irreversible complications (Meikle et al. 2004; Meikle 2007). The challenge of achieving a definitive LSD diagnosis is complicated by overlapping phenotypic spectra (Meikle et al. 2004; Meikle 2007). Investigations have, therefore, been developed to allow simultaneous analysis for a number of these related conditions (e.g. urine metabolic screens and enzyme analysis panels) (Nielsen et al. 2010). However, with current biochemical investigations, false negatives may occur, particularly with attenuated subtypes, (Meikle et al. 2004; Meikle 2007) hence alternative approaches deserve investigation. We suggest that detection of subtle facial features can be objectively quantified and may facilitate MPS screening and diagnosis.

Development of effective LSD therapies is hindered by disease rarity (Muro 2010), and some current therapeutic strategies remain suboptimal and costly. Insights from specific LSD registries have significantly contributed to knowledge of disease spectra and natural history, but incomplete data on long-term outcomes and cost-effectiveness has raised concerns of the utility of this approach (Hollak et al. 2011). These factors argue for studies with multicentre coordination that cross boundaries of individual therapies. 3D facial analysis lends itself to such studies as hardware costs are modest, there is minimal consumable cost, data can be attained non-invasively from multiple sites, and centralised analysis for existing registries is possible (Pastores et al. 2007). Lifelong treatments for some LSDs necessitates longitudinal outcome-monitoring regimes, hence the need for innovative monitoring strategies that are cost effective and preferably non-invasive. Additionally, the following factors support facial analysis as particularly suitable for very young individuals: (1) it poses no health risk, (2) capture time is fast in comparison to other medical imaging and (3) if required, image capture can be easily repeated until a suitable image is obtained.

3D facial analysis may be useful for investigating disease biology to identify factors underlying phenotypic expression and to trace causal links between genotypes, environmental factors and phenotypes. Genotype-phenotype correlations in individuals with MPS I are poorly understood and are only partly related to the frequency of private *IDUA* gene mutations (Terlato and Cox 2003). Therefore, analysis of spatially dense facial phenotypes may provide a novel avenue for resolving these genotype-phenotype relationships.

Phenomics, defined as the acquisition of high-dimensional phenotypic data that inherently spans multiple levels of an organism, has been suggested for exploring pathogenesis (Houle et al. 2010). When coupled with other scientific methods, 3D facial analysis has yielded insights into rare disease biology (Tobin et al. 2008). According to Houle et al. (2010), the three key elements that foster phenomic developments are technological development, statistical and analytical capabilities, and integration incentives. Our approach satisfies the first two criteria and the rarity of LSDs provides an impetus for the last. We argue that quantitative phenotyping approaches, such as dysmorphometrics, (Claes et al. 2012a) can augment the repertoire of scientific protocols traditionally applied to disease studies (Houle et al. 2010; Klingenberg 2002). Ultimately, the unique combination of the multisystem nature of LSDs, disorder-specific treatments and high-resolution multidimensional LSD facial data may provide a platform for new insights into facial and systems biology.

Conclusions

This exploratory study using dysmorphometrics supported the validity of non-invasive 3D quantification of facial profiles of individuals with MPS I. Accordingly, objective high-resolution determination of patterns of facial variants may facilitate delineation of the MPS I disease spectrum, including in those with subtle facial phenotypes. This variance-based approach, which is uniquely suitable to multicentre applications, provides the means to quantify LSD facial dysmorphology as a holistic entity. Discrete phenotypes hereby defined can support further investigations including correlations with other LSD-related endpoints, such as disorder-related complications, which may facilitate treatment monitoring. Furthermore, when combined with molecular approaches, it may allow for novel explorations of pathogenic processes in other metabolic and genetic syndromic conditions.

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Synopsis

Objective 3D facial phenotyping using dysmorphometrics provides prospective avenues for non-invasive treatment monitoring of metabolic conditions.

Authors' Contributions

SK wrote the manuscript with major input and revisions from all other authors. **MW** and **GB** were involved in drafting of the manuscript, while **PC**, **JG** and **PLS** provided valuable critical feedback. **PC** developed the fundamentals behind the anthropometric mask, the statistical face-space, dysmorphometrics, and the normal equivalent, with conceptual input from **MW**. **PC** also provided the facial mapping of the data and constructed the normative facial model of covariance. Finally, he ran the leave-one-out analysis to establish normative reference discordancy statistics. **GB** and **JG** provided clinical insight into investigated syndromes, rallied support from the MPS Society and fostered international collaborations. All authors read and approved the final manuscript.

Guarantor

As guarantor for this article, **GB** accepts full responsibility for this work and conduct of this study, has had access to the data, and controls the decision to publish.

Competing Interests

The authors declare that they have no competing interests.

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CASE REPORT

Orthotopic Liver Transplantation in an Adult with Cholesterol Ester Storage Disease

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Abstract Cholesterol ester storage disease (CESD) is a rare autosomal recessive lipid storage disorder associated with mutations of the gene encoding lysosomal acid lipase, manifestations of which include chronic liver disease and early atherosclerosis. Although normally presenting in childhood, severity is variable and the condition can occasionally remain undetected until middle age. Typical presentation is with asymptomatic hepatosplenomegaly and hyperlipidaemia, though the condition is probably underdiagnosed. Treatment is supportive and may include attention to cardiovascular risk factors. Phase I/II trials of enzyme replacement therapy are ongoing, but this approach remains experimental. We present the case of a 42-year-old woman diagnosed with CESD in childhood who ran an indolent course until re-presentation with cirrhotic hydrothorax. She underwent orthotopic liver transplantation but required re-transplantation for hepatic artery thrombosis. She remains well with excellent graft function 2 years later. Although atherosclerosis was apparent at assessment, and may have contributed to hepatic artery thrombosis, partial correction of the metabolic defect and restoration of liver function by transplantation together with ongoing medical therapy should permit reasonable survival over the longer term from both a liver and a vascular perspective. This is the first reported case of orthotopic liver transplantation for CESD in an adult, which was the only available option to improve survival. The case highlights the importance of monitoring patients with CESD through adulthood and suggests that liver replacement at a later stage may yet be indicated and remain of benefit.

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Introduction

Cholesterol ester storage disease (CESD) is a rare autosomal recessive lipid storage disorder caused by lysosomal acid lipase (LAL) deficiency; this results in chronic liver disease. LAL is responsible for lysosomal cleavage of ester bonds in a range of lipids, including cholesterol esters and triglycerides. Thus, lack of LAL activity leads to intralysosomal accumulation of lipid (Anderson et al. 1993). In humans, LAL is encoded by the LIPA gene located on chromosome 10. Complete loss-of-function mutations in LIPA lead to development of Wolman's disease; this presents in infancy with failure to thrive and is usually fatal before the age of 6 months (Anderson and Sando 1991). CESD arises from hypomorphic mutations in the

Table 1 Fasting lipid profile of a 42-year-old woman with CESD over 3 years from pregnancy through to recent liver transplantation. Statin therapy was commenced post-partum which improved LDL but not HDL cholesterol. The latter improved following liver transplantation

	Pregnancy	Pre-transplant	Post-transplant	Current
Cholesterol (mmol/L)	5.7	3.0	4.1	2.9
Triglyceride (mmol/L)	1.4	1.0	1.7	0.6
HDL cholesterol (mmol/L)	0.93	0.35	1.00	1.13
LDL cholesterol (mmol/L)	4.17	2.25	2.40	1.57

LIPA gene, which impair either expression or function of LAL. The majority of patients described to date possess a homozygous G to A substitution at location -1 of the splice donor site of exon 8 of LIPA (c.894G>A), which causes deletion of exon 8 and production of only 2–4 % normal enzyme (Muntoni et al. 2007). Heterozygote carrier frequencies have been estimated at 1 in 200 in northern Europe. However, genotypic heterogeneity is evident from reported compound heterozygotes with CESD (Muntoni et al. 2007; Pisciotta et al. 2009).

CESD usually presents in childhood or adolescence with hypercholesterolemia or hepatomegaly due to hepatic steatosis that eventually progresses to cirrhosis (Chatrath et al. 2009). Patients may succumb to complications relating to accelerated atherosclerosis or chronic liver disease, including hepatocellular carcinoma. Historically, most patients known to have CESD died before the age of 30 years (Leone et al. 1991; McCoy and Yokoyama 1991). However, it is increasingly recognised that milder forms of CESD can occur, raising questions regarding the true natural history of this disorder.

Case History

A 42-year-old woman with a known history of CESD presented with a 1-week history of progressive dyspnoea following administration of the H1N1 influenza vaccination. This was accompanied by more gradual abdominal distension. The patient had originally come to medical attention at the age of 6 years with hepatosplenomegaly, whereby CESD was diagnosed following liver biopsy. She defaulted from follow-up and remained well into adulthood. At the age of 39 years, she sought further specialist review during pregnancy. Leukocyte acid esterase (i.e. leukocyte LAL) activity was low at 93 μmol/g/h (350–2000) with an elevated plasma chitotriosidase activity of 222 μmol/L/h (4–120), reaffirming the diagnosis. Lipid profile after overnight fasting demonstrated cholesterol 5.7 mmol/L, triglyceride 1.4 mmol/L, HDL-C 0.93 mmol/L and LDL-C 4.17 mmol/L (Table 1). Pregnancy was uneventful and statin therapy was commenced post-partum. She defaulted from specialist follow-up, though continued statin therapy.

No additional risk factors for chronic liver disease could be elicited at time of admission. Examination and chest radiography demonstrated the presence of a moderate right pleural effusion. Laboratory values were as follows: serum bilirubin 45 μmol/L (<17), prothrombin time (PT) 16.2 s (9.8–12.6), serum creatinine 57 μmol/L (35–125), serum sodium 138 mmol/L (135–145) and urine sodium <5 mmol/L. Her Model of End-Stage Liver Disease score was 14 (Kamath et al. 2001), and United Kingdom End-Stage Liver Disease (UKELD) score 50.1 (Lewsey et al. 2006).

Pleural aspiration revealed a transudative effusion consistent with cirrhotic hydrothorax. Abdominal ultrasonography (US) demonstrated an enlarged nodular liver with patent portal and hepatic veins, gross ascites and 22 cm splenomegaly. Diuretics did not eliminate the requirement for regular thoracocentesis and a transjugular intrahepatic portosystemic shunt was felt unlikely to improve survival. Patients with end-stage liver disease and a UKELD score greater than 49 have previously been shown to benefit from liver transplantation in terms of their predicted survival at 1 year (Barber et al. 2007), so this together with the patient's diuretic resistant ascites led us to proceed with a formal liver transplant assessment.

Computed tomography (CT) confirmed the previous findings at US (Fig. 1, panel a). Multiple high-density calcified nodules were also noted. Further, there was significant calcification of the descending aorta and aortic arch (Fig. 1, panels b and c). Transthoracic echocardiography demonstrated mild left ventricular diastolic dysfunction but was otherwise normal; 9 min of exercise treadmill testing were completed without electrocardiographic changes. Psychiatric evaluation was satisfactory and the patient was listed for liver transplantation on the grounds of the accepted indication of diuretic-resistant ascites in the form of hydrothorax, further supported by a UKELD score greater than 49.

At operation, a mildly fatty, deceased donor allograft was implanted using duct-to-duct biliary anastomosis and cavo-cavoplasty; an infra-renal aortic conduit was fashioned as initial arterial inflow was impaired. Cold and warm ischaemic times were 9 h 10 min and 36 min, respectively. Standard post-operative immunosuppression with prednisolone, azathioprine and tacrolimus was administered. Explant histology showed cirrhosis and was consistent with the diagnosis of CESD. Numerous lipid-laden macrophages and Kupffer cells were present within sinusoids that were CD68 positive and diastase periodic acid Schiff resistant (Fig. 2). No additional form of chronic liver disease was apparent.

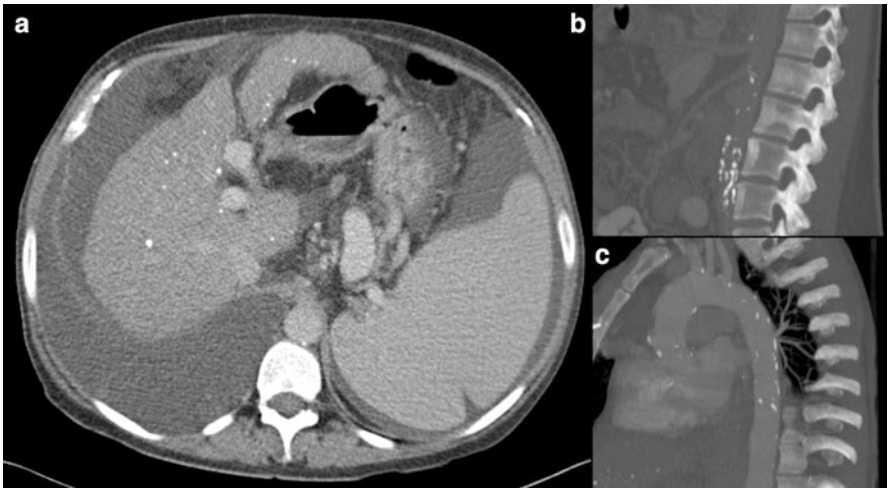

Fig. 1 CT images of a 42-year-old woman with end-stage liver disease due to CESD. Imaging obtained prior to liver transplantation. Post-contrast axial CT image of the upper abdomen showing a nodular liver containing multiple tiny foci of calcification in association with splenomegaly, ascites and a moderate right pleural effusion (**a**). Non-contrast sagittal CT images of the major arteries showing extensive calcification in the abdominal and thoracic aorta (**b** and **c**, respectively)

Initial recovery post transplantation was complicated by an episode of acute cellular rejection that resolved with intravenous methyl-prednisolone and the patient was discharged home 3 weeks following transplantation. Repeat enzyme analysis at discharge showed normalisation of plasma chitotriosidase activity (42 μmol/L/h) and persistent leukocyte acid esterase deficiency (39 μmol/g/h). Repeated fasting lipid profile revealed a significant rise in HDL cholesterol (Table 1).

Ten weeks post-transplant, during investigation for asymptomatic deranged liver function tests, imaging revealed unexpected hepatic artery thrombosis. Following clinical deterioration, the patient was subsequently re-listed and received a second transplant 3 months following her initial graft. Explant histology revealed an ischaemic liver with organised thrombus of the hepatic artery at the hilum. The thrombosed arterial conduit was left in situ and not examined histologically. Subsequent progress has been uncomplicated; the patient was formally anticoagulated with low-molecular-weight heparin for 3 months following re-transplant to reduce the risk of further hepatic artery thrombosis and is currently well on aspirin and statin therapy with excellent graft function almost 2 years following her second transplant.

In order to further characterise this patient's disorder, sequencing of LIPA was performed (Fig. 3). This revealed compound heterozygosity: c.[894G>A];[599T>C] (the common c.894G>A substitution described above and a T to C substitution at location 599 causing leucine to proline substitution). The second mutation is thought to disrupt alpha helical structure (Anderson et al. 1994). This compound heterozygous combination has been previously reported in siblings with CESD (Maslen et al. 1995).

Discussion

We have presented the first report of an adult liver transplantation for CESD. Our subject developed diuretic-resistant ascites with hydrothorax at the age of 42 years, having been diagnosed in childhood and run an indolent course during early adulthood. CESD has been previously diagnosed de novo in adulthood: Elleder et al. (1990) described CESD in two women aged 43 and 56 years, respectively, both of whom had asymptomatic hepatomegaly. One of these patients subsequently died of cerebrovascular disease. The same group subsequently described a 51-year-old man with a long-standing history of dyslipidaemia who died of cholangiocarcinoma (Elleder et al. 2000a). Autopsy findings demonstrated hepatomegaly with micronodular cirrhosis and disseminated cholangiocarcinoma; extensive severe atherosclerosis was also observed (Elleder et al. 2000b). Chatrath et al. (2009) reported a 43-year-old man diagnosed with CESD on liver biopsy performed with a view to staging hepatitis C infection. Further, they summarised the features of an additional 18 cases previously described. Reported deaths were either due to vascular disease or hepatic disease (liver failure or hepatobiliary malignancy).

No drug therapy exists for this condition, although normalisation of lipid profiles using cholestyramine or HMG-CoA reductase inhibitors has been reported (Leone et al. 1991; McCoy and Yokoyama 1991). Normalisation of the lipid profile does not necessarily prevent progression to hepatic failure in CESD, however, as seen in the case reported by Leone et al. (1995). Enzyme replacement therapy has shown promise in reducing lipid deposition in peripheral tissues in mouse and rat models of complete LAL deficiency analogous to Wolman's disease and is now

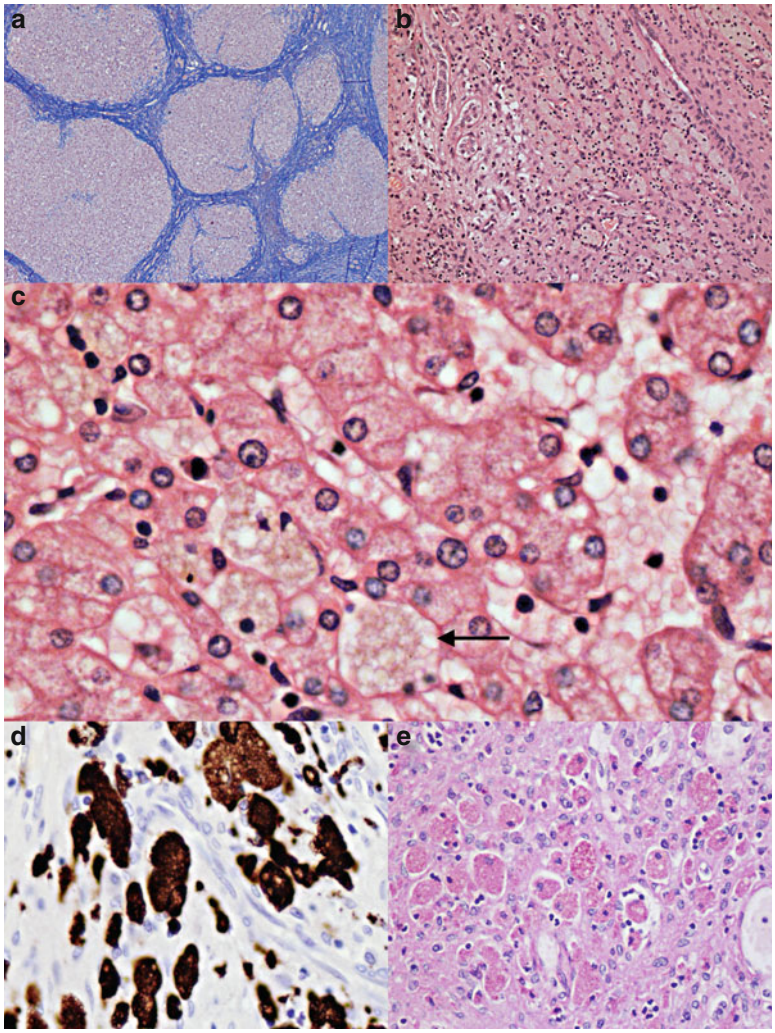

Fig. 2 Liver explant histology following transplantation for end-stage liver disease due to CESD. Chromotrope Aniline Blue staining (**a**) demonstrating established cirrhosis with complete nodules surrounded by broad fibrous septae (x40). Haematoxylin and Eosin (H&E)-stained section (**b**) demonstrating numerous foamy macrophages with tan-coloured cytoplasm within residual portal areas and fibrous septae (x200). H&E stained section (**c**) demonstrating lipid-laden, foamy hypertrophic Kupffer cells and sinusoidal macrophages (arrow) (x400). CD68 staining (**d**) showing strong positivity for intrasinusoidal macrophages and Kupffer cells (x400) which are periodic acid Schiff positive and diastase resistant (**e**) (x400)

being examined in phase I/II clinical trials for patients with CESD (Du et al. 2008; Enns 2012). Although this is not currently routine treatment and is unlikely to be of significant benefit once irreversible liver damage has occurred, this is an important research area, as is detection of CESD in adults with abnormal liver function.

Although successful liver transplantation for CESD has previously been reported in children and young adolescents (Arterburn et al. 1991; Ferry et al. 1991; Leone et al. 1995), this patient is far older than any of the previously reported cases. As a result of the increased cardiovascular mortality seen in CESD, and given that the patient had been untreated for the vast majority of her life, a major concern was the degree of established atherosclerosis and associated cardiovascular risk present. Although our patient had no overt signs of vascular disease, CT imaging had revealed extensive aortic calcification (Fig. 1). Furthermore, Doppler ultrasound imaging of the carotid arteries revealed dense plaques associated with mild stenoses. These findings of established atherosclerosis would be in keeping with her known CESD and may have been a factor in the development of post-transplant hepatic artery thrombosis; arterial anastomosis is technically more challenging in the presence of calcified atherosclerotic plaques, and plaque rupture around the site of anastomosis can occur. Despite these findings, we felt that there remained a clear benefit for liver transplantation.

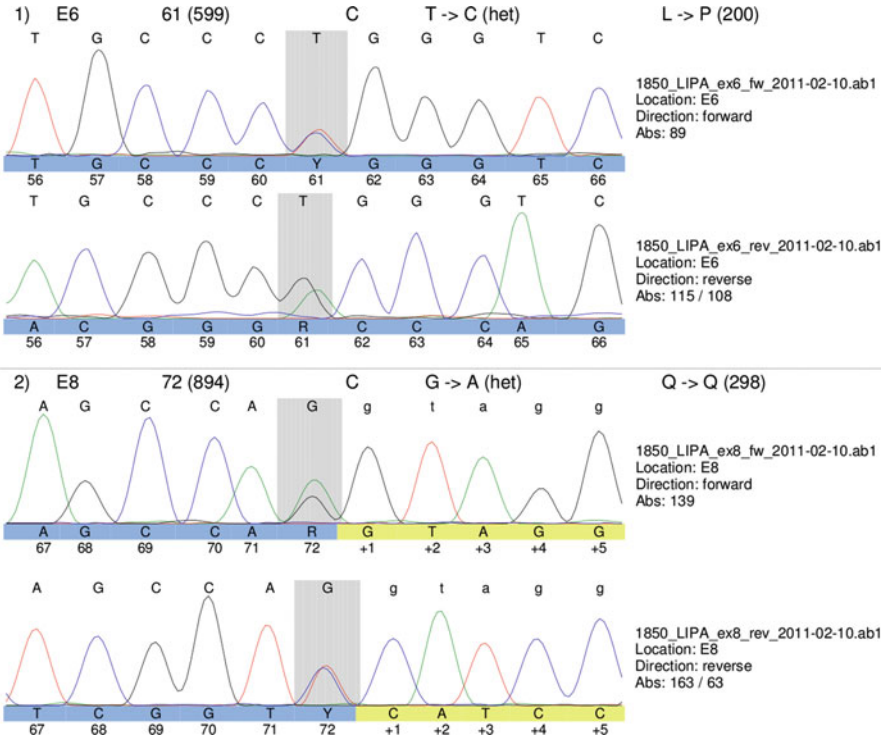

Fig. 3 DNA sequence chromatograms of a section of the *LIPA* gene from a 42-year-old woman with CESD. The grey boxes highlight heterozygous substitutions found in both forward and reverse directions at locations 599 (*top*, C substituted for wild-type T) and 894 (*bottom*, A substituted for wild-type G). The former is thought to result in disruption of alpha-helical structure, while the latter is found at a splice junction and usually causes deletion of exon 8, resulting in less than 10 % expression of normal protein (r.[894g>t,822_894delinsu])

Initial fasting lipid profiling demonstrated hypercholesterolemia with low HDL cholesterol (Table 1). Although statin therapy led to a reduction in total cholesterol, the low HDL cholesterol reversed only following liver transplantation. Multiple studies in healthy subjects have demonstrated the predictive importance of low HDL cholesterol in future cardiac and cerebrovascular outcome (Natarajan et al. 2010). Indeed a recent consensus statement on defining the metabolic syndrome has used a cut-off of less than 1.3 mmol/L as abnormal in a female population (Alberti et al. 2009). Normalisation of HDL cholesterol levels in CESD following commencement of HMG-CoA reductase inhibitors has previously been reported in pre-cirrhotic cases in the paediatric population (Leone et al. 1991), so it is likely that the low HDL cholesterol level observed here was related to impaired liver synthetic function. Liver transplantation may therefore be effective in reducing long-term vascular risk although this may be counterbalanced by the side effects of calcineurin inhibitors and the ongoing peripheral defect as evidenced by persistent leukocyte acid esterase deficiency.

In summary, we present the first published case of successful liver transplantation in an adult with CESD. The report highlights the challenges of concomitant vascular disease and the potential indolent nature of CESD in adulthood. The patient described here was lost to follow-up twice before presenting with severe decompensated liver disease. Our case therefore also emphasises the need for educating patients with asymptomatic but potentially progressive liver disease and for continued awareness amongst clinicians overseeing adult metabolic disorders. Two years following transplantation, the patient has remained free of vascular events, with excellent liver function and a normal lipid profile. Liver transplantation for CESD may therefore yield a significant survival advantage even well into adulthood. There is little evidence to support liver replacement other than in the context of liver failure. We can only speculate as to whether pre-emptive liver transplantation might ameliorate the mortality associated with vascular disease in CESD, particularly if HMG-CoA reductase inhibition might already offset this. Liver transplantation is therefore a viable option for adults with liver failure related to CESD; close monitoring of patients with CESD and liaison with tertiary hepatology services are important for the optimal timing of this intervention.

Synopsis

Regular follow-up of liver function in adults with cholesterol ester storage disease, an uncommon lipid storage

disorder, is an important consideration as orthotopic liver transplantation may be required to maintain long-term survival.

Conflicts of Interest and Financial Disclosures

The authors would like to thank Synageva Biopharma Corporation for providing the chromatograms in Fig. 3.

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RESEARCH REPORT

Inheritance of the m.3243A>G mutation

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Abstract The m.3243A>G is the most prevalent pathogenic mtDNA mutation but little is known about its inheritance. We studied 34 families containing 56 mother-child relations and 82 intersibling relations to investigate its transmission. We found a significant correlation between mother and child heteroplasmy levels ($r = 0.679$, $p < 0.001$). In mothers with a heteroplasmy level of below 25% we found 30% offspring without detectable mutation, while in mothers with a heteroplasmy level of above 25%, 100% of the offspring showed the m.3243A>G mutation. Heteroplasmy levels between siblings also correlated ($r = 0.512$, $p < 0.001$), but had limited extra predictive value because of outliers. These new data on inheritance of the m.3243A>G mutation might be of value in counseling patients and preventing transmission of the mutation.

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Abbreviations

mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
UEC	Urinary Epithelial Cells
MELAS	Mitochondrial myopathy, Encephalopathy, Lactate Acidosis, and Stroke-like episodes
MIDD	Maternally Inherited Diabetes and Deafness
PGD	Preimplantation Genetic Diagnosis

Introduction

Goto et al. first described the adenine to guanine transition at position 3243 of mitochondrial DNA (m.3243A>G) in the MT-TL1 gene encoding $tRNA^{LEU(UUR)}$ as the cause of mitochondrial myopathy, encephalopathy, lactate acidosis, and stroke-like episodes (MELAS) syndrome (Goto et al. 1990). Over time, more phenotypic variations of the m.3243A>G mutation have been reported, the most frequent being maternally inherited diabetes and deafness (MIDD) (van den Ouweland et al. 1992). Other variations include cardiac, ocular, and renal involvement (Lev et al. 2004; Lowik et al. 2005; Michaelides et al. 2008).

The m.3243A>G mutation is the most prevalent pathogenic mitochondrial mutation (Greaves and Taylor 2006) with a mutation prevalence ranging from 7.59 to 236/100.000 (Chinnery et al. 2000a; Majamaa et al. 1998; Manwaring et al. 2007).

Several cohort studies have been performed, containing mainly clinical and epidemiological data about carriers of the m.3243A>G mutation (Chinnery et al. 2000a; de Laat et al. 2012; Guillausseau et al. 2001; Katulanda et al. 2008; Ma et al. 2010; Majamaa-Voltti et al. 2006; Majamaa et al.

1998; Manwaring et al. 2007; Parsons et al. 2010) and some containing data on inheritance of the m.3243A>G mutation (Chinnery et al. 2000b; Frederiksen et al. 2006). In contrast to other mitochondrial mutations, sporadic presence of the m.3243A>G mutation is rare (Cree et al. 2009). Providing additional data on inheritance of mitochondrial mutations is essential to prevent transmission of these mutations. Poulton et al. reviewed the different possibilities to prevent transmission of maternally inherited mitochondrial diseases (Poulton et al. 2009). We describe a cohort of 34 families in which 82 carry the m.3243A>G mutation, focusing on 56 mother-child relations and 82 intersibling relations. The main purpose of this study is to indentify female carriers in whom transmission of the m.3243A>G mutation is present and in whom it is not definite.

Methods

All probands are patients of the Nijmegen Center for Mitochondrial Disorders at the Radboud University Nijmegen Medical Centre, diagnosed with the m.3243A>G mutation in muscle or blood. All patients participated in our cohort study for the m.3243A>G mutation (de Laat et al. 2012). This study was approved by the ethics committee of the Nijmegen-Arnhem region. Written informed consent according to the Helsinki agreement was obtained from all parents and patients ≥12 years.

Heteroplasmy levels where determined in urinary epithelial cells (UEC) in all participants using Pyrosequencing™ technology (Pyrosequencing, Uppsala, Sweden) as earlier described by Lowik et al. 2005. The pyrosequence reaction of the m.3234A>G mutation had a precision of 1.5%, and the mutation was detected from a heteroplasmy level of 5%. The detection limit for the MELAS mutation (m.3243A>G) was determined by serial dilution of a sample containing this mutation with wild-type mtDNA.

We analyzed the differences in heteroplasmy between mother and child and between familial siblings; the heteroplasmy levels of the older sibling were compared to that of the younger sibling. We used descriptive statistics to present the heteroplasmy levels in our patients, and Pearson's correlation coefficient was used to evaluate the relationship between the heteroplasmy levels between mother and child and between siblings.

Results

In our cohort of 82 patients from 34 families we identified 56 mother-child relations and 82 intersibling relations.

Of the 56 mother-child relations, ten mothers had one child, 14 mothers had two children and 6 mothers had 3 children. A total of 14 mothers that did not carry the m.3243A>G mutation were included, 11 mothers had a detectable mutation load but had a heteroplasmy level of below 25% and 5 mothers had a heteroplasmy level between 25% and 50%. There were no mothers with a heteroplasmy level above 50%. The rate of transmission of the mutation is visualized in Fig. 1a. Here we describe the transmission in more detail. In the mothers lacking the mutation, we found a detectable mutation in three children. These three patients were the only ones in their pedigree that had a detectable mutation; we therefore concluded that these patients were most likely to have a de novo mutation. We excluded these three families, because there was (so far) no inheritance of the mutation in these families. When we excluded these three families, a total of nine mothers were excluded. The five remaining mothers (with an undetectable heteroplasmy level) were sisters of a proband. In four cases, we found a detectable mutation in the mothers of these women. In one case the mother had passed away, so no heteroplasmy level could be determined.

The 11 mothers with a detectable mutation load but a heteroplasmy level below 25% had 19 children. Six of these children (32%) had no detectable mutation. In the 13 children where the mutation was present, 4 patients were between 5% and 25%, 5 patients were between 25% and 50%, and 4 patients were above 50% (resp. 61%, 64%, 75% and 93%).

The five mothers with a heteroplasmy level between 25% and 50% had nine children, all with detectable heteroplasmy levels. Three children were between 25% and 50%, three children were between 50% and 75% and three children were above 75%.

A correlation coefficient of 0.679 ($p < 0.001$) was found between the heteroplasmy levels of mothers and their children.

In our cohort, we indentified 82 intersibling relations. When we excluded the three families with the sporadic mutation, 63 intersibling relations remained. We divided this group into five subgroups depending on the heteroplasmy level of the oldest sibling, (resp. <5%, 5–25%, 26–50%, 51–75% and >75%). The relationship between the heteroplasmy levels in the siblings is indicated in Fig. 1b. Fifty percent of the oldest siblings with an undetectable mutation had a younger sibling with a detectable mutation. In these 10 siblings, the mother had a detectable mutation load in four cases; in the other six cases the heteroplasmy level of the mother was unknown. Of the 19 cases where the older sibling had a heteroplasmy level between 5% and 25%, the younger sibling had an undetectable mutation in 6 cases (32%). In the 24 cases where the older sibling had a heteroplasmy level higher than 25%, one younger sibling (4%) had an undetectable mutation. A correlation coefficient of 0.512 ($p < 0.001$) was found between the heteroplasmy levels of the siblings.

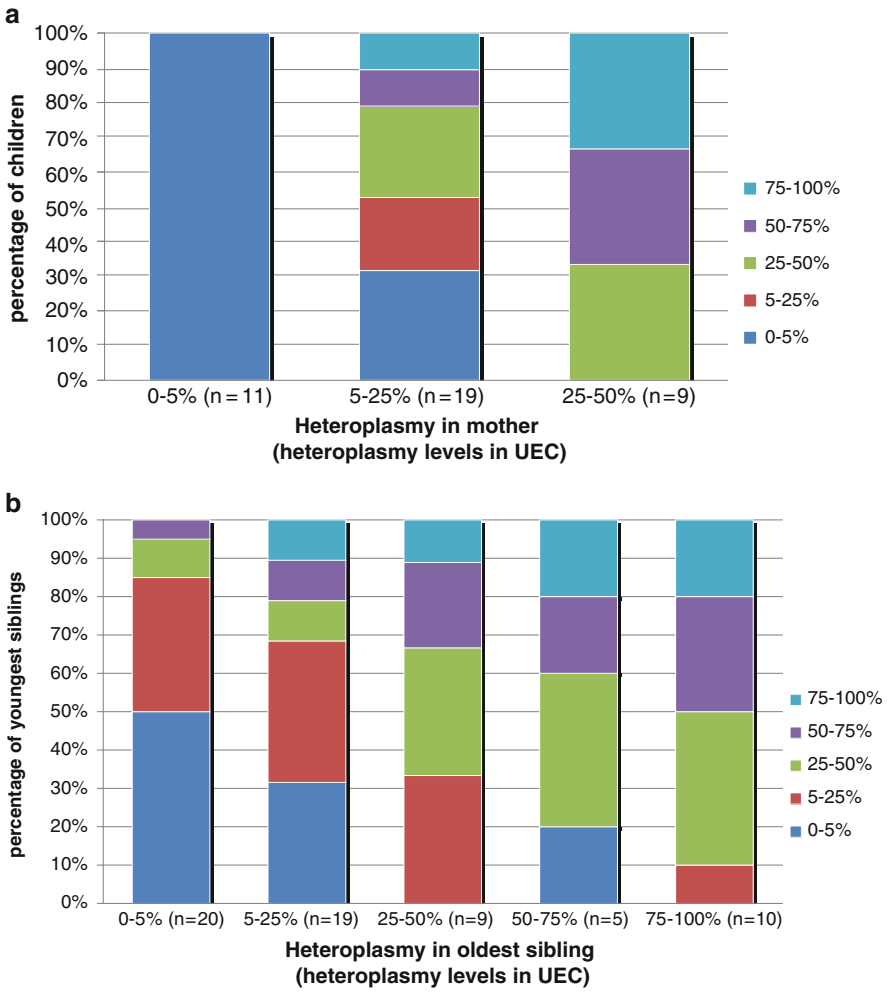

Fig. 1 (**a**) Heteroplasmy levels in children divided into three groups depending on the heteroplasmy level of their mother; *n* gives the number of mother child relations in every group. (**b**) Heteroplasmy levels in siblings divided in five groups depending on the heteroplasmy level of the older sibling; *n* gives the number of intersibling relations in every group

Discussion

Because of the unique characteristics of mitochondrial inheritance (mitotic segregation, heteroplasmy, and maternal inheritance), genetic counseling in mitochondrial diseases caused by an mtDNA mutation has always been very difficult (Cree et al. 2009). With this study we provide additional information regarding the inheritance of the m.3243A>G mutation. We used UEC to analyze heteroplasmy levels as different studies have shown a good relation between clinical phenotype and heteroplasmy in UEC (Whittaker et al. 2009). Besides, heteroplasmy in UEC is more stable compared to heteroplasmy in lymphocytes (de Laat et al. 2012; Ma et al. 2009). We must keep in mind that although there is a correlation between heteroplasmy levels in UEC and clinical symptoms, there are also patients with high levels of heteroplasmy that do not present with clinical symptoms during childhood. It is hypothesized, but as of yet unknown, whether these "dormant carriers" develop symptoms when they reach adulthood (de Laat et al. 2012).

We found a correlation of $r = 0.679$ ($p < 0.001$) between the heteroplasmy levels in mothers and their offspring, indicating that mothers with higher heteroplasmy levels have offspring with higher heteroplasmy levels. However, as indicated in Fig. 1a, there is quite a variance in the transmission of the m.3243A>G mutation. We demonstrated that mothers with a heteroplasmy level of above 25% transmit the mutation to all their offspring, providing clarity for patients. Thirty percent of offspring from mothers who have a heteroplasmy level below 25% will have undetectable heteroplasmy levels of the m.3243A>G mutation (using the current techniques). These findings support the use of such techniques as pre-implantation genetic diagnosis (PGD) to select for unaffected offspring in this group. In mothers with a heteroplasmy level of higher than 25%, all the offspring are affected, making it only possible to select for the embryo

with the lowest level of heteroplasmy using PGD. The only options that remain for these women is oöcyte donation or adoption to be 100% sure that there will be no transmission of the m.3243A>G mutation. A future possibility for these women could be nuclear transfer (Poulton et al. 2009).

Besides transmission from mother to child, we also investigated the relationship between heteroplasmy levels of siblings, in order to see if heteroplasmy levels in a child may provide extra information in predicting the heteroplasmy level of further progeny. Figure 1b indicates that the heteroplasmy level in the oldest sibling has some predictive value for younger siblings, but again there are outliers. In the group where the oldest sibling does not have a detectable mutation, there is still a 50% chance that the second sibling will have the mutation, and one patient even had a heteroplasmy level of above 50%. On the other side of the spectrum, we find an almost identical case in which the oldest sibling has a high (above 50%) level of heteroplasmy but the mutation could not be detected in the younger sibling. So, even though there is a correlation between the heteroplasmy levels in siblings ($r = 0.512$, $p < 0.001$), the presence of these outliers makes it difficult to offer genetic counseling to these families.

We conclude that the female carriers of the m.3243A>G mutation do not transmit the mutation to all of their offspring. We suggest that mothers with a heteroplasmy level below 25% could consider using techniques like PGD to prevent transmission in case of child wish. Long-term follow-up studies should be performed to examine the stability of the heteroplasmy values, to investigate whether PGD for women with heteroplasmy levels above 25% is feasible.

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Conflicts of Interest

Authors report no conflicts of interest.

Key Sentence/Synopsis

In mothers carrying an m.3243A>G mutation with a heteroplasmy level of below 25%, we found that 30% of the offspring were unaffected. This new data on inheritance of the m.3243A>G mutation will be of great help for counseling patients and preventing transmission of the mutation.

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RESEARCH REPORT

Recommendations on Reintroduction of Agalsidase Beta for Patients with Fabry Disease in Europe, Following a Period of Shortage

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Abstract The interruption of the manufacturing process of agalsidase beta has led to a worldwide shortage of this drug. In the EU, nearly all patients initially reduced their agalsidase beta dose, and many of these switched to agalsidase alfa (Replagal Shire HGT). The clinical consequences of this period of drug shortage need to be further evaluated. A gradual

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increase of agalsidase beta supply is now expected. This implies that patients could resume or even commence agalsidase beta treatment. Guidance for prioritization of patients is needed to support equitable distribution of agalsidase beta to EU member states. To achieve this, in absence of level I clinical evidence, a draft consensus proposal was initiated and distributed. No full consensus was achieved, as there is disagreement regarding the indications for switching patients from agalsidase alfa to agalsidase beta. Some physicians support the concept that the 1.0 mg/kg EOW dose of agalsidase beta is more effective than agalsidase alfa at 0.2 mg/kg EOW, while others believe that at recommended dose, the preparations are equivalent. In light of these difficulties and the uncertainties with respect to supply of agalsidase beta, recommendations were agreed upon by a subgroup of physicians. These current recommendations focus on prioritization of criteria indicative of disease progression.

Background

A viral contamination of Genzyme Corporation's production facility in June 2009 and subsequent ongoing manufacturing problems have caused an acute and prolonged shortage of agalsidase beta (Fabrazyme®) to approximately 30 % of prior global supply (Linthorst et al. 2011). This agalsidase beta shortage has resulted in anxiety among patients and those caring for Fabry patients, and its effects need to be studied thoroughly. In Europe and some other parts of the world, alternative treatment with another alpha galactosidase A preparation (agalsidase alfa, Replagal™ Shire Human Genetic Therapies, Inc.) is available and most patients in the EU subsequently switched to agalsidase alfa 0.2 mg/kg/EOW. The European Medicines Agency (EMA) reported a trend of increasing reports of adverse events during the shortage (EMA report dated 19 November 2011), which decreased following switch to agalsidase alfa or reinstitution of the full dose (1.0 mg/kg) of agalsidase beta. A subgroup seemed to have no obvious clinical deterioration due to the lowered dose. In a small study, clinical significant changes at lower dose could not be objectified, but biochemical analysis pointed towards recurrence of storage after switch to low-dose agalsidase beta and subsequent switch to agalsidase alfa (Smid et al. 2011), while another study reported increase in subjective symptoms (Australian State Fabry Disease Treatment Centers et al. 2011). These data are in line with earlier reports showing evidence of deterioration in a subset of patients after switching from agalsidase beta 1.0–0.3 mg/kg. (Lubanda et al. 2009) The EMA recommended monitoring patients closely when on lower than recommended dose of agalsidase beta and change to full dose or to agalsidase alfa in case of deterioration (EMA report 19 November 2011).

In the USA, only agalsidase beta is commercially available and patients have had prolonged treatment interruptions and/or received lower dosages of agalsidase beta than the recommended 1.0 mg/kg/EOW. Some patients also received agalsidase alfa on a compassionate basis or within research protocols.

The possibility to switch to agalsidase alfa as an alternative treatment resulted in unprecedented demand of agalsidase alfa and this led to fears of a shortage of agalsidase alfa in the last quarter of 2010. Although this never actually occurred, in 2010 recommendations on prioritization of enzyme replacement therapy (ERT) for naïve patients were agreed by a group of Fabry experts and

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patient representatives and these have subsequently been published (Linthorst et al. 2011). These recommendations emphasized that patients who had reversible disease manifestations should receive priority over those patients who may have more severe disease, but less reversible. We endorse this principle with respect to those patients who are already on treatment and for whom a change in ERT preparation is considered.

The manufacturer of agalsidase beta (Genzyme-Sanofi) has taken steps to secure future agalsidase beta production, and it is anticipated that in 2012, availability of agalsidase beta will gradually increase. These recommendations address how this extra agalsidase beta should be distributed within patients and the member states of the EU. An initiative was launched to try to achieve consensus within the group of treating physicians with respect to prioritization of patients. To achieve the broadest discussion, also physicians outside the EU were asked to comment.

Preference of agalsidase beta 1.0 mg/kg/EOW over agalsidase alfa 0.2 mg/kg/EOW (or vice versa) is not based on level I clinical evidence. Both preparations have shown clinical efficacy in patients, but it is also known that despite treatment with agalsidase beta or agalsidase alfa at the respective recommended doses, progression of disease occurs in a subset of patients. For instance, those with extensive cardiac fibrosis (Weidemann et al. 2009) or those with low estimated glomerular filtration rate (eGFR) or manifest proteinuria (Germain et al. 2007; Schiffmann 2005) at the initiation of ERT demonstrate ongoing progression of disease when compared to those without (severe) cardiac fibrosis or with preserved eGFR.

These recommendations are indicated only for the period of reduced agalsidase beta availability.

Methods

A draft proposal was written and subsequently distributed by email to treating physicians and patient organizations (represented by the board members of the Fabry International Network). All were asked for their opinions. Following review of these responses, the consensus document was finalized and agreed.

In these recommendations, dosages refer to the Summary of Product characteristics (SmPC) of agalsidase alfa (0.2 mg/kg) (Summary of Product Characteristics Replagal [Internet]. [cited 2012]) and agalsidase beta (1.0 mg/kg) (Summary of Product Characteristics Fabrazyme [Internet]. [cited 2012]). For agalsidase beta, based on the previously mentioned study, the SmPC states that while no definitive conclusion regarding the dose maintenance regimen can be drawn, the findings suggest that, after an initial debulking dose of 1.0 mg/kg every 2 weeks, 0.3 mg/kg every 2 weeks may be sufficient in some patients to maintain clearance of GL-3. Since the number of patients was small and long-term outcome is unknown, 0.3 mg/kg is not recommended. Thus, for clarity, in the following text, a licensed recommended dose means 0.2 mg/kg agalsidase alfa EOW and 1.0 mg/kg agalsidase beta EOW.

Results and Discussion

The initial draft was sent to 47 physicians, of whom 28 provided comments. With regard to the final recommendations, 26 were supportive, 1 could not support the recommendations, and the remainder ($N = 20$) did not respond ($N = 16$) or declined to participate in the discussion ($N = 4$). Those supportive are listed as co-authors. Some physicians indicated during the discussions that a formal prioritization was not feasible. This may relate to the many uncertainties both with respect to availability as well as to the usefulness of prioritization. Some physicians, outside of the EU, felt that they should not be part of this discussion. From others, mixed reactions were received indicating that a true consensus could not be achieved. The main disagreements were criteria for prioritization, uncertainty regarding whether some of these criteria were actually reversible by switching to another enzyme preparation and a desire to make individual decisions rather than having to adhere to recommendations. These recommendations are meant to provide the best possible advice for clinicians to use as guidance relating to the reintroduction of agalsidase beta.

ERT Current Situation

Following the release of the previous consensus document on prioritization of ERT-naïve patients in December 2010, and in accordance with EMA recommendations (EMA 22/Oct/2010), patients in the EU are primarily treated with 1.0 mg/kg/EOW agalsidase beta or with agalsidase alfa 0.2 mg/kg/EOW; only few cases are treated with agalsidase beta $<$ 1.0 mg/kg/EOW. Currently, extra agalsidase beta is expected to become available for an additional 100 patients in Q2 of 2012, and it is expected that in Q3 and Q4 quarter of 2012, availability will further increase. However, uncertainty remains regarding the level of the increase and the delay before all limitations on agalsidase beta will be lifted. Governmental financial issues (involuntary dose reduction) or physician/patient preference (voluntary choice) means that some patients are treated with a dosage of agalsidase beta $<$ 1.0 mg/kg (i.e. not the recommended dose), despite previous recommendations (Linthorst et al. 2011). To the best knowledge of the experts, this is limited to less than 15 cases in the EU. If a patient is treated with a lower than recommended dose, this should be done so in full

agreement between patient and physician, while maintaining close monitoring of therapeutic outcome. In those few patients where treatment with a lower dose of agalsidase beta is voluntary, signs of disease progression should be followed by a switch to agalsidase alfa or beta at recommended dose. It should be noted that while these recommendations are focused on the temporary shortage of agalsidase beta, there is abundant agalsidase alfa available for treatment.

Basic Principles of Treatment

1. Patients with Fabry disease can develop additional complications despite optimal treatment, while receiving comprehensive supportive therapy and ERT at licensed recommended dose.
2. There is currently no level 1 scientific evidence showing superiority of one enzyme preparation over the other (Vedder et al. 2007; Sirrs et al. 2010).
3. Patients should be treated with licensed recommended doses of agalsidase alfa and agalsidase beta.

Criteria for Prioritization

These criteria are based on the current severity of the disease, its rate of progression and the potential for reversibility of disease with ERT and not on age or gender (see also Table 1). Thus, the criteria apply for both the adult and paediatric population for male and female Fabry patients. These criteria only apply during the period of increased yet limited supply of agalsidase beta.

1. A switch to agalsidase beta 1.0 mg/kg/EOW should only be considered if there is a reasonable chance that this dose can be maintained. It is recommended that Genzyme-Sanofi build up a significant stock reserve to accommodate fluctuations in agalsidase beta production. A stock reserve of 3 months of treatment per patient is the minimum requirement for a patient to be able to switch.
2. Patients should be treated for at least 12 months with agalsidase alfa 0.2 mg/kg/EOW, before a switch to agalsidase beta 1.0 mg/kg /EOW can be considered.
3. A dose increase may especially be relevant in patients with anti-agalsidase antibodies. In these patients, a more robust reduction of (lyso)ceramide trihexoside (CTH) after dose increase is seen in plasma or urine as shown by various groups (Linthorst et al. 2004; Whitfield et al. 2005; Ohashi et al. 2007; Vedder et al. 2008; Van Breemen et al. 2011). However, it has not been documented to date that this additional reduction results in a more favourable clinical outcome. It is advised to screen for anti-agalsidase antibodies in males on ERT (irrespective of treatment or dose).
4. Patients should only be prioritized to switch to agalsidase beta 1.0 mg/kg/EOW treatment if they are demonstrating disease progression on agalsidase alfa 0,2 mg/kg, while there is still potential reversibility of affected organs, and the switch should be undertaken only after full discussion between the patient and physician.
5. Appropriate concomitant and adjuvant therapy (e.g. ACE-inhibitors or angiotensin receptor blockers, pain management, anti-arrhythmic drugs and devices, stroke prophylaxis) must always be considered for all patients (Summary of Product Characteristics Fabrazyme [Internet]. [cited 2012]; Summary of Product Characteristics Replagal [Internet]. [cited 2012]).

The following four steps are anticipated in the process of increase in agalsidase beta availability.

Step I: (Limited Increase in Agalsidase Beta)

All patients in step I include those receiving agalsidase beta <1.0 mg/kg/EOW on an involuntary basis, because of shortage of agalsidase beta (see Basic Principles of Treatment), regardless of whether they are deteriorating or stable. It is perceived that this group is very small. Patients on a lower dose of agalsidase beta can also switch to agalsidase alfa 0.2 mg/kg/EOW.

Step II (Additional Increase in Agalsidase Beta Supply)

All patients in step I have had the opportunity to be treated with recommended dose of agalsidase beta (1.0 mg/kg/EOW)

The following patients now qualify: patients treated with agalsidase alfa 0.2 mg/kg for at least 12 months who demonstrate signs of deterioration and where the physician, after full discussion with the patient, deems a switch to agalsidase beta necessary. Deterioration is defined as having occurred from start of ERT (does not need to have occurred during the agalsidase beta shortage):

(a) Severe neuropathic pains that cannot be satisfactorily controlled despite 3 months of maximum analgesic treatment, in agreement with neuropathic pain management protocols.
(b) Increase in left ventricular mass (at least 10 % on MRI or 20 % by ultrasound) despite treatment, while adult patients may have no or only mild cardiac fibrosis on MRI (fibrosis in only one left ventricle segment) (Weidemann et al. 2009).
(c) A 33 % increase in serum creatinin levels, or a significant decrease in renal function evidenced by means of measured GFR demonstrating progression from CKD I to CKD 2, but only while eGFR is above 30 ml/min.

Table 1 Priority stages for previously untreated Fabry patients (Taken from Linthorst et al. (2011))

Priority stage	Characteristics
High priority – children	Severe neuropathic pain unresponsive to a maximum trial of 3 months of optimal pain management (Filling-Katz et al. 1989; Weidemann et al. 2010)
	Persistent microalbuminuria defined by the median of three consecutive early morning urine samples
	24 h urinary protein >250 mg/day at presentation (consider simultaneous use of ACE inhibitors)
	In females, if microalbuminuria or proteinuria is the only clinical manifestation, a trial of ACE inhibitors for a period of 3–6 months in the first instance is recommended
	Age-adjusted left ventricular mass index ≥ 90th percentile
	TIA/stroke and/or MRI brain showing white matter lesions (subclinical or with clinical manifestations)
High priority – adults	Disease age of onset < 50 years
	Increased LVM without MRI evidence of extensive fibrosis
	GFR between 30 and 90 ml/min/1.73 m (Smid et al. 2011)
	Urine protein 0.3–1.0 g/24 ho[a]
	Severe neuropathic pain, if persistent after optimal pain management (Filling-Katz et al. 1989; Weidemann et al. 2010)
	Cerebrovascular disease
Intermediate priority	Less reversible disease
	Increased LVM with fibrosis
	GFR < 30 ml/min/1.73 m (Smid et al. 2011)
	Less severe disease:
	Disease age of onset > 50 years[b]
Low priority	End-stage cardiac disease (Weidemann et al. 2011)
	End-stage CNS disease, e.g. multi infarct dementia
	Multiple organ failure
	Life expectancy of less than 1 year due to other co-morbidities
	Those patients mildly affected (e.g. without LVH, acroparesthesias well controlled with optimal pain medication, eGFR > 90 ml/min/1.73 m (Smid et al. 2011))

[a] At presentation, with or without ACE inhibitors or angiotensin II receptor blockers

[b] Age of onset is the perceived age at which symptoms occurred which can be attributed to Fabry disease

- In case of serial serum creatinin measurements, at least 12 months of treatment should have been employed and two consecutive creatinin values at least 1 week apart should document a persistent increase in creatinin.
- After other causes of renal deterioration have been excluded,

(d) New (clinical) stroke/transient ischaemic attack (TIA) as documented by a neurologist not thought to be related to aging or other co-morbidities.

(e) Hospitalization due to cardiac disease (e.g. cardiac failure, rhythm disturbances, or additional interventions to treat these (such as pacemaker or Implantable Cardioverter-Defibrillator (ICD), CABG, valve surgery).

Step III (Further Increase in Availability)

All patients in step II for whom the physician/patient deems a switch necessary and have agreed to do so, have been switched. Now, the following patients can be considered for switch (again after full discussion between the patient and the treating physician):

(a) Patients currently treated with agalsidase alfa 0,2 mg/kg, where the physician/patient deems a switch to agalsidase beta necessary, but the patient is deteriorating (see above), but has an eGFR<30, is on dialysis, had a renal transplant or severe cardiac fibrosis (two or more left ventricle segments).

(b) Currently untreated patients belonging to the high priority of ERT naïve patients, as mentioned in the previous recommendations (see Table 1), for whom the treating physician has a preference of agalsidase beta 1.0 mg/kg over agalsidase alfa 0.2 mg/kg.

Step IV (Unlimited Supply of Agalsidase Beta)

No restrictions apply. Evaluate the possible benefits of switching back to agalsidase beta on an individual patient basis. We would advise that patients who are being treated

with doses other than the licensed recommended doses should be monitored with particular care.

There is no level 1 evidence comparing the effectiveness of agalsidase alfa and agalsidase beta at their recommended doses. We have stated above that patients receiving agalsidase alfa 0.2 mg/kg EOW for more than 1 year and are deteriorating could be considered for switch to agalsidase beta 1.0 mg/kg/day. While these recommendations focus on the agalsidase beta shortage and not on switching patients to other preparations in general, patients on agalsidase beta 1.0 mg/kg/EOW for a year and deteriorating could be considered for switch to agalsidase alfa 0.2 mg/kg EOW.

Genzyme Corporation-Sanofi, the manufacturer of agalsidase beta is willing to allocate increase in agalsidase beta supplies in accordance to these recommendations. However, this can only occur if physicians can provide information on the expected number of patients per country/region/treatment centre for whom they prefer to switch to agalsidase beta. We advise all treating physicians to remain in close communication with their local Genzyme-Sanofi representative in order to help them with the allocations.

Assessment of Patients and Storage of Samples for Future Biomarkers

There is only limited information on the efficacy on ERT with lower dosages of agalsidase beta or in patients who switched from agalsidase beta to agalsidase alfa (and back). Physicians caring for Fabry patients are strongly encouraged to carefully document changes in the course of the disease when treatment regimens are varied and to perform detailed physical examinations when patients are about to switch treatments. In addition, storage of samples before and after the switch (serum, plasma, urine) is strongly encouraged as these can be used for future analysis of biomarkers and antibodies. The current period of restricted enzyme supply may aid the Fabry community in gaining more understanding in the outcome of the various different treatment regimens confronting patients.

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CASE REPORT

cblE-Type Homocystinuria Presenting with Features of Haemolytic-Uremic Syndrome in the Newborn Period

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Abstract This study describes a *cblE* type of homocystinuria associated with haemolytic-uremic syndrome (HUS) features. We report on a male infant aged 43 days presenting with failure to thrive, hypotonia, pancytopaenia, HUS symptoms (microangiopathic haemolytic anaemia and thrombocytopaenia with signs of renal involvement) and fatal evolution. An underlying cobalamin disorder was diagnosed after a bone marrow examination revealed megaloblastic changes associated with hyperhomocysteinaemia. An urinary organic acid analysis revealed normal methylmalonic acid excretion. The *cblE* diagnosis was confirmed with a complementation analysis using skin fibroblasts and genetic studies of the *MTRR* gene. The patient treatment included parenteral hydroxocobalamin, carnitine, betaine and folinic acid, but there was no response. After the autopsy, the histopathological examination of the kidneys showed marked myointimal proliferation and narrowing of the vascular lumen. The central nervous system showed signs of haemorrhage that affected the putamen and the thalamus; diffuse white matter lesions with spongiosis, necrosis and severe astrogliosis were also observed. Microangiopathy was observed with an increase in vessel wall thickness, a reduction of the arterial inner diameter and capillary oedema. The signs of necrosis and haemorrhage were detected in the cerebellum, the cerebellar peduncles, the tegmentum and the bulbar olives.

In conclusion, cblE should be considered when diagnosing patients presenting with HUS signs and symptoms during the newborn period. Despite early diagnosis, however, the specific treatment measures were not effective in this patient.

Introduction

The *cblE* type of homocystinuria (OMIM236270) is a rare condition that is related to cobalamin metabolism, affects the 5-methyltetrahydrofolate-homocysteine methyltransferase reductase gene (MTRR, MIM 602568) and causes isolated hyperhomocysteinaemia (Wilson et al. 1999). To date, approximately 20 patients have been reported; therefore, the clinical spectrum of the disease must still be defined. The clinical hallmarks for the condition are megaloblastic anaemia, developmental delay, feeding difficulties and cerebral atrophy (Skovby et al. 2003). The presentation and diagnosis of *cblE* typically occurs in childhood, although manifestations in adulthood or in the first months of life have been reported (Müller et al. 2007; Zavadakova et al. 2002; Vilaseca et al. 2003). Vascular system involvement in these patients has been suggested (Zavadakova et al. 2002), although to our knowledge such findings have not been reported.

Published studies of the genetic causes of haemolytic-uremic syndrome (HUS) (Noris et al. 2005) have investigated issues such as complement component deficiencies (particularly the C3 fraction deficiency), deficiency of the complement regulatory protein in the alternative complement pathway (CD 46, factor I and factor H) (Zimmerhackl et al. 2006), and the *cblC* defect (Chenel et al. 1993; Geraghty et al. 1992; Kind et al. 2002; Russo et al. 1992). Additionally, only one case has reported an association between the *cblG* defect, HUS and pulmonary hypertension (Labrune et al., 1999). We believe that the association between the *cblE* type of homocystinuria and HUS has not been reported previously.

This paper reports on a new case of the *cblE* defect that presented after the newborn period with severe vascular involvement, as demonstrated by histopathological studies, HUS features and fatal evolution despite early treatment.

Material and Methods

Case Report

The patient was a male (born after an uncomplicated pregnancy) with a birth weight of 3.2 kg and normal body length and head circumference. The family history was uneventful. At 43 days of life, the patient presented with respiratory distress including cough, fever, poor feeding and pale grey skin colour. Haematological investigations revealed normocytic anaemia, thrombocytopaenia and some features of haemolysis: haemoglobin 4.2 g/dL, reference range (RR) 9–13.5; haematocrit 11%, RR 29–41; mean corpuscular volume 87 fl, RR 74–90; mean corpuscular haemoglobin concentration 32 pg/L, RR 25–35; platelets 20,000 u/mm^3, RR 150,000–500,000; reticulocyte count 1.3%, RR 1–2.5%; bilirubin 5.4 mg/dL, RR 0.2-1; lactate dehydrogenase 1162 UI/L, RR < 776; haptoglobin < 40 mg/L, RV 70–3100; and negative direct and indirect Coombs tests. An erythrocyte microscopic examination showed anisopoikilocytosis, Jolly corpuscles and some isolated schistocytes. The bone marrow aspiration revealed erythroid megaloblastic changes, granulocytic and dyserythropoietic features (karyorrhexis and pycnosis), and the megakaryocytic series was diminished (suggesting megaloblastic bone marrow changes due to ineffective erythropoiesis). The following renal function parameters were observed: haematuria, more than 100 red blood cells per high-power field; proteinuria, 30 mg/dL in a random urine sample, RV: 0–8; and hypertension (115/57) with slightly impaired renal function, serum urea 52 mg/dL, RV 7–45, serum creatinine 0.55 mg/dL; RV <0.5. C-reactive protein was normal (<5 mg/L). The patient presented with respiratory arrest and bradycardia while undergoing a transfusion of packed red cells and was admitted to the PICU. Biochemical analysis revealed homocystinuria (3,517 mmol/g creatinine, RV <20), hyperhomocysteinaemia (257 μmol/L, RV <7.5), a low plasma methionine level (2 μmol/L; RV 12–37), normal methylmalonic acid excretion and normal serum folate and cobalamin concentrations (data not shown); these data suggest a cobalamin genetic disorder. The treatment was initiated with hydroxocobalamin (1 mg per day administered intravenously for 4 days), folic acid (40 mg per day administered orally for 7 days), betaine (250 mg/kg/day for 2 days), carnitine (100 mg/kg/day intravenously every 8 h for 3 days) and pyridoxine (150 mg/day every 8 h for 3 days) with no significant clinical improvement. The patient had high blood pressure that was resistant to nifedipine and hydralazine boluses, decreased urine output, oedema, hyponatremia, proteinuria, haematuria and bloody diarrhoea. The thrombocytopaenia was persistent despite transfusions. Due to the severe neurological affectation, the patient expired despite mechanical respiratory support.

Magnetic resonance imaging showed severe impairment of the white matter (predominantly bifrontal) and a significant decrease in the grooves and ischaemic-haemorrhagia at the basal ganglia. A delay in myelination was observed. As shown in Fig. 1 (MRI axial T2 sections), we observed bilateral high intensity in the lenticular nuclei and the head of the caudate nuclei that was suggestive of haematic content.

After the autopsy, the pathological findings in the kidneys showed poor cortical-medullary differentiation, and the glomeruli and arterioles were affected histologically. The glomerular component showed thickened capillary walls with endothelial cell swelling and other glomeruli in which the dilatation of the capillaries was prominent. Some areas of mesangiolysis were also observed. The vascular changes

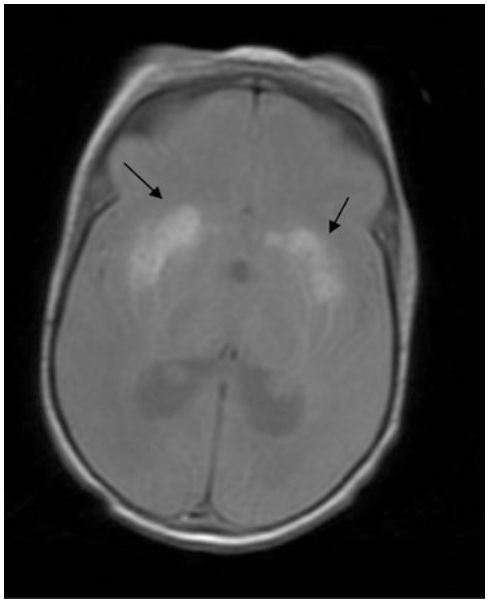

Fig. 1 Axial T2 section: An MRI of the brain shows bilateral high intensity in the lenticular nuclei and the head of caudate nuclei that is suggestive of haematic content (*black arrows*)

affected the intraglomerular arterioles with marked myointimal proliferation and narrowing of the vascular lumen (Fig. 2). In the central nervous system, haemorrhaging affected the putamen and the thalamus; we observed diffuse white matter involvement with spongiosis and necrosis, and severe astrocytosis. Microangiopathy was also observed with increased vessel wall thickness, reduced arterial inner diameter and capillary oedema. In the cerebellum, areas of necrosis and haemorrhage were detected: abnormalities in the cerebellar peduncles, the tegmentum and the bulbar olive were seen (Fig. 3).

Methods

Biochemical Analysis

A skin biopsy was performed under standard conditions, and cultured skin fibroblasts were obtained for biochemical studies. Methionine and serine synthesis were studied by [^{14}C] formate incorporation into amino acids after incubation with different OH-cobalamin quantities (Fowler et al. 1997). The uptake of [^{57}Co] labelled cobalamin and synthesis of cobalamin coenzymes was measured as previously referred (Zavadakova et al. 2002). The propionate incorporation in intact fibroblasts was measured after cell incubation with and without a high OH-cobalamin concentration (Willard et al. 1976). Complementation analysis of intact patient cells and fibroblasts treated with 40% polyethylene glycol was performed by the incorporation of ^{14}C-formate into methionine. As known complementation groups, the cblD, cblG and cblE cells were used.

Genetic Studies

The molecular analysis of the *MTRR* gene was carried out on genomic DNA and cDNA. The total mRNA and the genomic DNA were isolated from cultured fibroblasts from the patient using the MagnaPure system (Roche Applied Science, Indianapolis, IN), following the manufacturer's protocol. RT-PCR was performed using the SuperScript III First-Strand enzyme (Invitrogen, Carlsbad, CA). To identify mutations in the *MTRR* gene, a sequence analysis of its cDNA and genomic DNA was performed using the primers designed by the Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The sequence will be provided upon request. The identified changes were confirmed by sequencing the corresponding genomic DNA region and the parent samples to ensure that the changes were in different alleles and to rule out the presence of a large genomic rearrangement. For the genetic studies, written permission was obtained from the patient's parents. The ethical Committee of Hospital Sant Joan de Déu approved the study.

Results

The biochemical results are presented in Table 1. There was deficient formation of methionine in the cells grown in normal medium and under supplementation with increasing concentrations of OH-cobalamin with no increase of serine formation. The total content of the radioactive cobalamin was normal, and the methyl-form of cobalamin synthesis was low. The formation of the adenosyl-form of cobalamin was in the normal range. The ^{14}C propionate incorporation was within the normal values for the fibroblasts cultured with and without OH-cobalamin. Finally, complementation analysis of the patient fibroblasts confirmed that the patient belonged to the cblE complementation group (*MTRR* gene).

The patient harboured the two novel changes c.1324_1327 + 12del15 and c.125A>G (https://portal.biobase-international.com/hgmd) in the MTRR gene. The former mutation affected the normal splicing process of exon 9 (RT-PCR analysis in heterozygous state showed skipping of the entire exon 9), which generated a loss-of-function mutation (p.Leu442fs). The second change (p.Asp42Gly) was classified as 'damaging' by the PolyPhen computational prediction algorithm (genetics.bwh.harvard.edu/pph2).

Discussion

Here, we present a new case of the *cblE* defect with HUS features, which for the first time represents a severe phenotype with an associated fatal outcome. The HUS

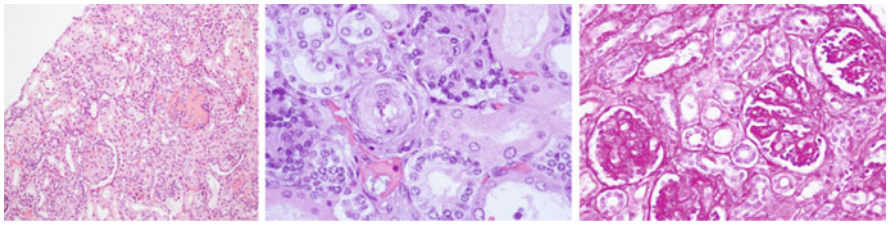

Fig. 2 The histopathological findings in the patient kidney. Microangiopathy was observed with thickened vessel walls and a reduction of the arterial inner diameter and capillary oedema. *Left panel*: glomeruli with thickening of the capillary walls and glomeruli with prominent capillary dilatation (×100). *Middle panel*: marked myointimal proliferation with narrowing of the lumen (×400) observed with haematoxylin-eosin staining. *Right panel*: PAS staining (×200) disclosed glomerular mesangiolysis

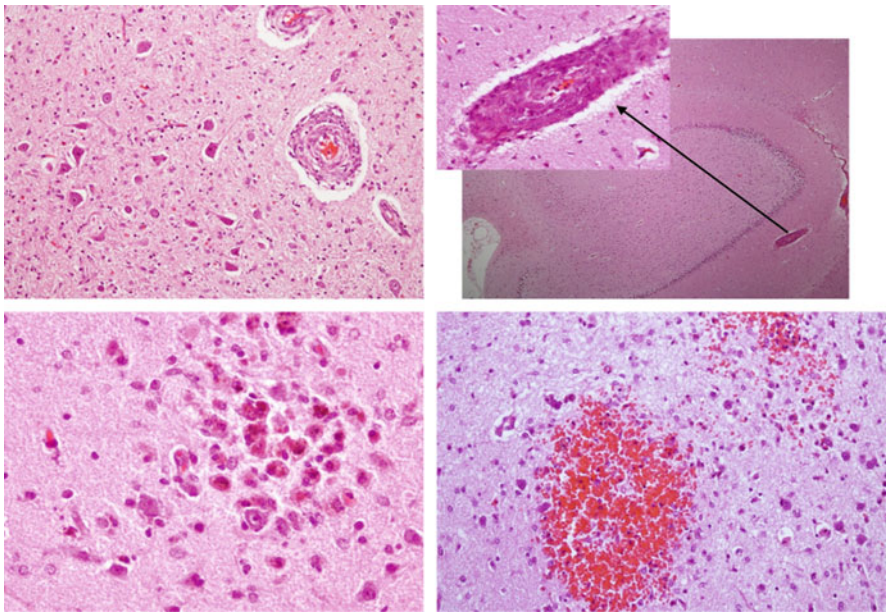

Fig. 3 The histopathological findings show microangiopathy in different areas of central nervous system. *Upper left panel*: cerebellar peduncles with myointimal proliferation of vessels. *Upper right panel*: vessel myointimal proliferation in the hippocampus. *Lower panels*: microhaemorrhage with a high content of macrophages containing hemosiderin, microglia activation (*left*) and recent bleeding in the cerebellum (*right*)

Table 1 Biochemical data in cultured skin fibroblasts from the case with cblE deficiency. A severe deficient methionine synthesis (first lane) was observed with low methylcobalamine (Me-Cbl) uptake (second lane), and normal propionate incorporation (third lane), indicating a cblE defect. For the complementation analysis (fourth lane), patient cells were mixed with known complementation groups with or without polyethyleneglycol (PEG) and methionine formed (nmol/mg protein/16 h) from ^{14}C-formate was calculated. Patient cells complemented with the cblD and the cblG mutatnt cells, but not with the cblE cell line, confirming the cblE complementation group of the patient

	Conditions	Patient	Parallel control
Methionine synthesis	OH–Cbl 0 µg/L	0.13 (nmol/16 h)	2.46 (nmol/16 h)
	OH–Cbl 1,000 µg/L	0.22 (nmol/16 h)	2.66 (nmol/16 h)
Me-Cbl uptake	% of total (^{57}Co)	10%	58%
Ado-Cbl uptake		24%	11%
Propionate incorporation	OH–Cbl 0 µg/L	11.7 (nmol/mg.16 h)	12.8 (nmol/mg.16 h)
	OH–Cbl 1,000 µg/L	13.8 (nmol/mg.16.h)	18.4 (nmol/mg.16 h)
Complementation	Difference		Positive control
Patient/cblD	(+PEG)–(–PEG)	+ 0.49	+ 0.47
Patient/cblE	(+PEG)–(–PEG)	–0.07	
Patient/cblG	(+PEG)–(–PEG)	+0.85	

diagnosis was based on the presence of microangiopathic haemolytic anaemia (some schizocytes were observed on the peripheral blood smear), thrombocytopaenia, renal involvement (haematuria and slightly impaired renal function) and microangiopathy in the kidneys with arteriolar rather than glomerular involvement (Giménez et al. 2008). Haemolysis was demonstrated by increased LDH and bilirubin levels and decreased haptoglobin concentrations. Considering these data (the presence of haemolysis, the morphological features of erythrocytes and the megaloblastic changes observed after the bone marrow examination) a plausible explanation for these findings could be haemolysis triggered by microangiopathy and an ineffective erythropoiesis due to a functional cobalamin deficiency. Haemolysis would explain why no megaloblastic changes appeared in the peripheral smear and the ineffective erythropoiesis why no increased reticulocyte values were observed.

In children, the prognosis is poor for HUS associated with other cobalamin disorders, and the mortality rate is high, even when symptoms present early (Chenel et al. 1993; Sharma et al. 2007). The rapid and fatal evolution of the condition despite early treatment with vitamins and cofactors was noteworthy in our case. It has been suggested that hydroxocobalamin treatment may cure HUS, but the severe neurological affectation in our case may have contributed to the fatal evolution (Chenel et al. 1993; Tuchman et al., 1988). It seems that the earlier the presentation the worse the prognosis; a 33% mortality rate has been documented in patients with the *cblC* defect that manifested in the newborn period (Rosenblatt et al. 1997; Rosenblatt Whitehead 1999). Neuroimaging findings and neuropathological studies supported the severity of the clinical evolution, and several brain areas were affected, most likely triggered by the vascular lesions. Microangiopathy in the kidneys and the central nervous system (affecting arteriola with vessel wall hyperplasia and decreased inner diameter and in the capillary circulation) was observed after the histopathological studies supported the relationship between the *cblE* defect and the vascular damage. An increase in plasma total homocysteine might provide a pathophysiological explanation for findings, as the impairment of vascular physiology by hyperhomocysteinaemia (involving different mechanisms) has been consistently demonstrated (Stamler et al. 1993; Hajjar 1993; Rodgers and Kane 1986). However, the pathophysiological mechanisms related to the association between hyperhomocysteinaemia and HUS is at present unknown, and this matter deserves further investigation.

At the gene level, the patient harboured two novel pathogenic changes according to the experimental data and the computational prediction algorithm. This pathogenicity was proven by the biochemical analysis of the patient fibroblasts, which confirmed an important reduction of methionine synthesis and methylcobalamin uptake, and also by the complementation analysis with the *cblE* fibroblasts.

In conclusion, *cblE*-type homocystinuria may be associated with hemolytic-uremic syndrome features with fatal outcome. Our patient exhibited vascular damage, possibly related to the severe hyperhomocysteinaemia.

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Synopsis

The vascular involvement in the kidneys and the central nervous system was demonstrated by the contribution of the *cblE* defect to patient mortality, despite early diagnosis and treatment.

Contribution Details

D. Palanca and J. Ortiz assisted with the clinical data collection and drafting of the manuscript. A. Garcia-Cazorla contributed to the neurological evaluation and the critical revision of the manuscript. C Jou, V. Cusí and M. Suñol interpreted the histopathological studies and assisted with the critical revision of the manuscript. T. Toll provided the differential diagnosis and assisted with the haematological data collection and the manuscript revision. B. Perez contributed to the molecular genetic analysis and interpreted the results. A. Ormazabal and R. Artuch provided the biochemical diagnosis and interpretation and assisted with the study design and drafting of the manuscript. B. Fowler assisted with the complementation studies and the interpretation of results.

Guarantor

Rafael Artuch

Conflict of Interest Statement

The authors declare no conflicts of interest.

Funding Details

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Ethics Approval

The parents of the infant in the index case signed an informed consent agreement in accord with the Helsinki Declaration of 1964 revised in Edinburgh in 2000. Our hospital ethics committee approved the study.

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RESEARCH REPORT

Mannose 6-Phosphate Conjugation Is Not Sufficient to Allow Induction of Immune Tolerance to Phenylalanine Ammonia-Lyase in Dogs

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Abstract The immune response to exogenous protein has been shown to reduce therapeutic efficacy in animal models of enzyme replacement therapy. A previously published study demonstrated an immunosuppressive regimen which successfully induced immune tolerance to α-L-iduronidase in canines with mucopolysaccharidosis I. The two key requirements for success were high-affinity receptor-mediated enzyme uptake, conferred by mannose 6-phosphate conjugation, and immunosuppression with low-dose antigen exposure. In this study, we attempted to induce immune tolerance to phenylalanine ammonia-lyase by producing a recombinant mannose 6-phosphate conjugated form and administering it to normal dogs according to the previously published tolerance induction regimen. We found that the recombinant conjugated enzyme was stable, could bind to the mannose 6-phosphate receptor with high affinity, and its uptake into fibroblast cells was mediated by this receptor. However, at the end of a tolerance induction period, all dogs demonstrated an antigen-specific immune response when challenged with increasing doses of unconjugated phenylalanine ammonia-lyase. The average time to seroconvert was not significantly different among three separate groups of test animals ($n = 3$ per group) and was not significantly different from one group of control animals ($n = 3$). None of the nine test group animals developed immune tolerance to the enzyme using this method. This suggests that high-affinity cellular uptake mediated by the mannose 6-phosphate receptor combined with a previously studied tolerizing regimen is not sufficient to induce immune tolerance to an exogenous protein and that other factors affecting antigen distribution, uptake, and presentation are likely to be important.

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Competing interests: None declared

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Introduction

The intravenous administration of recombinant human enzyme replacement therapy has revolutionized the treatment of several lysosomal storage diseases. Recombinant enzyme is currently in clinical use for Gaucher disease; Fabry disease; mucopolysaccharidosis I, II, and VI; and Pompe disease (Kakkis et al. 2001; Wraith et al. 2004; Muenzer et al. 2006; Harmatz et al. 2005; Barton et al. 1991; Eng et al. 2001; Schiffmann et al. 2000). Enzyme replacement therapy improves many aspects of these disorders and in many cases has enabled patients to lead more normal lives.

Administration of recombinant proteins often produces an antibody response, which can have clinical or therapeutic consequences. An immune response to these protein antigens occurs in most patients being treated with replacement enzyme but is usually well tolerated clinically (Wang et al. 2008). There is evidence, however, that antibodies to replacement enzyme can reduce the effectiveness of treatment (Sifuentes et al. 2007; Brooks et al 1997,

1998; Turner et al. 2000; Dickson et al. 2008). Modulation of the immune response to the recombinant protein is therefore an important consideration in optimizing enzyme replacement therapy.

Previous studies by our laboratory (E.D.K.) showed that immune tolerance to recombinant human α-L-iduronidase (rhIDU) could be induced in dogs with mucopolysaccharidosis I (MPS I) using a 60-day regimen of the immunosuppressive drugs cyclosporin A and azathioprine in combination with 12 weekly intravenous (IV) rhIDU infusions at a low dose (Kakkis et al. 2004). After completing this regimen, animals did not produce antigen-specific antibodies when challenged with a full treatment dose of rhIDU up to 6 months after the induction period, thereby demonstrating immune tolerance to rhIDU. There were two key requirements for the successful induction of tolerance using this regimen. The first requirement was achieving a sufficient cyclosporin A serum level prior to starting the low-dose tolerizing rhIDU infusions. Study animals that failed to develop cyclosporin A serum trough levels of at least 350 ng/mL or that were not treated with immunosuppression consistently developed strong antibody responses to rhIDU (Kakkis et al. 2004). The second requirement was efficient cellular uptake of rhIDU, which was shown to depend on mannose 6-phosphate conjugation of the enzyme. Mannose 6-phosphate (M6P) modification of N-linked carbohydrates occurs on α-L-iduronidase and other lysosomal enzymes and allows high-affinity receptor-mediated uptake via the mannose 6-phosphate receptor (Dahms et al. 1989). When treated with the above immunosuppressive regimen, dogs failed to develop immune tolerance to dephosphorylated rhIDU or to ovalbumin, a glycoprotein lacking mannose 6-phosphate modification, but could be induced to develop immune tolerance to recombinant human α-glucosidase, an enzyme containing high levels of mannose 6-phosphate on its N-linked carbohydrates (Kakkis et al. 2004). We hypothesized that we could induce immune tolerance to other exogenous proteins by producing an M6P conjugated form and then administering low doses of the modified protein along with the above outlined immunosuppressive regimen.

Classic phenylketonuria is an autosomal recessive disease caused by deficiency of phenylalanine hydroxylase in humans that result in elevated phenylalanine levels and a spectrum of subsequent clinical findings including severe cognitive impairment (Mitchell et al. 2011). Phenylalanine ammonia-lyase (PAL), a key enzyme in plant and fungus phenylpropanoid metabolism, catalyzes the degradation of phenylalanine to *trans*-cinnamic acid and ammonia (Koukol and Conn 1961; MacDonald and D'Cunha 2007) and has been studied as a potential alternative therapy to reduce phenylalanine levels in phenylketonuria (Sarkissian et al. 1999). When PAL was administered in an animal model of phenylketonuria, it was shown to induce a vigorous immune response that degraded the protein (Sarkissian et al. 1999, 2008). The ability to induce immune tolerance to PAL could improve its therapeutic use in humans as well as provide a method for improving the safety and efficacy of recombinant protein therapy with a wide variety of nonmammalian immunogenic protein antigens.

In this report, we tested the hypothesis that mannose 6-phosphate conjugation is necessary to allow induction of immune tolerance to an exogenous protein by producing recombinant phenylalanine ammonia-lyase conjugated with an M6P-containing group and administering it to dogs in low doses along with the previously published immunosuppressive tolerizing regimen.

Materials and Methods

Production of Purified Recombinant PAL

We expressed recombinant phenylalanine ammonia-lyase (RtPAL) in *Escherichia coli* transformed with the yeast *Rhodosporidium toruloides* PAL gene modified to introduce a lysine residue via an R91K amino acid substitution. The protein was secreted in a rich culture medium upon IPTG (isopropyl-β-D-thio-galactoside) induction and purified by hydrophobic interaction followed by anion-exchange chromatography. Purified RtPAL (76.9 kDa) was buffer-exchanged by ultrafiltration/diafiltration using a 30 kDa molecular weight cutoff polyethersulfone membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany) into Dulbecco's Phosphate-Buffered Saline (D-PBS) (VWR International, Radnor, PA) adjusted to pH 6.9 with hydrochloric acid (HCl).

Conjugation of RtPAL With a Mannose 6-Phosphate Carrier

N-succinimidyl-S-acetylthioacetate (SATA) (Thermo Fisher Scientific Inc., Rockford, IL), a sulfhydryl-containing linker that conjugates to primary amines, was added to 4 mg/mL of D-PBS-buffered RtPAL in dimethyl sulfoxide (DMSO) (Sigma-Adrich, St. Louis, MO) in a 15:1 mole ratio (SATA:RtPAL) and incubated for 2 h at room temperature to form the RtPAL-SATA conjugate. DMSO and excess SATA were removed by gel filtration using a Zeba desalt column (Thermo Fisher Scientific Inc.) pre-equilibrated with D-PBS adjusted to pH 7.2 with HCl. The purified product was then deacetylated to produce a deprotected sulfhydryl group on SATA by addition of hydroxylamine hydrochloride ($Cl^-H_3N^+OH$) (Thermo Fisher Scientific Inc.) to 2.3 mg/mL

of RtPAL-SATA in a 100:1 mole ratio ($Cl^-H_3N^+OH$:RtPAL-SATA) and incubated at room temperature for 75 min.

BPM6, a proprietarily synthesized molecule consisting of two carbohydrate chains terminated by mannose 6-phosphate groups and connected to a primary amine backbone, was prepared for conjugation to RtPAL-SATA by treatment with succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Thermo Fisher Scientific Inc.) in DMSO in a 15:1 mole ratio (SMCC:BPM6) and incubated at room temperature for 45 min to form BPM6-SMCC. Freshly prepared BPM6-SMCC was immediately added to the above prepared RtPAL-SATA in a 15:1 mole ratio (BPM6-SMCC:RtPAL-SATA) to form conjugated RtPAL-BPM6 using standard cross-linking chemistry, incubated first for 2 h at room temperature and then overnight at 4°C. The final product RtPAL-BPM6 was purified by gel filtration and formulated for injection at 1.78 mg/mL in 10 mM Tris (tris(hydroxymethyl)aminomethane) + 140 mM NaCl pH 7.5.

Characterization of RtPAL-BPM6

Protein concentrations were determined by A280 absorbance assay using 0.51 Lg^{-1} cm^{-1} as extinction coefficient. Protein endotoxin levels of the formulated material were undetectable (<1 EU/mL) as measured using a Limulus amebocyte lysate spectrophotometric assay (Charles River, Wilmington, MA). Enzymatic activity was measured according to a previously published method using optical absorption at 290 nm to monitor the production of *trans*-cinnamic acid when RtPAL-BPM6 was added to a buffered solution containing L-phenylalanine (Wang et al. 2005).

Molar substitution ratios (MSR) were determined by separate colorimetric assays for primary amines, using Ellman's reagent, and for free sulfhydryl groups, using 2,4,6-trinitrobenzenesulfonic acid (TNBSA) (Thermo Fisher Scientific Inc.), according to the manufacturer's protocols. The MSR homogeneity was investigated with isoelectric focusing (IEF) on a Novex pH 3–7 IEF gel (Invitrogen, Carlsbad, CA), and conjugate phosphorylation was identified after digestion with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Protein aggregation and molecular weight shift upon conjugation were monitored by SDS PAGE gel electrophoresis.

Mannose 6-Phosphate Receptor Binding

To characterize RtPAL-BPM6 binding to the mannose 6-phosphate receptor, a Nunc MaxiSorp ELISA plate (Thermo Fisher Scientific Inc.) was first coated with 4 μg/mL of soluble MPR (Center for Advanced Biotechnology and Medicine, UMDNJ, Piscataway, NJ) in pH 9.5 carbonate buffer and incubated overnight at 4°C, then washed with D-PBS with 0.1% polysorbate-20, and finally blocked at 37°C for 1 h in D-PBS with 2% BSA and 0.05% polysorbate-20. The blocking solution was discarded and test samples consisting of unconjugated RtPAL, the intermediate RtPAL-SATA, or the conjugated product RtPAL-BPM6 were loaded into plate wells in duplicate and incubated at 37°C for 1 h. Samples of each species were initially prepared at 2 nM concentrations in D-PBS with 2% BSA and 0.05% polysorbate-20 and then subjected to serial twofold dilutions to 0.03125 nM. After washing the plate, we loaded HRP-conjugated rabbit anti-RtPAL IgG (BioMarin Pharmaceutical, Inc., Novato, CA), diluted to 1:5000 in the same buffer, into plate wells and incubated at 37°C for 30 min. Following a final plate wash, wells were incubated at 37°C for 15 min with TMB peroxidase substrate (Bio-Rad Laboratories, Hercules, CA), then 2 N H_2SO_4 was added to stop the HRP-TMB reaction. Finally, absorbance at 450 nm was measured using a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The mean A_{450} values of duplicate wells were plotted versus sample protein concentration to yield binding curves, and single-reciprocal linear transformation (Hanes-Woolf method) and linear regression fit were performed to calculate the Kd (dissociation constant) value.

Cellular Uptake via the Mannose 6-Phosphate Receptor

Human fibroblast cells were treated with 2nM FITC-labeled RtPAL or RtPAL-BPM6 and incubated for 4 h in a tissue culture incubator (37°C, 8% CO_2) and then visualized using confocal fluorescence microscopy to determine intracellular uptake. Additional fibroblast cell incubations were performed with RtPAL-BPM6 in the presence of either free mannose 6-phosphate or free glucose 6-phosphate to test the specificity of MPR-mediated uptake. Fibroblast cell nuclei were counterstained with DAPI (diamidino-2-phenylindole).

Animal Background

The study subjects were twelve normal beagles, approximately 6 months old and weighing between 8–12 kg, housed and maintained under the care of a veterinarian at the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The study was reviewed and approved by the institutional animal care and use committee.

Tolerance Induction Regimen

The tolerance induction regimen consisted of treatment with the immunosuppressive drugs cyclosporin A (CsA) (Novartis)

and azathioprine (Aza) (DSM Pharmaceuticals) and tolerizing weekly enzyme infusions as previously described (Kakkis et al. 2004). The CsA dose started at 37.5 mg/kg/day divided twice per day and was adjusted to reach a target serum trough level of $\geq$ 400 ng/mL. Starting on the first morning of CsA treatment, 5 mg/kg Aza was given once every other day. Weekly intravenous infusions of RtPAL-BPM6 at low dose (0.056 mg/kg) began after 18 days of immunosuppression and continued for 12 weeks. After the third weekly RtPAL-BPM6 infusion, the doses of CsA and Aza were halved every 2 weeks and then discontinued at week 7. At week 13, we began a weekly antigenic challenge dose of unconjugated RtPAL and monitored for the development of an anti-RtPAL IgG antibody response. The RtPAL dose was doubled every week to a goal of 0.5 mg/kg/week at week 16 and was continued at this level for an additional 5 weeks. One week after the final infusion at week 21, serum was collected and infusions discontinued. In the original tolerance induction study, dogs that were successfully tolerized to rhIDU did not produce an antigen-specific IgG response when challenged in this manner (Kakkis et al. 2004).

Determination of Specific Antibody Titers in Canine Serum

Serum samples for antibody analysis were collected weekly prior to infusions and frozen at -20°C for subsequent analysis. Anti-RtPAL IgG antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA) method. In brief, serum samples were first diluted 1:50 then subsequent 1:3 serial dilutions were incubated on assay plates containing wells with adsorbed RtPAL. Specific binding of anti-RtPAL IgG antibodies to the coated wells was detected using a monkey anti-canine IgG secondary antibody conjugated to HRP and measuring absorbance after incubation with a substrate. An absorbance cutpoint was generated for each assay plate based on comparison with pooled serum from untreated animals. The optical density (OD) values of the serial dilutions were compared with this plate cutpoint, and the highest dilution factor that generated OD value above the plate cutpoint was reported as the titer for this sample. The time to seroconversion was defined as the number of elapsed weeks at which an anti-RtPAL IgG antibody titer greater than 1:50 was observed.

Statistical Analysis

Analyses were performed with SYSTAT statistical analysis software (Systat Software, Inc., Chicago, IL). Means and standard deviations were calculated according to standard formulas. We performed analysis of variance (ANOVA) with a post-hoc Tukey-Kramer test, and $p < 0.05$ was considered significant.

Monitoring

The safety of the tolerance regimen and recombinant enzyme infusions was assessed with serum chemistries to evaluate liver and renal function, urinalysis, cyclosporin levels during tolerance induction, physical examination, daily weight, and recording of adverse events. Blood samples were collected every 4 weeks for a complete blood count and chemistry profile. Urinalysis with reagent strips was also performed every other week on a fresh urine sample to monitor parameters such as proteinuria and hematuria. During infusions with the recombinant enzyme, the dogs were monitored for anaphylactic reactions. There were no adverse events in dogs in any experimental group, and all animals survived the study.

Results

Successful stepwise conjugation of RtPAL to the mannose 6-phosphate carrier BPM6 (Fig. 1) was demonstrated by TNBSA assay and Ellman's assay. RtPAL is a homotetramer containing 30 lysine residues per monomer, some of which are buried in the protein core, which serve as the primary amine sites for conjugation to SATA. The free primary amine molar ratios as determined by the TNBSA assay were 14.8 (RtPAL), 11.3 (RtPAL-SATA), and 11.1 (RtPAL-BPM6), indicating that an average of 3.5 lysine molecules per RtPAL monomer were no longer free after the SATA conjugation step. The free sulfhydryl molar ratios, indirect measures of SATA conjugation through the presence of deprotected SATA sulfhydryl groups, were 0.2 (RtPAL), 3.4 (deacetylated RtPAL-SATA), and 0.6 (RtPAL-BPM6), indicating that RtPAL had been conjugated to an average of 3.2 SATA molecules per monomer. Additionally, this result showed that an average of 2.8 SATA ligands per RtPAL monomer had been successfully deacetylated and subsequently bound after the addition of BPM6-SMCC, resulting in loss of previously detected free sulfhydryl groups.

Conjugate homogeneity at each step of the RtPAL-BPM6 production process was inferred from IEF electrophoresis (Fig. 2, panel a), which showed an expected distribution of isoelectric point (pI) values for each product corresponding to the distribution of MSR values about the average for each product. The absence of overlap in the pI distribution of RtPAL (Fig. 2, lane 1, panel a) and RtPAL-SATA (Fig. 2, lane 2, panel a) indicated that all RtPAL molecules had reacted with at least one SATA molecule. The decreasing pI values seen as stepwise conjugation proceeds from RtPAL, to RtPAL-SATA, to RtPAL-BPM6 (Fig. 2, lane 3, panel a) are consistent with successive loss of positively charged primary amines (e.g., lysine residues

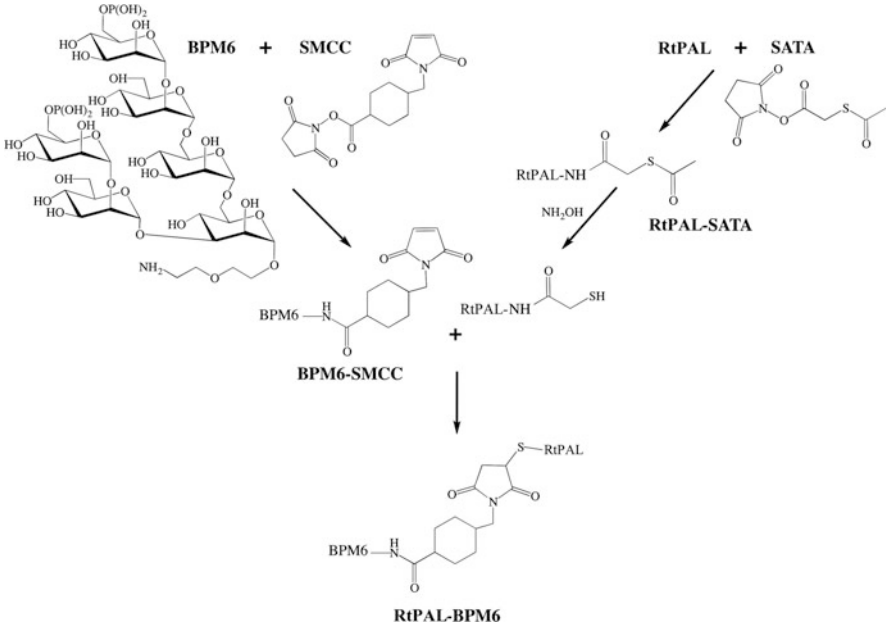

Fig. 1 RtPAL-BPM6 stepwise conjugation scheme

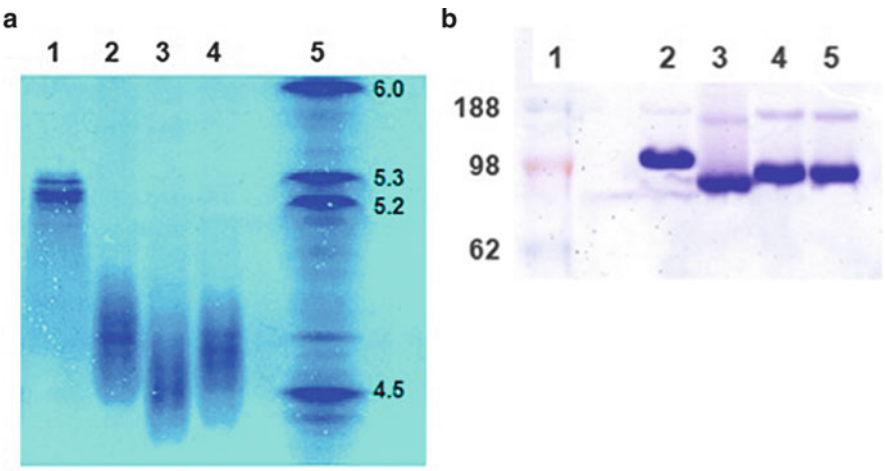

Fig. 2 Isoelectric focusing (IEF) gel electrophoresis (panel **a**) of intermediate products in RtPAL-BPM6 stepwise conjugation: RtPAL (lane 1), RtPAL-SATA (lane 2), RtPAL-BPM6 (lane 3), RtPAL-BPM6 treated with alkaline phosphatase (lane 4), and IEF Marker (lane 5). NativePAGE Novex® 3-12 % Bis-Tris Gel (panel **b**): molecular weight marker (lane 1), RtPAL (lane 2), RtPAL-SATA (lane 3), RtPAL-BPM6 (lane 4), and RtPAL-BPM6 treated with alkaline phosphatase (lane 5)

on RtPAL) and addition of negatively charged phosphate groups (e.g., mannose 6-phosphate groups on BPM6). Treatment of RtPAL-BPM6 with alkaline phosphatase is expected to dephosphorylate BPM6 at the terminal mannose 6-phosphate groups and produce a shift to a higher pI (Fig. 2, lane 4, panel a). Taken together, these data are consistent with successful stepwise conjugation of RtPAL to form RtPAL-BPM6. Under native conditions, gel electrophoresis (Fig. 2, panel b) showed monomeric products with the expected molecular weight ranges.

We tested the RtPAL-BPM6 conjugate for stability in vitro using IEF and gel electrophoresis following storage at 4°C for up to 2 months and multiple freeze-thaw cycles and found the conjugation to be preserved (data not shown). However, all enzymatic activity of RtPAL was lost during the first conjugation step to form RtPAL-SATA, and the decrease in activity appeared to be a direct function of the SATA:RtPAL molar ratio used in the reaction (Fig. 3). While modification of RtPAL with other bulkier groups has been shown to induce a limited loss of activity, the small

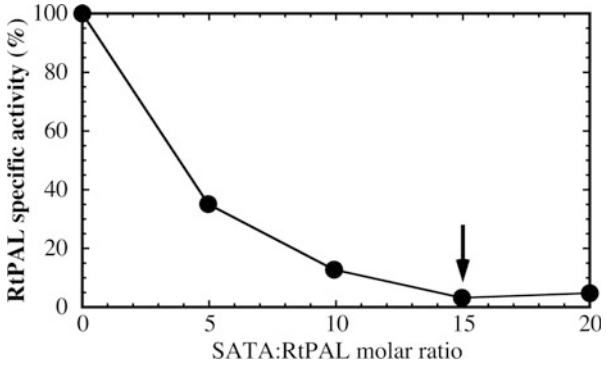

Fig. 3 RtPAL enzymatic specific activity (percentage of unconjugated RtPAL activity) measured as a function of the amount of SATA added (SATA:RtPAL molar ratio) in the first step of the RtPAL conjugation scheme. The SATA:RtPAL molar ratio used in the conjugation reaction described in text was 15:1 (*arrow*)

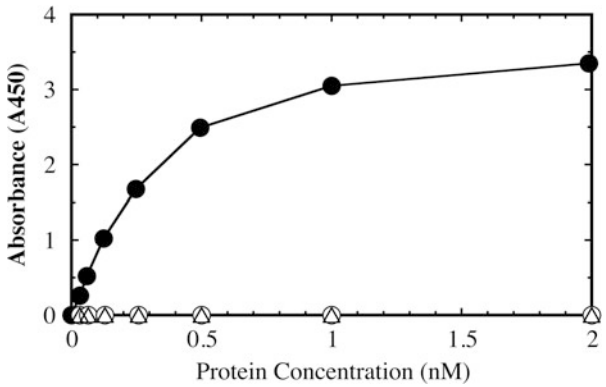

Fig. 4 Mannose 6-phosphate receptor (MPR)-coated ELISA plate wells incubated with RtPAL-BPM6 (*solid circles*), RtPAL (*open circles*), or RtPAL-SATA (*open triangles*), showing saturable binding of RtPAL-BPM6 with a calculated Kd of 0.4 nM

size of the SATA molecule may facilitate its penetration and conjugation at sites within the core of the tetramer where it can induce changes in enzyme folding, substrate binding, or substrate affinity (Wang et al. 2005).

Conjugated RtPAL-BPM6 exhibited saturable binding to the mannose 6-phosphate receptor. The Kd value, defined as the sample protein concentration resulting in 50% of maximal binding, was calculated to be 0.4 nM (Fig. 4). Neither unconjugated RtPAL nor RtPAL-SATA bound to MPR-coated wells, demonstrating that the terminal mannose 6-phosphate residues on BPM6 confer MPR binding specificity.

We observed MPR-mediated cellular uptake of the recombinant conjugated enzyme when human fibroblast cells were incubated with fluorescently labeled RtPAL-BPM6. There was noticeably increased intracellular fluorescence (Fig. 5, panel a) as compared with negligible fluorescence when the cells were incubated with unconjugated labeled RtPAL (Fig. 5, panel b). Intracellular fluorescence decreased markedly when fibroblast cells were incubated with RtPAL-BPM6 in the presence of free mannose 6-phosphate (Fig. 5, panel c), consistent with inhibition of RtPAL-BPM6 uptake via the MPR owing to excess M6P. By contrast, there was no apparent inhibition of MPR-mediated uptake when fibroblast cells were incubated with RtPAL-BPM6 in the presence of free glucose 6-phosphate (Fig. 5, panel d).

To test our hypothesis that high-affinity intracellular uptake via the MPR permits immune tolerance induction to RtPAL, we administered low-dose RtPAL or RtPAL-BPM6 to normal beagles concurrently treated with an immunosuppressive tolerance induction regimen. In the first experiment, six dogs were assigned to two groups, a control group ("Group 1," $n = 3$) and a test group ("Group 2," $n = 3$). The test group (Group 2) received the previously published tolerance induction regimen consisting of immunosuppression until week 7 and tolerizing infusions of RtPAL-BPM6 weekly for 12 weeks, while the control group (Group 1) received identical RtPAL-BPM6 infusions without immunosuppression (Kakkis et al. 2004). After the 12-week tolerance induction period, both groups of dogs were challenged with increasing doses of the unconjugated

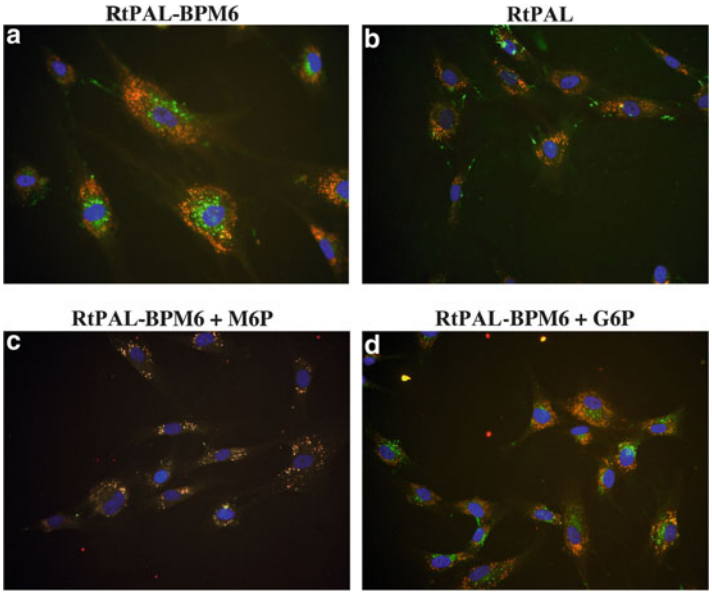

Fig. 5 Fibroblast cells incubated with 2nM FITC-labeled RtPAL-BPM6 (*green*, panel **a**) or unconjugated FITC-labeled RtPAL (*green*, panel **b**). Fibroblast cells were also incubated with FITC-labeled RtPAL-BPM6 in the presence of free mannose 6-phosphate (M6P, panel **c**) or free glucose 6-phosphate (G6P, panel **d**). Fibroblast cell nuclei are counterstained with DAPI (*blue*), and background intracellular autofluorescence is also visible (*red*)

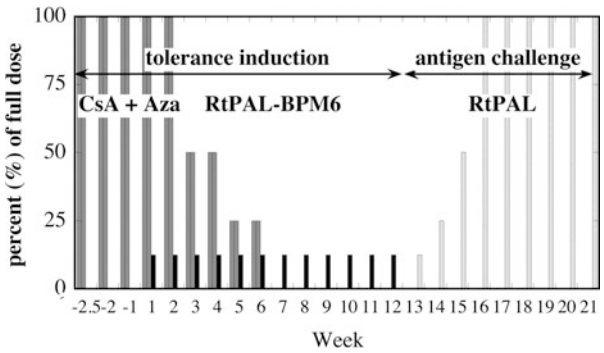

Fig. 6 Schematic diagram of tolerance induction (weeks 1–12) and antigen challenge (weeks 13–21) regimen. Cyclosporin A (CsA) and Azathioprine (Aza) dosing are indicated by the full height gray bars marked weekly. After 18 days of immunosuppression, test group dogs began receiving tolerizing low dose weekly infusions of RtPAL-BPM6 (black bars, beginning at week 1). After 2 weeks of combined full dose immunosuppression and low dose tolerizing enzyme infusion, immunosuppressive dosing was decreased to half the full dose beginning at week 3, then to one fourth the full dose beginning at week 5, and then discontinued at week 7. At week 12, tolerizing low dose weekly RtPAL-BPM6 infusions were discontinued, then at week 13 antigen challenge with unconjugated RtPAL (*striped bars*) was started and doubled every week to the goal dose at week 16, then continued at that dose until week 21

antigen RtPAL, and anti-RtPAL IgG antibody levels were measured (Fig. 6). Tolerized dogs were predicted to have minimal or no change from anti-RtPAL baseline levels while non-tolerant dogs demonstrated seroconversion with enzyme-specific antibody production. Seroconversion was defined as a specific anti-RtPAL IgG antibody titer of $>$ 1:50.

Control dogs all developed a rising anti-RtPAL IgG titer soon after starting the low-dose RtPAL-BPM6 infusions in the absence of immunosuppressive treatment (Fig. 7), with an average time to seroconversion of 4.7 weeks (Table 1). The titers reached a plateau by week 7 and remained roughly constant for the rest of the observation period. There was no sustained significant change in titer when the control group was challenged with increasing weekly doses of RtPAL beginning at week 13, and these dogs had no evidence of a systemic inflammatory response, hypersensitivity reaction, or anaphylaxis. In comparison, test dogs in Group 2 had a relative delay in seroconversion, developing rising anti-RtPAL IgG titers at an average of 7.3 weeks (Table 1) when the immunosuppressive portion of the tolerance regimen ended (Fig. 7). The difference in average

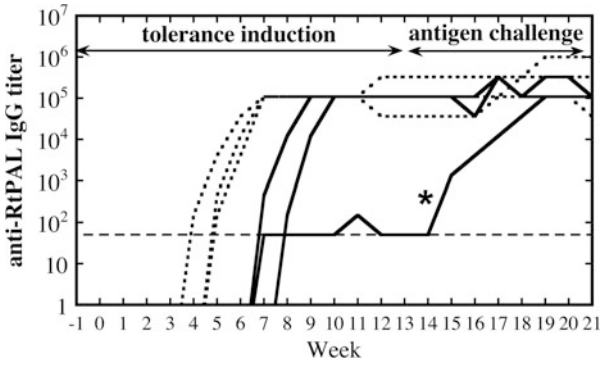

Fig. 7 Anti-RtPAL IgG antibody titers (OD units/μL) measured by ELISA for control ("Group 1," *dotted lines*) and test ("Group 2," *solid lines*) dogs during the tolerance induction phase (weeks 1–12) and the antigen challenge phase (weeks 13–21). Test group dogs received the last dose of immunosuppression at week 6, and all dogs began receiving unconjugated RtPAL at week 13. Seroconversion cutoff titer of 1:50 is marked by the thin dashed line. One dog in the test group (*) showed a delay in developing a rising antibody titer after the end of immunosuppression

Table 1 Average time to seroconversion, defined as the time at which an anti-RtPAL IgG antibody titer above 1:50 was detected in the serum of dogs treated with tolerizing infusions of RtPAL-BPM6 or RtPAL while receiving immunosuppressive therapy lasting 7 or 9 weeks (rows 2–4). The first row shows the control group, which was treated with tolerizing enzyme infusions and no immunosuppression

	Study group	Tolerizing infusion (0.056 mg/kg/ week)	Weeks of tolerizing infusions	Duration of immunosuppresion (weeks)	Weeks of full dose CsA + Aza	Time to seroconvert (average weeks ± SD)
IMMUNE TOLERANCE REGIMEN– PUBLISHED DURATION	Group 1 ($n = 3$)	RtPAL-BPM6	12	N/A	N/A	4.7 ± 0.6
	Group 2 ($n = 3$)	RtPAL-BPM6	12	7	2	7.3 ± 0.5 ($p = 0.89$)
IMMUNE TOLERANCE REGIMEN– LONGER DURATION	Group 3 ($n = 3$)	RtPAL-BPM6	12	9	4	7.3 ± 5.5 ($p = 1.0$)
	Group 4 ($n = 3$)	RtPAL	12	9	4	8.7 ± 7.1 ($p = 0.98$)

p-values correspond to comparison of average times to seroconvert between test groups (rows 2–4) and control group (row 1); all p-values for comparison of average times to seroconvert between test groups were nonsignificant

time to seroconversion between the test and control groups was not statistically significant (Table 1). One dog in Group 2 (*, Fig. 7) showed a marked delay in developing a rising antibody titer and maintained a low seropositive anti-RtPAL IgG titer even after immunosuppression was discontinued at week 7. When challenged with increasing doses of unconjugated RtPAL, this test dog began producing anti-RtPAL IgG antibodies after week 13, although at a slower rate than other animals.

To improve the success of tolerance induction, we tested the effect of extending the initial immunosuppressive period. A second test group ("Group 3," $n = 3$) received the same tolerizing infusions with RtPAL-BPM6 and an additional 2 weeks of full dose CsA and Aza (weeks 1–4, immunosuppression discontinued at week 9), and a third test group ("Group 4," $n = 3$) received tolerizing infusions with RtPAL-SATA as well as the additional 2 weeks of full dose CsA and Aza (Table 1). Group 3 dogs also developed anti-RtPAL IgG antibodies during the tolerance induction period with an average time to seroconversion of 7.3 weeks that was not significantly different from the control group. Group 4 was added as a necessary control, to determine whether RtPAL-SATA without conjugation to BPM6 could induce immune tolerance. Group 4 dogs developed anti-RtPAL IgG antibodies during the tolerance induction period as expected, with an average time to seroconversion of 8.7 weeks that also did not represent a statistically significant difference from Group 1.

Discussion

Our results show that BPM6-conjugated RtPAL contains approximately 3 BPM6 molecules per RtPAL monomer, is stable for injection after prolonged storage, and is taken up in vitro by fibroblast cells by an MPR-mediated

mechanism, but that animals receiving tolerizing infusions of RtPAL-BPM6 according to a previously published tolerance induction regimen do not develop immune tolerance to RtPAL. In addition, extending the duration of immunosuppressive treatment during the tolerance induction period did not improve the success of tolerance induction or significantly delay the average time to seroconversion. Among all groups of animals studied, the administration of immunosuppressives to the test groups during the tolerance induction period was the only clinically relevant distinguishing factor. However, the longer average time to seroconversion in each test group was not a statistically significant difference in comparison with the control group.

Multiple methods have been studied to reduce the immunogenicity of PAL while maintaining its enzymatic activity. These include structural modification with polyethylene glycol (PEG) and engineering resistance to chymotrypsin for oral delivery (Wang et al. 2005; Sarkissian et al. 2008; Kang et al. 2010). These methods rely on evasion or bypassing of the immune response to a foreign and immunogenic therapeutic protein. Inducing immune tolerance to PAL by increasing cellular uptake with mannose 6-phosphorylation has not been previously studied as a method to improve therapeutic efficacy.

The cation-independent MPR is an integral membrane protein that also binds insulin-like growth factor II and functions in the sorting of newly synthesized lysosomal enzymes as well as endocytosis of extracellular lysosomal enzymes and proteins bearing M6P (Kornfeld 1992; Gary-Bobo et al. 2007). This receptor has been found on a variety of cells including macrophages and was shown to mediate uptake of M6P-conjugated bovine serum albumin, suggesting that conjugating immunostimulant molecules with M6P could lead to uptake by and activation of macrophages (Roche et al. 1985). However, it remains unclear to what extent the MPR is expressed on antigen-presenting dendritic cells in the periphery, which are thought to be required for T-cell-dependent B cell activation by taking up, processing, and presenting antigen to T helper cells in the context of major histocompatibility complex II (MHC II) (Sauerborn et al. 2010).

While it is not known whether M6P modification increases uptake of the conjugated protein by antigen-presenting cells, it is likely that this modification alters the distribution of the conjugated protein among the target tissue and immune system compartments. Immunogenicity of biopharmaceuticals depends on structural parameters such as sequence variation and glycosylation as well as pharmacokinetic parameters such as route of administration, dosage, and duration of exposure (Schellekens 2002). Thus the effectively altered exposure kinetics conferred by M6P conjugation may be important in forming immune tolerance although it is clear that this is not sufficient.

It is interesting to note that in two animals from the first test group (Group 2, Table 1) receiving the 7-week course of immunosuppression, the rise in antigen-specific IgG titers closely followed discontinuation of immunosuppression. This timeline could suggest that antigen presentation and T cell activation may not have been blocked by the immunosuppression, but rather B cell proliferation and antibody production were reversibly suppressed. In the single animal from Group 2 that showed a relative delay in mounting an antigen-specific IgG response until challenged with unconjugated RtPAL, tolerance to RtPAL-BPM6 may have been achieved transiently but was not sustained upon exposure to the more immunogenic RtPAL. Since high-affinity cellular uptake mediated by BPM6 conjugation did not appear to reduce exposure to the immune system in the first two animals, it is possible that this RtPAL modification altered the structure of the conjugated protein in a manner that initially reduced its immunogenicity to the third dog in Group 2. Thus when this dog was exposed to the unmodified RtPAL antigen starting at week 13, it developed a rising anti-RtPAL IgG titer over an immunologically appropriate timescale. This sort of structural alteration is consistent with the observed loss of RtPAL activity upon conjugation with BPM6.

Our research shows that PAL can be successfully expressed in a recombinant form and conjugated to the M6P carrier BPM6, and that this modification enables high affinity binding to the MPR and specific MPR-mediated uptake into human fibroblast cells. Conjugating RtPAL with BPM6 was not sufficient to allow induction of immune tolerance in animals receiving immunosuppression and tolerizing infusions of RtPAL-BPM6 according to the regimen described in Kakkis et al. 2004. PAL is not an endogenous canine enzyme and so there is no naturally occurring central immune tolerance, as would be the case for a self-antigen. Further study is needed to determine a method to induce immune tolerance to RtPAL.

Acknowledgments This study was supported by a grant from BioMarin Pharmaceutical, Inc., which also provided RtPAL for this study. We thank Catalina Guerra, Jenny Dancourt, and Barbara Villareal for their technical assistance.

Synopsis

We produced mannose 6-phosphate-conjugated recombinant phenylalanine ammonia-lyase (PAL), administered it to normal dogs using a previously published tolerizing regimen, and found that, despite demonstrated M6P receptor binding and cellular uptake, all test animals failed

to develop immune tolerance to PAL and produced a marked antibody response.

Competing Interest Statement

The coauthors TL, BZ, PT, SC, JF, and JFL are employees of BioMarin Pharmaceutical. EDK is a former employee of BioMarin and is now Chief Executive Officer of Ultragenyx Corporation.

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RESEARCH REPORT

Association of Dopamine Receptor Gene Polymorphisms with the Clinical Course of Wilson Disease

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Abstract *Background:* Dopamine receptor D2 (*DRD2*) polymorphisms are proposed to be important factors in the presentation of neuropsychiatric symptoms in many disorders, including decreased striatum levels of dopamine D2 receptors in Wilson disease. The present study investigated the association between *DRD2* gene polymorphisms and clinical manifestation of Wilson disease.

Methods: Analyzing data from 97 symptomatic Wilson disease patients, we investigated the *DRD2* gene polymorphisms rs1800497, rs1799732, and rs12364283. We assessed the polymorphisms impact on the phenotypic presentation of the disease.

Results: Generally, the *DRD2* gene polymorphisms had no impact on the hepatic or neuropsychiatric clinical presentation of Wilson disease. However, rs1799732 deletion allele carriers with neuropsychiatric symptoms had earlier onset of WD symptoms by almost 6 years compared with individuals without this allele (22.5 vs. 28.3 years; $P < 0.05$). This unfavorable effect of the rs1799732 polymorphism was even more pronounced among adenosine triphosphatase 7B gene (*ATP7B)* p.H1069Q homozygous patients, in whom carriership of the deletion allele was related to earlier initial neuropsychiatric manifestation by 14 years (18.4 vs. 32.2 years; $P < 0.01$).

Conclusions: Genetic variation of *DRD2*, specifically the rs1799732 polymorphism, may produce an earlier clinical presentation of Wilson disease neuropsychiatric symptoms and signs that occur in the course of dopaminergic system impairment due to copper accumulation in the brain. We speculate that this effect may be due to the impact of *DRD2* polymorphism on dopamine D2 receptor density, but further studies are needed to understand the mechanisms of such phenotypic effects.

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Competing interests: none declared

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Introduction

Wilson disease (WD) is an inherited copper metabolism disorder leading to copper accumulation in many tissues (mainly the liver and brain) with secondary damage to affected organs (Roberts and Schilsky 2008; Ala et al. 2007; Ferenci et al. 2003, 2007). WD is associated with a wide spectrum of symptoms (hepatic, neurological, psychiatric, and others) as well as great variability in clinical presentation and outcome (Roberts and Schilsky 2008; Ala et al. 2007; Ferenci et al. 2003, 2007; Schilsky et al. 1994). Although these differences remain largely unexplained, several factors are known to impact clinical presentation of WD, including gender (Schilsky et al. 1994; Litwin et al. 2012a) and genotype (Stapelbroek et al. 2004; Gromadzka et al. 2005, 2006). It is also suspected that WD presentation may be influenced by polymorphisms in the genes encoding prion-related protein, methylenetetrahydrofolate reductase, interleukin-1 receptor antagonist, and apolipoprotein-E

Table 1 *DRD2* polymorphisms and their possible clinical significance

Polymorphism	Possible clinical significance	Comment
rs1800497 *ANKK* TaqIA	The A1 allele frequency is higher in patients with neuroleptic malignant syndrome (Suzuki et al. 2001). The A1 allele is associated with tardive dyskinesia, Parkinson's disease (data are conflicting), and late onset hallucination induced by treatment with levodopa (Noble 2003; Makoff et al. 2000; Olivieri et al. 2000).	The A1 allele may be associated with decreased D2 receptors in striatum (Noble 2003).
rs1799732 −141 C Ins/Del	The Del allele frequency is higher in patients with neuroleptic malignant syndrome (mechanism not known; data conflicting) (Kishida et al. 2004; Farde et al. 1995). The Del allele is significantly associated with poorer antipsychotic drug response (Zhang et al. 2010).	Data are conflicting regarding the impact on D2 receptors density in striatum Deletion (Del+) may be associated with increased number of D2 receptors (Jonsson et al. 1999) or may have no impact on DRD2 density (Ritchie and Noble 2003).
rs12364283 DRD2 Ex8	*DRD2 Ex8* A/A genotype is associated with increased anxiety, depression, and suicide attempts after detoxification treatment in alcohol-dependent patients, with reduced response to dopamine D2 agonists (apomorphine) and to D2 antagonists (tiapride) (Samochowiec et al. 2000).	The E8A/A allele may be associated with reduced DRD2 expression (Samochowiec et al. 2000).

(Merle et al. 2006; Gromadzka et al. 2011a, b; Schiefermeier et al. 2000; Litwin et al. 2012b). Other genes like antioxidant-1 (Atox-1), copper metabolism gene *MURR1* domain containing proteins (COMMD), and X-linked inhibitor of apoptosis (XIAP) (Simon et al. 2008; Weiss et al. 2006; Burstein et al. 2005; Weiss et al. 2010) have also been suggested as WD modifiers. Nonetheless, phenotype-related differences in WD manifestation are still mainly unknown.

In WD, most of the neuropsychiatric symptoms are due to basal ganglia dysfunction secondary to copper accumulation (Magalhaes et al. 1994; Schlaug et al. 1994; Nyberg et al. 1982). Pathology studies in WD revealed reduced striatal dopamine and hydroxylase tyrosine levels (Vallone et al. 2000; Nyberg et al. 1982; Mousseau et al. 1993) and animal studies have indicated decreased dopamine receptor 2 (DRD2) during copper overloading (de Vries et al. 1986). Single photon emission computerized tomography (SPECT) and positron emission tomography (PET) studies have shown postsynaptic dopaminergic deficit (loss of D2 receptors in striatum) in WD patients (Oder et al. 1996; Westermark et al. 1995) as well as presynaptic nigrostratial dopaminergic damage (Jeon et al. 1998). WD patients also exhibit reduced bindings of dopamine ligands to dopamine receptors on lymphocytes probably due to dopamine receptors damage during copper intoxication (Członkowski and Członkowska 1984; Członkowska et al. 1987).

Polymorphisms in the *DRD2* gene and the related ankyrin repeat and protein kinase–containing protein (*ANKK*) gene – including *ANKK* TaqIA (rs1800497), *DRD2* PROM −141 C Ins/Del (rs1799732), and *DRD2* Ex8 (rs12364283) – impact the dopamine receptor D2 density in the striatum with a high clinical significance in the etiology of many neuropsychiatric disorders, especially involuntary movements (Table 1) (Wu et al. 2006; Noble 2003; Thompson et al. 1997; Kisihida et al. 2004; Suzuki et al. 2001; Tan et al. 2003; Zhang et al. 2010; Ritchie and Noble 2003; Farde and Nordstrom 1993; Farde et al. 1995, 1997; Tinsley et al. 2009).

We hypothesized that these polymorphisms may represent an important predicting factor for phenotypic manifestations of WD. The aim of the present study was to determine, in a large group of WD patients, the relationships among these three important DRD2-related single nucleotide polymorphisms (SNPs) and WD presentation.

Methods

We studied 97 WD symptomatic patients (42 men and 55 women) who had received a confirmed diagnosis from the Institute of Psychiatry and Neurology in Warsaw, Poland, between 1988 and 2010. This study was approved by the local ethics committee and informed consent was provided by all study subjects. The diagnoses were based on clinical symptoms, abnormal copper metabolism (decreased levels of serum ceruloplasmin and serum copper, and increased 24-h urine copper excretion), presence of the Kayser-Fleischer ring, and, in many cases, genetic examination. If the diagnosis was not certain, it was confirmed by measuring Cu-64 incorporation into ceruloplasmin after 24 and 48 h. None of the examined patients were treated with neuroleptics or drugs that could interfere with dopamine metabolism (dopamine agonists, levodopa),

Table 2 SNPs, primers, and PCR products

SNP ID	Position	Primer and sequence	PCR product	RE	DNA variant	Allele	Fragment size (bp)
rs1800497	11 q23.2	F: 5'-CTT GCC CTC TAG GAA GGA CAT	310	Taq1	T/C	T	310
	ANKK					C	180
		R: 5'-ACC TTC CTG AGT GTC ATC AAC C					130
rs1799732	11 q23.1	F: 5'-CAA CCC TGG CTT CTG AGT CC	207	MvaI	−141 ins/del	C	207
	Promoter	R: 5'-GAG CTG TAC CTC CTC GGC GAT C				–	177
rs1236428	11 q23.1	F: 5'-GCC TGT CCT CCC CGG CTC TG	349	Hpa II	A/G	A	349
	Ex 8	R: 5'-GGC AGT GAG GAG CAT GGA GCC AAC				G	282

as such treatment could produce neuropsychiatric manifestations, such as drug-induced movement disorders.

Symptomatic WD patients were defined as patients with clinical signs of WD at onset and/or diagnosis. The hepatic symptoms and signs were assessed based on a detailed questionnaire that included data on fatigue, weight loss, leg edema, jaundice, abdominal swelling, hematemesis, hemorrhages, and fulminant liver failure. Laboratory examinations included ultrasound examinations of the liver and spleen, gastroscopy, and assessments of aminotransferases, alkaline phosphatase, bilirubin, INR, and albumen that were available from medical history and records. The evaluation of neuropsychiatric symptoms and signs was also based on a detailed questionnaire addressing salivation, dysphagia, speech, writing and gait disturbances, involuntary movements, adynamia, epileptic seizures, mood disorders, anxiety, and cognitive impairment (Litwin et al. 2012a).

The age at WD symptom onset/diagnosis was assessed based on patient history, symptoms and signs of WD, and/or available medical documentation in addition to clinical and laboratory investigations.

WD genotyping was determined by polymerase chain reaction (PCR) as reported previously (Gromadzka et al. 2005, 2006) and was assessed according to *ATP7B* genotype (homozygous p.H1069Q/p.H1069Q, compound heterozygous p.H1069Q/other mutation, or negative for the p.H0169Q mutation). Polymorphisms were determined by PCR and restriction fragment length polymorphism analysis of *ANKK* TaqIA (rs1800497), *DRD2* PROM −141 C Ins/Del (rs1799732), and *DRD2* Ex8 (rs12364283) as previously described (Grandy et al. 1989; Hori et al. 2001; Samochowiec et al. 2000). Investigated SNPs, primers, and PCR products are presented in Table 2. One hundred (50 males and 50 females) unrelated, matched, healthy controls were used for SNP comparison and to check the Hardy–Weinberg equilibrium.

Due to the clinical significance of DRD2 in neuropsychiatric disorders, further analysis included a comparison of the distributions of the three polymorphisms between patients with and without neuropsychiatric symptoms and signs, and between patients with neuropsychiatric presentation and patients with dystonic symptoms (most severe neurologic presentation). All analyses of significance were repeated in the set of patients homozygous for the H1069Q mutation, a more homogenous WD patient group.

Statistical Analysis

All data were analyzed using Statistica version 9. The mean, range, percentage, and SD were noted for descriptive summary statistics. Quantitative variables were compared using the Mann–Whitney *U* test. Categorical variables were compared between groups using the chi-square test and Fisher's test; $P < 0.05$ was considered statistically significant. For the multiple comparisons, hypothesis testing was performed using the Bonferroni correction (the *P*-value divided by the total number of pairwise comparisons) to correct for the chance that in multiple comparisons the null hypothesis would be rejected by chance. For three polymorphisms, the level of significance was equal to 0.008.

Results

Polymorphisms and WD Clinical Manifestations

In our group of 97 symptomatic patients, 31 had both neuropsychiatric and hepatic manifestations at onset, 32 had only neurological, and 34 had only hepatic symptoms and signs. In total, 63 patients had neuropsychiatric symptoms; among them, 21 had dystonia.

In WD patients and control subjects, no significant deviation from the Hardy–Weinberg equilibrium form was found for *ANKK* TaqIA (rs1800497; WD patients, A1/A1 = 2, A1/A2 = 33, A2/A2 = 62; control subjects, A1/A1 = 3, A1/A2 = 28, A2/A2 = 69; chi-squared = 0.006, degrees of freedom (d.f). = 1, $P < 0.093$), *DRD2* PROM −141 C

Table 3 Distribution of neuropsychiatric and dystonia symptoms and signs in WD patients according to *DRD2* polymorphism

	ANKK TaqI (A1 allele)		*DRD2* PROM −141 C deletion		*DRD2* Ex 8 (A/A vs. A/G and G/G)	
Patients symptoms and signs:	**A1**	**A2**	**Del−**	**Del+**	**A/A**	**A/G and G/G**
	($n = 35$)	**($n = 62$)**	**($n = 83$)**	**($n = 14$)**	**($n = 54$)**	**($n = 43$)**
Not neuropsychiatric ($n = 34$)	14 (41 %)	20 (59 %)	31 (91 %)	3 (9 %)	20 (59 %)	14 (41 %)
Neuropsychiatric ($n = 63$)	21 (33 %)	42 (66 %)	52 (82 %)	11 (17 %)	34 (54 %)	29 (46 %)
Neuropsychiatric with dystonia ($n = 21$)	9 (43 %)	12 (57 %)	18 (86 %)	3 (14 %)	11 (52 %)	10 (48 %)
Neuropsychiatric without dystonia ($n = 42$)	12 (29 %)	30 (71 %)	34 (80 %)	8 (20 %)	23 (54 %)	19 (46 %)

Data do not sum to 100 % due to rounding errors. There were no statistically significant differences according to polymorphism or WD clinical form.

Table 4 Age of symptom onset in patients with presence/absence of neuropsychiatric signs and symptoms according to *DRD2* polymorphism

	ANKK Taq1 (A1 allele)		*DRD2* PROM −141 C deletion		*DRD2* Ex 8 (A/A vs. A/G and G/G)	
Clinical manifestation and age of symptoms and signs onset	**A1**	**A2**	**Del−**	**Del+**	**A/A**	**A/G and G/G**
	($n = 35$)	**($n = 62$)**	**($n = 83$)**	**($n = 14$)**	**($n = 54$)**	**($n = 43$)**
All WD patients 25.1 ± 8.7 ($n = 97$)	23.5 ± 7.6 ($n = 35$)	26.09 ± 9.3 ($n = 62$)	25.6 ± 8.9 ($n = 83$)	22.0 ± 7.0 ($n = 14$)	25.1 ± 8.1 ($n = 54$)	25.1 ± 9.3 ($n = 43$)
Not neuropsychiatric 21.2 ± 8.05 ($n = 34$)	20.5 ± 7.6 ($n = 14$)	21.6 ± 8.4 ($n = 20$)	21.2 ± 8.0 ($n = 31$)	20.3 ± 10.4 ($n = 3$)	19.5 ± 5.5 ($n = 20$)	22.4 ± 9.3 ($n = 14$)
Neuropsychiatric 27.3 ± 8.4 ($n = 63$)	25.5 ± 7.1 ($n = 21$)	28.2 ± 9.0 ($n = 42$)	28.3 ± 8.5 ($n = 52$)	22.5 ± 6.5[a] ($n = 11$)	26.3 ± 9.1 ($n = 34$)	28.4 ± 7.5 ($n = 29$)

[a] Significant difference in age at onset of symptoms ($P = 0.035$) between *DRD2* PROM −141 C del, Del + positive vs. negative patients.

Ins/Del (rs1799732; WD patients, Ins/Ins = 83, Ins/Del = 14, Del/Del = 0; control subjects Ins/Ins = 80, Ins/Del = 20, Del/Del = 0; chi-squared = 0.614, d.f. = 1, $P < 0.433$), and *DRD2* Ex8 (rs12364283; WD patients, A/G = 52, A/A = 40, G/G = 5; control subjects, A/G = 43, A/A = 52, G/G = 5; chi-squared = 1.234, d.f. = 1, $P < 0.266$). We did not detect an impact of these polymorphisms on the clinical manifestation of WD at onset (Table 3).

Polymorphisms and Age at First WD Symptom Onset

The mean age of all patients at the first signs of WD was 25.1 ± 8 years (range 7–57 years). We found a significant association only between rs1799732 polymorphism and age of onset of WD neuropsychiatric symptoms – carriers of the deletion (Del+) allele of the −141 C Ins/Del polymorphism presented earlier onset of WD neuropsychiatric signs by 6 years compared with Del − carriers (22.5 vs. 28.3 years; $P = 0.035$; Table 4).

Homozygous p.H1069Q Patients: Polymorphisms, Clinical manifestation, and Age of Symptom Onset

WD genotyping of 97 symptomatic WD patients revealed that 43 patients were homozygous for the p.H1069Q mutation, 36 patients were compound heterozygous, and 18 patients were negative for the p.H1069Q mutation. We did not detect an impact of polymorphisms on the clinical WD manifestation in p.H1069Q homozygous patients (Table 5). Due to the very small group of patients that were homozygous for p.H1069Q mutations in the dystonic group ($n = 3$), we did not assess these patients separately. However, among WD p.H1069Q patients, we detected a statistically significant effect of the *DRD2* −141 C Ins/Del polymorphism. In this homogenous group, Del + allele carriers presented earlier onset of any WD symptoms by 9 years (20.1 vs. 29.4 years; $P = 0.019$); furthermore, the subset of these patients with neuropsychiatic signs presented with symptom onset 14 years earlier (18.4 vs. 32.2 years; $P = 0.001$; Table 6).

Table 5 Distribution of neuropsychiatric symptoms and signs in WD patients according to *DRD2* polymorphism in 43 p.H1069Q homozygous patients

	ANKK Taq1 (A1 allele)		*DRD2* PROM −141 C deletion		*DRD2* Ex 8 (A/A vs. A/G and G/G)	
Clinical manifestation (symptoms and signs) ($n = 43$)	**A1 ($n = 16$)**	**A2 ($n = 27$)**	**Del− ($n = 36$)**	**Del+ ($n = 7$)**	**A/A ($n = 24$)**	**A/G and G/G ($n = 19$)**
Not neuropsychiatric ($n = 15$)	6 (40 %)	9 (60 %)	13 (86 %)	2 (13 %)	8 (53 %)	7 (46 %)
Neuropsychiatric ($n = 28$)	10 (35 %)	18 (65 %)	23 (82 %)	5 (18 %)	16 (57 %)	12 (42 %)

Data do not sum to 100 % due to rounding errors. There were no statistically significant differences according to polymorphism and WD clinical form.

Table 6 Age of symptom onset in all WD patients, and according to the presence/absence of neuropsychiatric symptoms and signs and to *DRD2* polymorphism in p.H1069Q homozygous patients

	ANKK Taq 1 (A1 allele)		*DRD2* PROM −141 C deletion		*DRD2* Ex 8 (A/A vs. A/G and G/G)	
Clinical manifestation and age of symptoms and signs onset (years)	**A1 ($n = 16$)**	**A2 ($n = 27$)**	**Del− ($n = 36$)**	**Del+ ($n = 7$)**	**A/A ($n = 24$)**	**A/G and G/G ($n = 19$)**
All WD patients ($n = 43$) 28.1 ± 9.2	25.5 ± 7.8 ($n = 16$)	29.2 ± 9.6 ($n = 27$)	29.4 ± 8.8 ($n = 36$)	20.1 ± 6.2[a] ($n = 7$)	27.8 ± 8.1 ($n = 24$)	27.9 ± 9.8 ($n = 19$)
Not neuropsychiatric ($n = 15$) 25.2 ± 7.6	22.6 ± 8.0 ($n = 6$)	27.0 ± 7.2 ($n = 9$)	25.3 ± 7.6 ($n = 13$)	24.5 ± 10.6 ($n = 2$)	27.6 ± 8.8 ($n = 8$)	22.5 ± 5.4 ($n = 7$)
Neuropsychiatric ($n = 28$) 29.7 ± 9.7	27.3 ± 7.5 ($n = 10$)	31.1 ± 10.7 ($n = 18$)	32.2 ± 8.8 ($n = 23$)	18.4 ± 4.0[b] ($n = 5$)	30.9 ± 7.3 ($n = 16$)	28.8 ± 11.3 ($n = 12$)

[a] Statistically significant difference ($P = 0.019$) in age at onset of symptoms between *DRD2* PROM −141 C del, Del + positive and negative patients (all WD patients).

[b] Statistically significant difference ($P = 0.001$) in age at onset of symptoms between *DRD2* PROM −141 C del, Del + positive and negative patients (patients with neuropsychiatric symptoms and signs).

Discussion

In the present investigation, we identified a significant impact of the *DRD2* PROM −141 C Ins/Del (rs1799732) polymorphism on WD clinical neuropsychiatric presentation. We were thus able to partially confirm our initial hypothesis that changes in dopaminergic neurotransmission due to *DRD2* polymorphism could be important for clinical neuropsychiatric manifestation. Carriers of the Del + allele of the −141 C Ins/Del polymorphism presented earlier onset of WD neuropsychiatric symptoms by almost 6 years compared with the Del variant. This unfavorable effect of the −141 C Ins/Del polymorphism was even more pronounced in WD p.H1069Q homozygous patients, as the Del + allele carriers presented earlier onset of any WD symptoms by nearly 9 years and neuropsychiatric symptoms by nearly 14 years. Our analysis suggests that the Del + effect was restricted to neuropsychiatric manifestation. Additionally, we did not find any correlation between WD manifestation and the two other studied polymorphisms *ANKK* TaqIA (rs1800497) and *DRD2* Ex8 (rs12364283). The p.H1069Q effect observed in our study could be explained by the fact that compared to other mutations, p.H1069Q *ATP7B* exerts a relatively mild effect on functions of ATPase 7B (Stapelbroek et al. 2004; Gromadzka et al. 2005) and it is possible that the WD phenotypes of patients possessing more severe *ATP7B* mutations are modulated by other factors to a lesser degree.

In WD, most neuropsychiatric symptoms are due to basal ganglia copper accumulation and the secondary damage to affected structures (dystonia, parkinsonism, and others) or prefrontal cortex disturbances (Magalhes et al. 1994; Schlaug et al. 1994; Nyberg et al. 1982; Oder et al. 1993; Seniow et al. 2002), as both of these areas involve the dopaminergic system (Vallone et al. 2000). Autopsies and radiological and laboratory investigations of WD cases have found reduced striatal dopamine and hydroxylase tyrosine levels (Nyberg et al. 1982), as well as reduced dopamine D2 receptor density (Oder et al. 1996; Schlaug et al. 1994). However, treatment with dopamine agonists or levodopa had no effect, probably due to the presence of both pre- and postsynaptic dopaminergic damage (Frankel et al. 1989; Jeon et al. 1998). The density of DRD2 postsynaptic receptors tends to increase during anti-copper treatment (Schlaug et al. 1994), suggesting that the

dopamine D2 receptor pathway may be critical to WD clinical presentation. Furthermore, *DRD2* polymorphisms are predictive of the dopamine receptor D2 density in the striatum (Noble 2003; Ritchie and Noble 2003), which may also affect WD neuropsychiatric manifestation.

Based on the previously reported clinical significance of the polymorphisms *ANKK* TaqIA and *DRD2* Ex8 (Table 1), we thought that the decreased DRD2 density in the striatum in TaqI A1 allele carriers or reduced expression of DRD2 in Ex8 A/A genotype may worsen dopaminergic neurotransmission leading to earlier onset of neuropsychiatric WD signs, but our data did not confirm this hypothesis. The *ANKK* TaqIA and *DRD2* Ex8 polymorphisms had no impact on WD clinical presentation. We also did not identify a relationship between dystonic manifestation of WD and *ANKK* TaqI or *DRD2* Ex8 polymorphisms as we had expected. This lack of a detected association may be due to our small patient sample, or there may be a different etiology of neuropsychiatric symptoms in WD via other mechanisms involving DRD2.

Previous reports of the clinical significance of the *DRD2* −141 C Ins/Del polymorphism have been conflicting (Table 1). According to some studies (Jonsson et al. 1999; Zhang et al. 2010) Del + carriers may have higher numbers of DRD2 receptors in the striatum. A decrease of postsynaptic DRD2 is usually observed during WD (Oder et al. 1996; Schlaug et al. 1994), and increase is observed during chelating treatment (Schlaug et al. 1994). According to these observations, we should have found a protective effect of the Del + allele on neuropsychiatric presentation (increased number of DRD2) in WD, but we did not. On the contrary, we found that the Del + genotype accelerated the onset of neuropsychiatric symptoms. This unfavorable effect of the Del + allele may be explained by other mechanisms – like changes in receptor affinity, changes in receptor structure, interactions with other genes or environmental factors, or D2 receptor hyposensitivity, leading to a decreased effect of dopaminergic transmission. Another possible mechanism with such an effect could be connected with the impact of the *DRD2* −141 C Ins/Del polymorphism on the number of presynaptic D2 receptors (Vallone et al. 2000). According to such a hypothesis, Del + allele carriers could present increased numbers of such receptors, thus providing WD patients with stimulation of D2 presynaptic receptors with low doses of dopamine, which could further reduce dopamine release and dopaminergic cell firing and finally reduce locomotor functions and lead to neuropsychiatric WD presentation (Vallone et al. 2000). Further studies to confirm such hypotheses are very important, because such information could help establish group of WD patients in whom treatment with higher dose of levodopa would be beneficial (Del + carriers). Future investigations of this topic should include assessment of DRD2 density in radiological studies.

Our study has a few limitations. The first is the small number of patients included and the further limited number of SNPs analyzed. However, it should be noted that WD is a rare disease, and the number of patients in our study is very similar to that in many other WD or *DRD2* gene polymorphism studies (Schiefermeier et al. 2000; Kishida et al. 2004). This is the first pilot study of *DRD2* gene polymorphism in WD, so we tried to assess the most important *DRD2* SNPs in neuropsychiatric disorders (especially movement disorders). Furthermore, data are conflicting regarding the impact of the *DRD2* −141 C Ins/Del polymorphism on DRD2 density; without more specific studies (PET), we cannot confirm the etiology of the impact of the Del + allele on WD neuropsychiatric presentation. In the present report, we can only hypothesize about the impact on DRD2 expression based on some previous studies (Zhang et al. 2010; Jonsson et al. 1999), but these possibilities should be further investigated especially in WD.

In summary, our findings suggest that the *DRD2* −141 C Ins/Del polymorphism affects WD neuropsychiatric presentation, probably through disruption of the balance of dopamine neurotransmission that makes these patients more sensitive to basal ganglia intoxication by copper accumulation. Further studies of *DRD2* SNPs with dopamine receptor imaging in WD patients are needed to better understand the mechanisms of such phenotypic effects.

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We have no relevant financial interest in the Submitted Publication material.

Conflict of Interest

The authors declare no potential conflict of interest relevant to this article.

Documentation of Author Roles

1. Anna Członkowska: research project – conception; manuscript preparation – review and critique
2. Grażyna Gromadzka: research project – execution; statistical analysis – review; manuscript critique review

3. Tomasz Litwin: research project – conception and organization, execution; statistical analysis – design, execution; manuscript preparation – writing of the first draft, review
4. Jerzy Samochowiec: DRD2 polymorphism analysis
5. Anna Grzywacz: DRD polymorphism analysis
6. Andrzej Członkowski: manuscript review and corrections

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RESEARCH REPORT

Growth in Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency

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Abstract Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency is an inborn error of fatty acid metabolism that affects the degradation of long chain fatty acids and causes insufficient energy production and accumulation of toxic intermediates. The treatment consists of a diet low in fat, with supplementation of medium-chain triglycerides that bypass the metabolic block. In addition, frequent feeds and extra carbohydrates are given during febrile illnesses to reduce lipolysis. Hence, this diet differs from the general dietary recommendations for growing children. Furthermore, the Swedish dietary instructions for fat intake in LCHAD deficiency are given in grams, which differ from most guidelines that recommend fat intake as percentage shares of total caloric intake.

Aims: To assess growth in patients with LCHAD deficiency, in relation to dietary treatment and to evaluate if overweight/obesity is more common than in the normal population.

Results: The growth velocity showed acceleration after diagnosis and the start of treatment, followed by a period of stable or decelerated growth. The majority of the patients developed overweight to a greater extent than children without LCHAD deficiency. Several patients also went through a phase of obesity. Data on final height (FH) showed that three out of five patients had grown according to their genetic potential.

Conclusions: Regular and frequent follow-up and careful monitoring of weight are essential to avoid the development of overweight and obesity. The Swedish dietary instructions defining fat intake in total grams per day may be an alternative approach to achieve a moderate total caloric intake.

Abbreviations

BW	Birth Weight
E%	Energy percentage
EFA	Essential fatty Acid
FH	Final Height
FH SDS	Final Height Standard Deviation Score
IUGR	Intra Uterine Growth Restriction
LCF	Long Chain Fatty Acid
LCHAD	Long-chain 3-hydroxyacyl-CoA dehydrogenase
MTP	Mitochondrial trifunctional protein
SGA	Small for gestational age
TH	Target Height
TH SDS	Target Height Standard Deviation Score

Introduction

LCHAD deficiency (OMIM 609016) was first described in 1989 (Wanders et al. 1989 ; Wanders et al. 1990a; Hagenfeldt et al. 1990) in children who presented with lethargy and hypoketotic hypoglycemia after a short period of fasting often in association with gastroenteritis or other febrile infections. The diagnosis is usually established within the first year of life, often when nightly feedings are weaned (Saudubray et al. 1999; Tyni et al. 1997; Spiekerkoetter et al. 2009a). Some patients present with acute symptoms such as coma, cardiac arrest, and hypoketotic hypoglycemia, while others present with unspecific symptoms such as failure to thrive, poor weight gain, and vomiting. Common clinical findings are liver enlargement, muscular hypotonia, and hypertrophic cardiomyopathy. The mortality rate is high before diagnosis and treatment (den Boer et al. 2002). Measurements of acylcarnitines in plasma or nonesterified dicarboxylic and 3-hydroxy fatty acids in plasma and organic acids in urine and mutation analysis (Hagenfeldt et al. 1995; IJlst et al. 1994) are part of the diagnostic procedure. The most common mutation is G1428C in the α-subunit of the trifunctional protein (IJlst et al. 1996; IJlst et al. 1994; Sims et al. 1995).

Obstetric complications such as acute fatty liver of pregnancy, HELLP syndrome, preeclampsia and growth retardation are common when the fetus is affected with LCHAD deficiency, possibly as a result of placental insufficiency (Tyni et al. 1998a; Wilcken et al. 1993; Yang et al. 2002). The long-term outcome is variable, but a constant feature is retinal pigmentations progressing to impaired retinal function (Fahnehjelm et al. 2007; Tyni et al. 1998b). Peripheral neuropathy and psychomotor delay have been described in some patients (Bennett et al. 2000; den Boer et al. 2002; Saudubray et al. 1999; Spiekerkoetter et al. 2009a, b; Tyni et al. 1997; Wanders et al. 1990b).

The aim of the treatment is to minimize the necessity of energy production from long-chain fatty acids of both exogenous and endogenous origin, and thereby avoid accumulation of toxic intermediates of the defective β-oxidation. The diet, low in fat content and hence long-chain fatty acids from normal food, is supplemented with medium-chain triglycerides (MCT fat) and thereby bypasses the enzymatic defect. Supplementation with essential fatty acids is necessary to avoid deficiency (Gillingham et al. 1999; Gillingham et al. 2003; Spiekerkoetter et al. 2009a); however, management may vary considerably (Potter et al. 2012). To inhibit lipolysis, frequent feeds are necessary. During catabolic events such as febrile infections, anabolic treatment with an intravenous glucose infusion may be necessary. Even after the diagnosis has been established and treatment started, the patients may have episodes of metabolic decompensation, especially during infections or fasting, with muscle pain, muscular hypotonia, and elevated serum CK and aminotransferases.

The Swedish dietary instructions for children with an LCHAD deficiency differs from most international guidelines as they are given in grams of long-chain fatty acid intake (Shaw and Lawson 2001) instead of as a percentage of the total energy intake (Gillingham et al. 1999; Gillingham et al. 2003; Spiekerkoetter et al. 2009b, 2010).

The diet in LCHAD is extreme and in disagreement with general nutritional recommendations for growing children. Height may be negatively affected by the low-fat diet or the disease itself, and there is a risk for weight gain with high carbohydrate intake, short fasting periods and continuous night feeds. In addition, a rapid weight gain may result in acceleration in height. This study was designed to examine how the Swedish dietary treatment affects growth, height, and weight development in children with LCHAD deficiency, with particular emphasis on growth patterns, final height, and development of overweight and/or obesity over time.

Materials and Methods

Study Population

Data were retrospectively collected from 10 patients. The age at diagnosis varied between 2 days and 13 months (mean 6.1 months). One patient was diagnosed in the neonatal period before any symptoms had developed because of a family history. The oldest patient has been treated for more than 20 years. The diagnosis was based on increased levels of 3-OH dicarboxylic acids in urine and 3-OH fatty acids in plasma and was confirmed by mutation analysis. Seven patients were homozygous for the common LCHAD deficiency mutation G1528C; the other three were compound heterozygous for G1528C.

The study protocol was approved by the Ethics Committee, and informed consent was given by the patients and families.

Clinical Follow-Up

The patients were followed regularly at our centers. Information on height, weight, and biochemical parameters (plasma 3-OH fatty acids, plasma carnitine, liver enzymes, creatine kinase, and fatty acid profile) was collected and analyzed. Information on clinical presentation and follow-up was collected from the medical records. The number of episodes when the child had fever or muscular pain and had been treated with extra carbohydrate intake, either orally at home or as glucose infusions at home, were noted (Table 1).

Dietary Treatment

Dietary treatment with a low intake of LCT fat was commenced at the time of the diagnosis in all children. The amount of LCT fat in grams was adjusted according to Swedish nutritional minimum requirement recommendations in correlation with age (Shaw and Lawson 2001). Parents were taught how to calculate their child's LCT intake in grams, to ensure that it did not exceed the maximum prescribed quantity. The carbohydrate and protein intake was not adjusted in detail. Changes in weight or height curves were followed to assess the caloric intake. Observed stagnation or acceleration in weight was compensated for by a moderate increase or reduction of the carbohydrate and protein intake. In addition, the parents were asked to complete food diaries at least once yearly with records of everything their child ate, specified in grams, during 3 consecutive days.

Sufficient amounts of essential fatty acids were given according to Swedish recommendations, predominantly as walnut oil containing a minimum of nonessential fats. The majority of the children (8 out of 10) received docosahexaenoic acid (DHA) supplementation to maintain DHA plasma levels just above or within the upper reference range. In addition, the diet was enriched with vitamins and minerals. All but one of the children had a percutaneous gastrostomy (PEG) with continuous night feeds, predominantly given as a low-fat formula containing whey protein, carbohydrates, MCT fat, vitamins, minerals, and trace elements. The fasting periods were limited to 3–4 h, depending on age. In order to reduce lipolysis, two children were given uncooked corn starch. During febrile infections, a carbohydrate-rich supplement or intravenous glucose infusion was administered. Only patients with a carnitine deficiency were given supplements of 25–50 mg/kg/day, which were omitted during acute decompensations.

Growth

Height and weight were measured and compared to Swedish reference data (Wikland et al. 2002) and plotted as height SDS and BMI SDS. For children born preterm, height and weight SDS were corrected for gestational age up to age 2 years. Overweight was defined as iso-BMI $\geq$ 25 kg/m^2 and obesity as iso-BMI $\geq$ 30 kg/m^2 (Cole et al. 2000; Han et al. 2010; Lindgren et al. 1995). Parental heights were recorded and the target heights (TH) (Tanner et al. 1970) were calculated. In order to assess whether the children achieved the final height (FH) according to their genetic potential, TH SDS was compared to FH SDS. The age at menarche for the girls and their mothers was recorded. The onset of puberty for the boys was assessed by observing start and duration of the pubertal growth spurt in the charts. For patients who had reached FH, the height of the pubertal spurt was calculated and compared with the Tanner reference (28 $\pm$ 8 cm for the boys, 25 $\pm$ 8 cm for the girls) (Tanner et al. 1976). Birth weight SDS were obtained from the growth charts while the exact SDS of height, weight, BMI, and target height were calculated by the NordiNet® IOS pediatric database and the results used to generate the SDS growth curves.

Results

Clinical Follow-Up

After the confirmed diagnosis and dietary intervention was started, no patients died. Data of birth weight, pregnancy complications, symptoms at diagnosis, and number of episodes with increased carbohydrate intake are listed in Table 1.

Dietary Treatment

The food diaries showed that the reported fat intake adhered quite well to the recommended diet. Table 2 illustrates a characteristic day for a 5-year-old girl with LCHAD deficiency, following the Swedish recommendations. Regrettably we were unable to calculate the dietary intake for the patient group over time, since not enough data was obtained due to noncompliance and/or missing food diaries.

In order to compare the fat intake in grams to other recommendations emphasizing fat restriction as E%, the results from the food diaries of two patients of different age, gender, weight, physical activity levels, and body composition were converted to E%. Calculations were done over several years in the same patients and showed that the reported intake in grams for the two patients was equivalent to 13–24 E% of fat, distributed as 8–19 E% MCT and

Table 1 Birth data and clinical information. Four children are born preterm (33 %), all BW are corrected for gestational age, and two patients are SGA, one borderline SGA, and one has BW SDS −1.8. Preeclampsia is present in three pregnancies (30 %). *NS* No symptoms at diagnosis, *S* Clinical symptoms but no acute illness at diagnosis, *SS* Severe symptoms at diagnosis with elevated liver enzymes and cardiomyopathy and/or seizures. The number of episodes when the child had been treated with extra carbohydrate intake, either orally or as glucose infusions at home, were noted

Patient	Gestational age (weeks)	Birth weight (g)	Birth weight SDS (corrected for gestional age)	Caesarian section	Maternal hypertension	Preeclampsia	Neonatal hypoglycemia	Symptoms at diagonis	Number of episodes with extra carbohydrate intake	Night feeds
1	37	2,165	−2.3	Yes	Yes		Yes	SS	>10	Yes
2	38	3,070	−0.				Yes	SS	>10	Yes
3	38	2,865	−1.0	Yes	Yes	Yes		SS	>5	
4	35	2,016	−1.8		Yes	Yes	Yes	SS	<5	Yes
5	35	1,910	−2.1		Yes		Yes	S	>10	Yes
6	38	3,510	1.0					NS	>5	Yes
7	38	3,260	0.3				Yes	SS	<5	Yes
8	29	1,101	−1.5	Yes	Yes	Yes		S	<5	Yes
9	31	1,309	−2.0	Yes			Yes	S	>5	Yes
10	38	3,165	0.2				Yes	S	>10	Yes

Table 2 Dietary intake in grams from a standard day for a 5-year-old girl with an LCHAD deficiency

Time	Food	Amount (g)
8 am	Skim milk (<0.015 fat)	175
	Polenta	30
	Jam	20
	Liquigen	10
11 am	Potatoes	120
	Chicken filet	70
	Carrot	30
	Onion	10
	Bouillon (fat-free)	100
	Cornstarch	5
	Liquigen	10
2 pm	Fruit yogurt (0.05% fat)	200
	Corn flakes	15
	Liquigen	10
5 pm	Lentils	50
	Potatoes	50
	Tomatoes	60
	Onion	20
	Paprika	30
	Bouillon (fat-free)	100
	Skim milk (<0.01%)	175
	Liquigen	10
7 pm	Banana	110
9 pm to 6 am	Monogen (blended)	300
	Walnut oil	2.5
	Fish oil[a]	1.5

[a] Providing 330 mg DHA

4–5 E% LCT. As expected, the children with LCHAD deficiency had a lower total fat intake than recommended for growing children.

Growth

The growth trajectories illustrated that the majority of patients had an initial height and BMI acceleration after the diagnosis, followed by a stabilization or deceleration in height SDS after a couple of years (Figs. 1 and 2). Eight patients (7 girls, 1 boy; patients 1 and 4–10) developed overweight before 6 years of age. Six patients (5 girls, 1 boy; patients 1, 4–6, 8, and 10) had an iso- BMI of slightly more than 30 kg/m^2 for a 1–5-year period. Seven patients (5 girls, 2 boys; patients 1, 2, 5, 6, and 8–10) had an iso-BMI $\geq$ 25 kg/m^2, and one of these patients (patient 10) had an iso BMI $\geq$ 30 kg/m^2 at the latest assessment. The other three patients (2 girls, 1 boy; patients 3, 4, and 7) had normal weight for age at the most recent examination (Figure 2). No patients were underweight at any time, apart from patient 2 who showed a different growth pattern than the other patients (Fig. 2), with a fall in BMI below − 2 SDS at 3 years of age, after which the patient showed a markedly increased weight gain. He had poor appetite and was physically very active throughout his younger years, followed by recurrent episodes of muscular pain and elevated CK levels treated with increased energy intake. In addition, this patient had a combination of complex treatment resistant seizures. Following gastrostomy at 5 years of age and commencement with vigabatrin, his weight increased, a side effect known to be associated with vigabatrin therapy (Chadwick 1999). Patient 1 had a rapid weight gain for a short period (+ 4 SDS between 3 and 6 years) when she had recurrent infections.

Five patients had reached FH (patients 1–5). FH was within ± 1.0 SDS of TH in three patients (patients 2, 4, and 5) and slightly below target in two patients (patients 1 and 3). Patient 3 had critical hypoglycemia at the time of diagnosis and developed neurological sequele, contractures, and epilepsy with poor seizure control.

Four out of ten patients (1,4,5,9) (40 %) had a BW between −1.8 and −2.3 SDS when correcting for gestational age (Table 1). Three patients had had menarche (patients 1, 4, 5). One girl (patient 1) had an early puberty, treated with a GnRH agonist between 8 and 10 years of age. Her pubertal growth spurt was normal (24 cm) (Tanner et al. 1976) but she did not reach TH. Patient 4 had menarche at the same age as her mother (at 14 years) while patient 5 had menarche 2 years earlier than her mother (at 11 years). The onset of puberty for the boys was within the normal age. One out of five patients (patient 3) had a subnormal height gain during adolescence, compared to the Tanner reference (Tanner et al. 1976). Mean female FH was 164 cm, constituting – 0.6 SDS, and mean male FH 183 cm, equal to 0.4 SDS. Mean TH SDS for all patients was 0.6 SDS, indicating that this patient population was comparable to the normal population.

Discussion

In this Swedish cohort of ten children with LCHAD deficiency, growth trajectories showed increased growth during a period of months to years after the diagnosis, followed by a period of stable or decelerated growth. The majority of the patients developed overweight and, in addition, obesity during various periods. The growth trajectories were comparable to those of normal children with overweight or obesity (He and Karlberg 2001). Three out of five patients reached FH within their TH; consequently, it seems that neither the disorder itself nor the dietary treatment affected FH negatively.

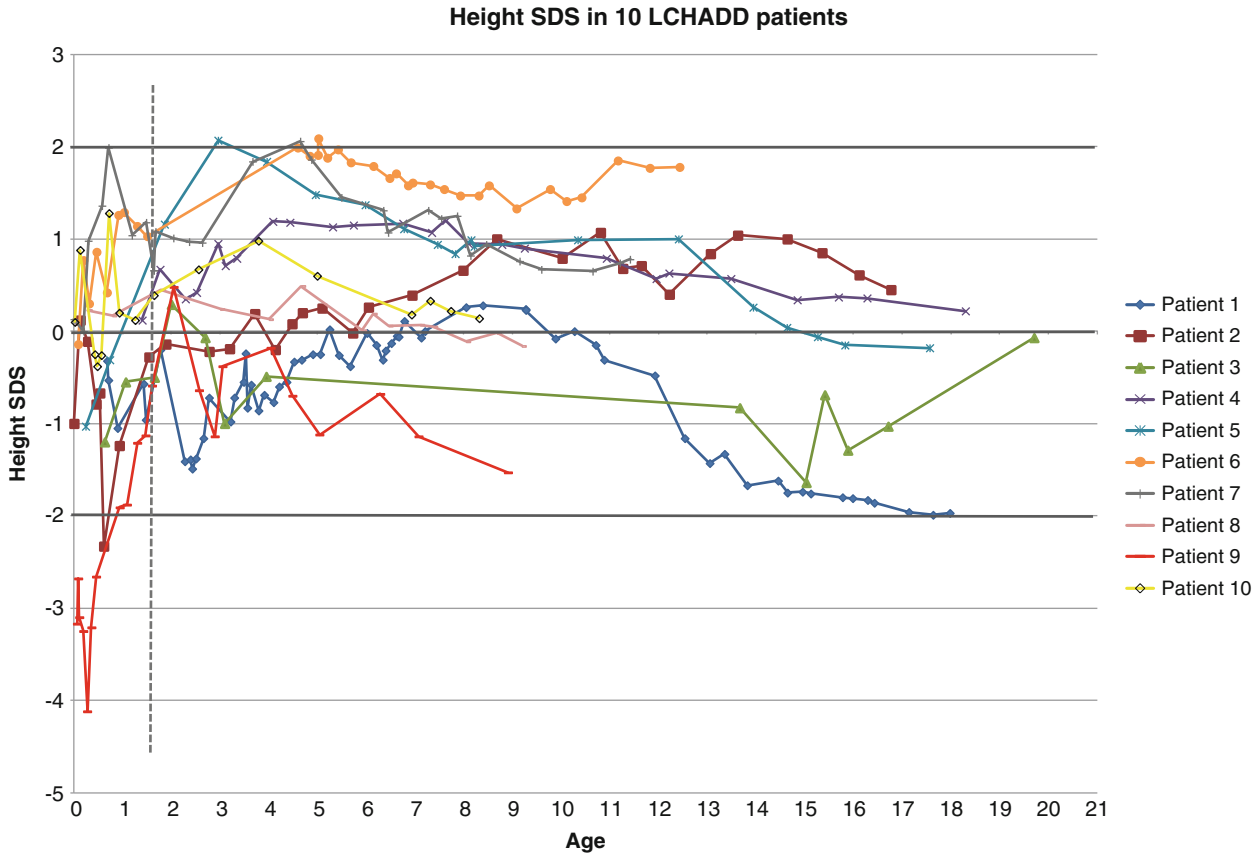

Fig. 1 Height SDS in 10 patients with an LCHAD deficiency. The dotted vertical line illustrates the time at which all patients were diagnosed and dietary treatment was started (13 months). The growth trajectories for height SDS show an initial accelerated growth in childhood after the time of diagnosis

The Swedish dietary instructions (Shaw and Lawson 2001) for LCT intake were given in grams, in an attempt to achieve metabolic control but avoid overfeeding and obesity. This differs from recent dietary guidelines (Spiekerkoetter et al. 2009b, 2010; Gillingham et al. 1999) that recommend indicating LCT intake as E%. Hence calculating grams of fat instead of recommending fat intake as E% may be an alternative approach to decrease LCT intake since E% will allow a larger amount of LCT the higher the total calorie intake is. In order to compare the intake of LCT fat in patients adhering to the Swedish recommendations with the generally more common instructions in E%, we recalculated the food diaries from two patients. These two examples illustrate that the reported LCT intake is somewhat lower than that recommended by Spiekerkoetter et al. (25–30 E% from fat with 20–25 % as MCT and 5–10 % as LCT) (Spiekerkoetter et al. 2009b) as well as Gillingham (10–20 E% as MCT and 10 E% from LCT) (Gillingham et al. 2003). In this retrospective study, there was no method to really know what the child actually ingested over time. The nutritional diaries give a subjective 3-day snapshot of the patients' life, often recorded during a period when the child was well (Lichtman et al. 1992). In addition, the available data was not sufficient to allow for more detailed calculations, but the data retrieved indicated good compliance with given dietary recommendations. Previous results indicate that patients with an LCHAD deficiency may have increased lipolysis as soon as after 3–4 hours of fasting (Halldin et al. 2007) underscoring the importance of minimizing the fasting periods and recommending night feeds. This differs from the recommendations by Spiekerkoetter et al., who suggest maximum fasting periods of 10–12 hours at night from 4 years of age (Spiekerkoetter et al. 2009b).

In our cohort, six out of ten patients (60 %) were overweight and one out of ten (10 %) obese at the most recent assessments. However, six patients had a transient period of obesity that regressed to overweight as they became older. In comparison, a Swedish study of healthy schoolchildren aged 8–9 showed that 11 % of the girls and 15 % of boys were overweight, and 3 % of the girls and 4 % of the boys were obese (Rasmussen et al. 2004). This indicates that overweight/obesity was overrepresented in our cohort. Analogous to our findings, Gillingham et al.

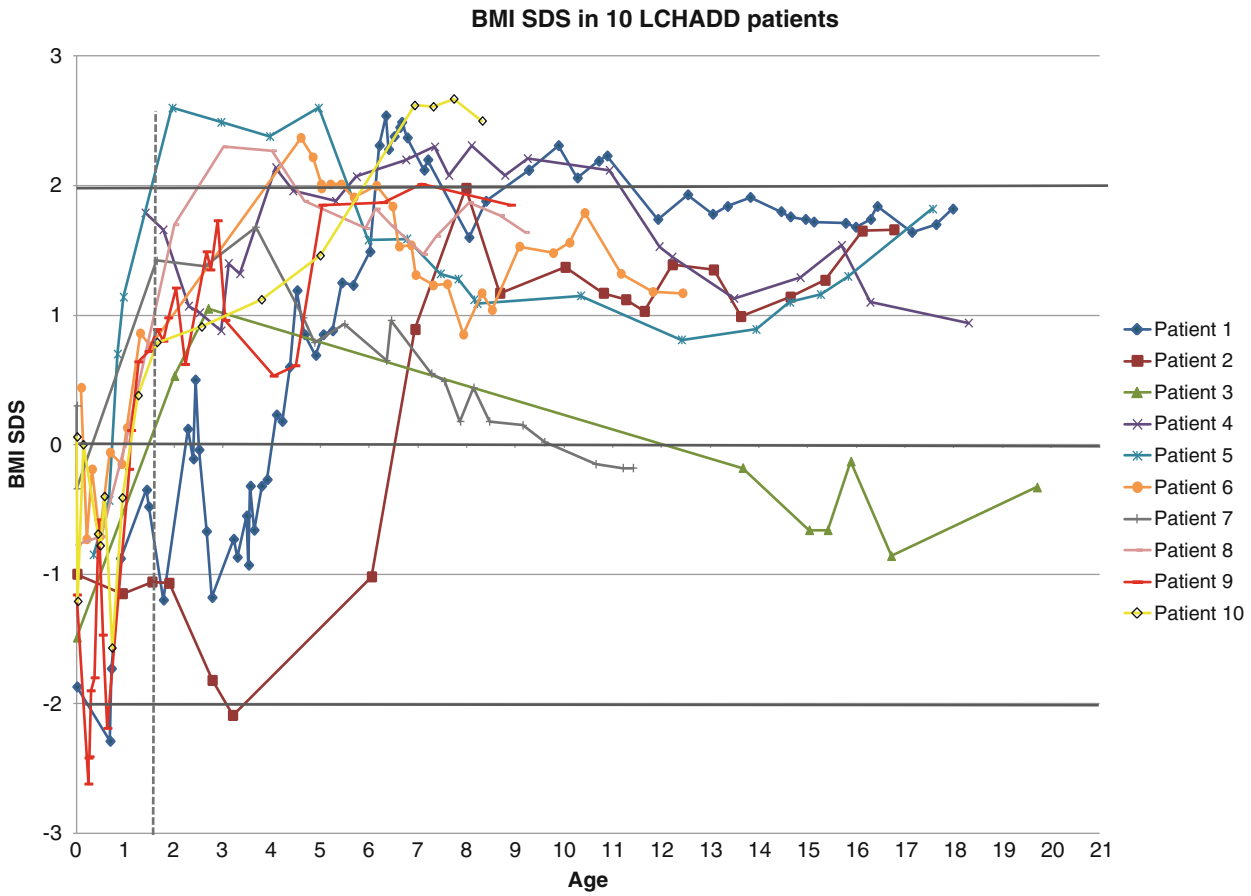

Fig. 2 BMI SDS in 10 patients with LCHAD deficiency. The dotted vertical line illustrates the time at which all patients were diagnosed and started on dietary treatment (13 months). Growth trajectories for BMI SDS show early BMI acceleration after the time of diagnosis and development of overweight/obesity in childhood. The majority of patients have a BMI SDS of over ± 1 at the latest assessments

show an obesity rate of 30 % in nine children with LCHAD deficiency (Gillingham et al. 2007), while Lund et al. do not report the weight development of their patients (Lund et al. 2010).

It is noteworthy that the severe lipid restriction results in a relatively high carbohydrate content which is interesting from an overweight/obesity perspective. There is emerging evidence that the rate of carbohydrate absorption after a meal, the glycaemic index, may affect weight gain (Brand-Miller et al. 2009). Foods with high glycaemic index that result in an initial period of high blood glucose and insulin levels, followed by a rapid fall in blood glucose is often associated with reduced satiety and excessive caloric intake (Ludwig 2002, 2007). On the other hand, low GI foods produce a more consistent blood glucose and insulin release and consequently increased satiety, but may in addition promote fat oxidation and decrease lipogenesis (Brand-Miller and Buyken 2012; Kong et al. 2011; Thomas et al. 2007). This subject warrants future studies since it is not obvious how the glycaemic index could be used to improve the situation for LCHAD patients.

Overweight/obesity in childhood is associated with accelerated linear growth, earlier pubertal maturity, and a subnormal height gain in adolescence, and in the end not affecting FH (Dunger et al. 2005; Garn and Haskell 1959; He and Karlberg 2001; Shalitin and Phillip 2003). Two out of three girls in our study had an early puberty, possibly caused by the increased weight gain in childhood. A rapid weight gain in infancy may also cause insulin resistance (Dunger et al. 2005). To the best of our knowledge, insulin sensitivity has not been studied in LCHAD-deficient patients, although one study suggests that children with LCHAD/MTP-deficiency and adiposity have normal insulin sensitivity based on normal insulin secretion (Gillingham et al. 2007). Three out of five patients reached FH within 1 SDS of TH and mean FH was – 0.6 SDS (girls)/0.4 SDS (boys), which indicated adequate growth in the majority of patients. Two patients did not reach TH, likely due to complicating factors other than the fatty acid disorder itself, as one patient developed early puberty and the other had contractures affecting the height measurements.

Earlier reports have demonstrated increased frequencies of IUGR of 47 % (Tyni et al. 1998a) and 43 % (Yang et al. 2002) in patients with LCHAD/MTP defects, not only explained by preeclampsia-related conditions. In this cohort, four out of ten patients (40 %) had some degree of IUGR. Preeclampsia developed in three out of ten pregnancies (30 %) (Table 1), which is consistent with several previous reports (den Boer et al. 2002; Tyni et al. 1998a; Yang et al. 2002). Birth weight is significantly associated with childhood growth (Li et al. 2004). However, this could not be studied in this small patient population.

The causes of obesity and overweight are certainly multifactorial, although the primary determinants of weight gain are excessive caloric intake or insufficient physical activity (Prentice and Jebb 1995) (Han et al. 2010). In LCHAD-deficient patients, there is an obvious risk for overfeeding prompted by the dietary treatment per se, the frequency of meals during the day, the continuous night feeds and short fasting periods as well as altered resting energy expenditure due to the inborn error of metabolism. Parents learning that their child has a disorder with risk for hypoglycemic episodes, treated with frequent feeds, may initially feel insecure and overcompensate resulting in increased weight gain following diagnosis. Children with LCHAD deficiency may be less physically active due to muscular weakness or pain and/or neurological deficits. The increase in BMI seen throughout early childhood may also reflect the increased caloric intake during infections. In the general population, children with parents that have an elevated BMI are at risk of developing obesity (Danielzik et al. 2002; Xu et al. 2011). The children with LCHAD deficiency differ in that they have a specific diet, different from the rest of the family. However, it is likely that hereditary and/or social factors such as attitude to food and physical activity may influence weight development in the patient when other family members have an increased BMI.

Childhood obesity is associated with a higher risk of morbidity in childhood and adolescence (Daniels 2009) with serious disability and premature death in adulthood (Bjorge et al. 2008; Reilly and Kelly 2010). Planned weight reduction is difficult in patients with an LCHAD deficiency, since this is associated with a risk of metabolic decompensation (Gillingham et al. 1999; Hagenfeldt et al. 1990; Halldin et al. 2007; Houten and Wanders 2010). Hence, it is challenging but crucial for the clinician to prevent the development of overweight and obesity. It is necessary to adjust the diet and energy intake and encourage physical activity to achieve normal weight without the development of complications. In conformity with the care of patients with other inborn errors of metabolism, it is essential to evaluate whether the therapeutic effects on the derangements of the disorder justify a strict dietary regimen or whether the patients would have a better quality of life with more liberal dietary recommendations.

Conclusions

Dietary treatment in LCHAD deficiency presents a challenge. The necessity of frequent feeds, high contents of carbohydrates, and periods of high energy intake to maintain good metabolic control increases the risk of overweight and obesity. In this cohort of LCHAD-deficient patients, an accelerated growth during the first years after the diagnosis and start of treatment followed by a period of stable or decelerated growth was seen. The patients developed overweight more often than in the general population, but few were obese despite overnight feeds. To improve metabolic control and weight development, we recommend frequent regular clinical follow-ups and extra vigilance concerning diet and weight. It is possible that giving recommendations for LCT fat intake in grams instead of E% may facilitate weight control and it is important to evaluate the appropriate length of fasting periods to avoid overcompensation.

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RESEARCH REPORT

Fabry Disease in Latin America: Data from the Fabry Registry

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Abstract The purpose of these analyses was to characterize demographic and baseline clinical characteristics of Latin American patients with Fabry disease compared to that of patients in the rest of the world. Observational data reported to the Fabry Registry were obtained from untreated patients or prior to treatment with enzyme replacement therapy. As of October 1, 2010, 3,752 patients were enrolled in the Fabry Registry worldwide, including 333 patients within Latin America. Latin American patients tended to be younger than Fabry Registry patients enrolled in the rest of the world: mean current age 35.5 years versus 39.2 years for men ($p < 0.05$ by t-test), mean age 37.8 years versus 43.6 years for women ($p < 0.05$ by t-test). A smaller percentage of Latin American patients have received enzyme replacement therapy, compared to patients in the rest of the world: 67% versus 80% for men, and 19% versus 39% of women, respectively. Thirty-one percent of men and 22% of women in Latin America reported experiencing a significant cardiovascular, renal, or cerebrovascular event, at a mean age of 35 ± 12.6 years in men and 44 ± 12.3 years in women. Cardiovascular events were the most common type of initial clinical event among men and women in Latin America. The medical community in Latin America should be aware of Fabry disease as a possible cause of renal or cardiac dysfunction. Increased awareness will facilitate prompt diagnosis and initiation of treatment.

Background

Fabry disease is a rare X-linked lysosomal storage disorder caused by decreased or absent activity of the enzyme α-galactosidase A (α-Gal A) (Brady et al. 1967). This condition results in the accumulation of glycolipids – mainly globotriaosylceramide (GL-3) in lysosomes from endothelial

cells, fibroblasts, pericytes from dermal tissue, heart, kidney, and the autonomic nervous system (Desnick et al. 2001; Kolodny and Pastores 2002). The global incidence of Fabry disease is has been estimated as 1:40,000 males (Desnick et al. 2001) and as 1:117,000 in the general population (Meikle et al. 1999). However, patients who have atypical or few symptoms may not be diagnosed with Fabry disease, which makes it difficult to determine a precise incidence or prevalance of the disease. . Furthermore, newborn screening studies have suggested that the prevalence of various types of mutations in the gene encoding α-Gal A may actually be much higher (for review, see [Germain 2010]).

Because Fabry disease is X-linked, men usually experience more severe manifestations than women (Desnick et al. 2001). However, a significant portion of heterozygous women also develop symptoms (Wilcox et al. 2008). The first symptoms of Fabry disease often appear during childhood, including acroparesthesias, abdominal pain, diarrhea, hypohidrosis or anhidrosis, corneal verticillata, and angiokeratoma (Desnick et al. 2001; Hopkin et al. 2008; Nguyen et al. 2005). The most serious complications of Fabry disease, including renal failure, cardiovascular events, and stroke usually manifest during adulthood (Desnick et al. 2001; Germain 2010; Wilcox et al. 2008). The increased availability of renal replacement therapy over the past several decades has extended the lifespan of Fabry patients (Branton et al. 2002; Mehta et al. 2009; Waldek et al. 2010). Cardiovascular disease is now the most commonly reported cause of death among both men and women with Fabry disease (Mehta et al. 2009; Waldek et al. 2010). The life expectancy (calculated from birth) for patients with Fabry disease is 58.2 years for men and 75.4 years for women, respectively (Waldeck et al. 2009).

The clinical diagnosis of Fabry disease can be difficult, because its signs and symptoms are often mistaken for other, more common diseases. In males, the demonstration of deficient enzymatic activity of α-galactosidase A in plasma or leukocytes can be used to confirm diagnosis (Brady et al. 1967). Enzyme activity can also be analyzed in dried blood spots on filter paper (Chamoles et al. 2001), which facilitates the screening of large populations. However, positive results obtained by this method should be corroborated by analysis of enzyme activity in plasma or leukocytes. Measurement of enzymatic activity is less useful in female heterozygotes, who often have α-galactosidase A activity levels within the normal range. Genotyping should be used for diagnostic confirmation in heterozygous females (Linthorst et al. 2008). Furthermore, it is important that physicians obtain a complete medical history and perform a detailed pedigree analysis as the adverse consequences associated with delayed diagnosis include not only medical morbidity, but also psychological damage, loss of educational opportunities, and loss of economic productivity (Gold et al. 2002; Cole et al. 2007).

Currently, the only specific treatment for Fabry disease is enzyme replacement therapy (ERT) with recombinant human α-Gal A (Eng et al. 2001; Schiffmann et al. 2001). Two forms of ERT are commercially available: agalsidase alfa (Shire Human Genetic Therapies, Inc. Cambridge, MA, USA) and agalsidase beta (Genzyme, a Sanofi company, Cambridge, MA, USA). The Fabry Registry is a global observational database that compiles clinical and laboratory data from patients with Fabry disease; it was established to further investigate the long-term effects of ERT and the natural progression of Fabry disease in a larger population. The purpose of these analyses was to compare key demographic and clinical characteristics of untreated Fabry Registry patients in Latin America (LATAM) to that of untreated patients in the rest of the world (ROW).

Methods

All patients with Fabry disease are eligible to enroll in the Fabry Registry, regardless of age, gender, symptoms, or whether or not they are receiving ERT from any commercial source. Patient and physician participation is voluntary and all patients sign informed consent forms that have been reviewed and approved by local Institutional Review Boards/Ethics Committees. The Fabry Registry provides participating physicians with a recommended schedule of key clinical and laboratory assessments (http://www.fabryregistry.com). However, treating physicians determine the actual frequency of assessments according to a patient's individualized needs.

Fabry Registry patients in LATAM included those in Argentina, Brazil, Chile, Colombia, Mexico, Peru, Uruguay, and Venezuela. Patients in the ROW included all Fabry Registry patients except those in LATAM.

These analyses include data reported to the Fabry Registry as of October 1, 2010. Clinical parameters included in the analysis were chronic kidney disease (CKD) stage, urine protein to creatinine ratio, and left ventricular posterior wall thickness. Data were collected from treatment-naïve patients or, if a patient was treated, baseline data were analyzed (i.e., prior to the initiation of ERT). Patients whose ERT status was not reported to the Fabry Registry (7 men and 1 woman in LATAM) were not included in the clinical event analyses. Clinical parameter data were ascertained prior to and up to 1 month after the first agalsidase beta infusion, among adults who ever received agalsidase beta as a source of ERT (≥18 years old at the time of first infusion).

Clinical events that occurred prior to the initiation of ERT or in untreated patients were included in these analyses. Cardiovascular clinical events were defined as myocardial infarction, cardiac syncope, congestive heart failure, angina pectoris, or significant cardiac procedures (e.g. pacemaker or other implantable cardiac device placement, bypass, stent

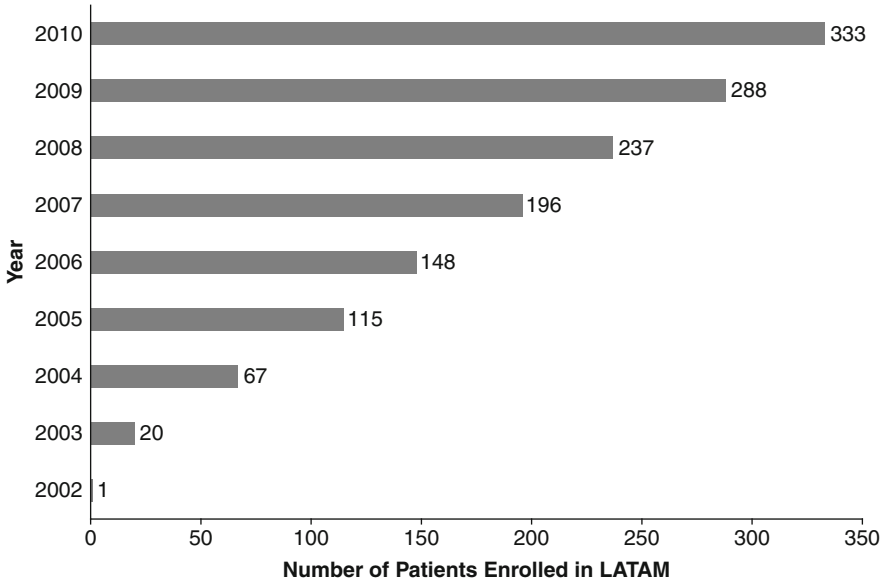

Fig. 1 Enrollment in the Fabry Registry within LATAM by year. Data shown represent cumulative enrollment of LATAM patients in the Fabry Registry as of December 31 of each year except 2010, which shows enrollment as of October 1, 2010

placement, valve replacement, transplantation), or arrhythmias. Arrhythmias may have been reported to the Fabry Registry as cardiovascular events, or detected by ECG in a follow-up visit or reported in a patient's medical history. Cerebrovascular events were defined as stroke. Renal events were defined as receiving chronic dialysis (40 days or longer) or kidney transplantation. Estimated glomerular filtration rate (eGFR) was calculated in mL/min/1.73 m^2 from serum creatinine values, using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Levey et al. 2009).

To better understand the population of patients from LATAM, demographic characteristics of the patients enrolled in LATAM were compared to that of the patients enrolled in the Fabry Registry in the ROW. Tests of the null hypothesis of equal distributions between the cohorts were conducted by the *t*-test. Statistical analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC). Demographics and clinical characteristics of the cohorts are presented using summary statistics. An alpha-level of 0.05 was used as the cut-point to determine statistical significance.

Results

Enrollment

As of October 1, 2010, 333 patients were enrolled in the Fabry Registry within LATAM and 3,419 patients were enrolled in the ROW. Enrollment in the Fabry Registry in LATAM has steadily increased since the first patient was enrolled in this region in 2002 (Fig. 1).

Demographics

Table 1 shows that patients in LATAM were significantly younger than patients enrolled in the ROW. As of October 1, 2010, the mean age of men in LATAM was 35.5 years versus 39.2 years for men in the ROW ($p = 0.0054$); the mean age of women in LATAM was 37.8 years versus 43.6 years for women in the ROW ($p < 0.0001$). Thirteen percent of Fabry Registry patients enrolled in LATAM were children (<18 years old as of October 1, 2010), compared to 11% of patients in the ROW. The mean age at the time Fabry disease was diagnosed was similar among patients in LATAM and patients in the ROW by gender, but men tended to be diagnosed at a younger age than women in both populations (Table 1).

ERT Treatment Status

A substantially smaller percentage of women in LATAM have ever received ERT, compared to LATAM men (19% versus 67%), as shown in Fig. 2. Among both genders, patients in LATAM were less likely to receive ERT than patients in the ROW. Seventy percent of LATAM men have received ERT, compared to 80% of men in the ROW ($p = 0.0008$); 19% of LATAM women have received ERT, compared to 39% of women in the ROW ($p < 0.0001$) (Fig. 2).

Table 1 Summary of demographic characteristics of Fabry registry patients in LATAM and the ROW

	LATAM		ROW	
	Males	Females	Males	Females
Patients in the Registry, *n*	166	167	1679	1740
Current Age (years), *n*	166	167	1678	1739
Mean ± SD	35.5 ± 13.71[a]	37.8 ± 17.81[b]	39.2 ± 16.81	43.6 ± 17.87
Median (min, max)	34.4 (5, 78)	36.2 (5, 85)	41.4 (1, 85)	44.5 (1, 90)
Age distribution, *n* (%)				
Pediatric (<18 years)	17 (10.2)	27 (16.2)	210 (12.5)	151 (8.7)
Adult (≥18 years)	149 (89.8)	140 (83.8)	1,468 (87.4)	1,588 (91.3)
Unknown	0	0	1 (0.1)	1 (0.1)
Age at diagnosis of FD (years), *n*	161	160	1,662	1676
Mean ± SD	28.6 ± 13.35	32.3 ± 17.15	27.3 ± 17.28	33.6 ± 18.16
Median (min, max)[c]	28.3 (2, 62)	30.7 (0, 77)	25.3 (0, 81)	32.8 (0, 82)

Data shown represent that reported to the Fabry Registry as of October 1, 2010.

[a] $p = 0.0054$ for men in LATAM versus men in the ROW, by *t*-test

[b] $p < 0.0001$ for women in LATAM versus women in the ROW, by *t*-test

[c] Patients with a prenatal age at diagnosis were considered to have an age at diagnosis of zero.

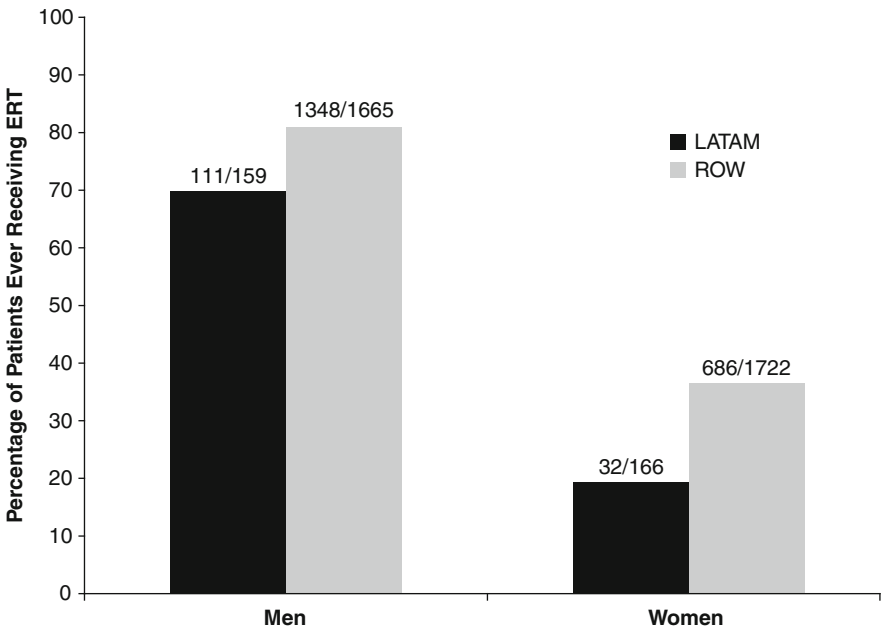

Fig. 2 ERT treatment status among men and women enrolled in the Fabry Registry in LATAM and in the ROW. Percentages were calculated based on the number of patients who reported ever receiving ERT from any source, excluding patients whose ERT status was not reported to the Fabry Registry. The numbers above each bar represent the number of patients who had reported receiving ERT/total number of patients in each category. For men, LATAM vs ROW $p = 0.0008$; for women, LATAM vs ROW $p < 0.0001$, by Chi-square test

Clinical Events

Clinical events were evaluated in the 325 patients from LATAM who had a known ERT status. Thirty-one percent of men (50 of 159) and 22% of women (37 of 166) in LATAM reported experiencing a significant cardiovascular, renal, or cerebrovascular event, as defined in Methods. The mean age at the time of the first clinical event was 35 ± 12.6 years in men and 44 ± 12.3 years in women. Cardiovascular events were the most common type of initial clinical event among men and women in LATAM.

Table 2 Summary of clinical events and age at first clinical event

		Males	Females
Number of patients		159	166
Patients reporting any type of clinical event	*n* (%)	50 (31.4)	37 (22.3)
Age at first event (years)	Mean (SD)	35.4 (12.58)	44.3 (12.31)
	Median (min, max)	36.3 (0, 66)	47.2 (13, 71)
Patients reporting cardiovascular event	*n* (%)	30 (18.9)	34 (20.5)
Age at first cardiovascular event (years)	Mean (SD)	37.8 (15.19)	45.5 (12.50)
	Median (min, max)	42.5 (0, 73)	47.6 (13, 71)
Patients reporting renal event	*n* (%)	24 (15.1)	4 (2.4)
Age at first renal event (years)	Mean (SD)	35.6 (10.93)	41.4 (6.84)
	Median (min, max)	35.9 (19, 66)	39.6 (36, 51)
Patients reporting stroke event	*n* (%)	8 (5.0)	3 (1.8)
Age at first stroke event (years)	Mean (SD)	41.2 (14.40)	40.1 (8.42)
	Median (min, max)	39.6 (24, 74)	42.3 (31, 47)

Data are from LATAM patients who had reported whether or not they had ever received ERT. All data were collected prior to initiation of ERT or are from patients who had not received ERT. Patients whose ERT status was unknown (7 men and 1 woman) were excluded from these analyses. Data shown represent that reported to the Fabry Registry as of October 1, 2010

Cardiovascular Manifestations

As shown in Table 2, 19% of men (30 of 159) and 21% of women (34 of 166) in LATAM reported a cardiovascular event, at a mean age of 38 and 46 years, respectively. The most common type of cardiovascular event was arrhythmia (any type), which was reported by 16% of men (26 of 159) and 19% of women (32 of 166). Arrhythmias included those reported to the Fabry Registry as cardiovascular events as well as those detected during ECG exams or reported in a patient's medical history. Less common cardiovascular events included significant cardiac procedures (8 of 159 men [5%] and 2 of 166 women [1%]), myocardial infarction (5 of 159 men [3%] and no women), angina pectoris (4 of 159 men [3%] and 3 of 166 women [2%]), and heart failure (1 of 159 men [0.6%] and 1 of 166 women [0.6%]). Echocardiographic exam data were reported for 163 patients in LATAM. Among these, 18% percent of men (29 of 72) and 15% of women (25 of 91) exhibited left ventricular hypertrophy (LVH).

Renal Manifestations

Fifteen percent of men in LATAM (24 of 159) had a renal event (kidney transplant or chronic dialysis), at a mean age of 36 ± 10.9 years (Table 2). Two percent of women in LATAM (4 of 166) had a renal event, at a mean age of 41 ± 6.8 years. Among these patients, 10 of the 24 men and 2 of the 4 women had kidney transplants.

Cerebrovascular Manifestations

Five percent of men (8 of 159) and 2% of women (3 of 166) in LATAM reported having a stroke, at a mean age of 41 ± 14.4 years and 40 ± 8.4 years, respectively. One percent of men (2 of 159) and 5% of women (8 of 166) reported a transient ischemic attack (TIA).

Pre-Treatment Clinical Characteristics

Among the adult Fabry Registry patients in LATAM who reported receiving agalsidase beta treatment, 115 had reported some type of baseline echocardiographic and/or renal data to the Fabry Registry. Prior to beginning agalsidase beta treatment, among patients with available data, 38% of men (15 of 40) and 35% of women (7 of 20) exhibited left ventricular hypertrophy (Table 3). This is lower than the percentage of adults in the rest of the world who had left ventricular hypertrophy prior to beginning agalsidase beta treatment (57% of men and 48% of women, Table 3). Baseline renal function data (CKD stage as determined by eGFR) were available for 84 adult patients who later received ERT with agalsidase beta (Table 3). Prior to beginning treatment, 19 of 62 adult men in LATAM (31%) were in CKD Stages 3–5 (eGFR <60 mL/min/1.73 m^2). Four of 22 adult Latin American females (18%) were in CKD Stages 3–5 prior to beginning treatment. These are similar to the percentages of men and women in the ROW

Table 3 Summary of pre-treatment renal and cardiac characteristics of adult Fabry registry patients in LATAM and the ROW

	Adults in Latin America (LATAM)		Adults inrest of world	
	Males	Females	Males	Females
Adult patients who ever received agalsidase beta, *N*	86	29	1001	511
Pre-treatment left posterior wall thickness (LPWT), *n* (%)	40	20	572	343
LPWT < 12 mm	25 (62.5)	13 (65.0)	248 (43.4)	178 (51.9)
LPWT ≥ 12 mm, indicating left ventricular hypertrophy (LVH)	15 (37.5)	7 (35.0)	324 (56.6)	165 (48.1)
Age at first report of LVH (years)				
Mean (SD)	34.5 (11.94)	44.8 (12.49)	39.4 (11.5)	46.5 (12.2)
Median (min, max)	32.7 (20, 69)	47.9 (21, 72)	39.2 (18, 81)	47.3 (18, 76)
Pre-Treatment CKD Stage, n (%)	62	22	840	440
Stage 1 (≥90 mL/min/1.73 m^2)	32 (51.6)	10 (45.5)	383 (45.6)	210 (47.7)
Stage 2 (≥60–< 90 mL/min/1.73 m^2)	11 (17.7)	8 (36.4)	187 (22.3)	153 (34.8)
Stage 3 (≥30–< 60 mL/min/1.73 m^2)	8 (12.9)	3 (13.6)	147 (17.5)	56 (12.7)
Stage 4 (≥15–< 30 mL/min/1.73 m^2)	4 (6.5)	1 (4.5)	55 (6.5)	11 (2.5)
Stage 5 (<15 mL/min/1.73 m^2)	7 (11.3)	0	68 (8.1)	10 (2.3)
Pre-treatment urine protein to creatinine ratio, *n* (%)	21	9	452	280
<0.3 g/g	6 (28.6)	5 (55.6)	157 (34.7)	120 (42.9)
≥0.3–< 1 g/g	6 (28.6)	2 (22.2)	113 (25.0)	63 (22.5)
≥1–< 3 g/g	4 (19.0)	0	129 (28.5)	62 (22.1)
≥3 g/g	5 (23.8)	2 (22.2)	53 (11.7)	35 (12.5)

Data are from adults who ever received agalsidase beta (≥18 years old at the time of the first infusion) and reflect data available as of 01 October 2010. Data obtained prior to and up to 1 month after the first agalsidase beta infusion were included in these calculations. eGFR (estimated glomerular filtration rate) was calculated in mL/min/1.73 m^2 from serum creatinine values, using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation

who were in CKD Stages 3–5 before beginning agalsidase beta treatment (Table 3). Relatively few patients in LATAM had pre-treatment urinary protein data reported to the Fabry Registry; 9 of 21 men and 2 of 9 women reported a urinary protein to creatinine ratio ≥ 1 g/g prior to beginning agalsidase beta treatment.

Discussion

As the largest registry that tracks clinical data for patients with Fabry disease, the Fabry Registry is a unique source of valuable information about this disorder. The objective of these analyses was to better characterize the growing population of Fabry Registry patients in LATAM.

Enrollment in LATAM has steadily increased over the past 8 years, with 333 patients enrolled in LATAM as of October 1, 2010. Compared to Fabry Registry patients in the ROW, a higher percentage of Fabry Registry patients in LATAM are children and the overall mean current age is younger in LATAM than in the ROW. It is possible that larger average family size in LATAM compared to the ROW could contribute to this difference, as diagnosis of one family member could lead to the screening of a higher number of younger family members. However, the younger current age in LATAM versus the ROW was not accompanied by a corresponding difference in age at diagnosis. It is not clear why a statistically significant difference was not observed in age at diagnosis, but there is a great deal of variation and a large standard deviation in the reported age at diagnosis. In any case, the high percentage of young patients enrolled in the Registry is very encouraging, as it suggests that physicians in LATAM are diagnosing Fabry disease in young patients. However, it is also important to regularly monitor these young patients; children in LATAM tend to have clinical data reported to the Fabry Registry less frequently than adults (Fabry Registry database).

As in the ROW, women in LATAM were less likely to receive ERT than men. Although some women have relatively mild symptoms of Fabry disease, others experience substantial manifestations of Fabry disease, including serious clinical events (Wilcox et al. 2008). Within LATAM, 37 of 166 women had a clinical event during the natural history period (i.e., prior to initiating any ERT). Overall, Fabry Registry patients in LATAM were less likely to have received ERT than patients in the ROW. This may be related to the fact that ERT became widely commercially available earlier in many countries in the ROW than in LATAM and may also be a reflection of differences in access to healthcare.

Overall, 31% of men and 22% of women in LATAM experienced a clinical event during the natural history period, which is similar to what has been reported for the global Fabry Registry population (31% of men and 20% of women) (Wilcox et al. 2008). The most common initial events reported by both genders were cardiovascular events, which is also similar to what has been reported for the overall Fabry Registry population (Wilcox et al. 2008). A retrospective chart review of 447 untreated patients also reported that cardiovascular events were the most common type of event in both genders (Schiffmann et al. 2009). The most common type of cardiovascular event reported was arrhythmia; overall, 18% of patients in LATAM reported some type of cardiac arrhythmia. This is consistent with other reports describing arrhythmias as the most common and earliest type of cardiac manifestation of Fabry disease (Linhart et al. 2007; Schiffmann et al. 2009; Wilcox et al. 2008). Among the LATAM patients who received agalsidase beta and for whom baseline echocardiographic data were reported, a smaller percentage displayed left ventricular hypertrophy, compared to patients in the ROW. This may be related to the fact that the patients in LATAM were younger than patients in the ROW.

Fifteen percent of men and 2% of women in LATAM required renal replacement therapy, through either dialysis or a kidney transplant, at a mean age of 36 years for men and 41 years for women. This is remarkably similar to what has been reported for the overall Fabry Registry population. Ortiz et al. reported that 14% of untreated men and 2% of untreated women in the global Fabry Registry required dialysis or a kidney transplant at a men age of 39 years (men) and 40 years (women) (Ortiz et al. 2010). Unfortunately, relatively few patients in LATAM reported detailed renal clinical data to the Fabry Registry, in terms of eGFR and/or urinary protein levels. The data that are available suggest that similar percentages of patients in LATAM and in the ROW exhibit CKD Stage 3 or higher prior to initiating ERT (approximately 30%).

A small percentage of patients in LATAM reported a stroke: 5% of men and 2% of women at a mean age of 41 years and 40 years, respectively. This is generally consistent or slightly lower than what has been reported for the overall Fabry Registry population; Sims et al. reported that 7% of untreated men and 4% of untreated women experienced a stroke (Sims et al. 2009). In the global Fabry Registry population, the age at first stroke was 40 for men and 46 for women (Sims et al. 2009).

Many of the manifestations of Fabry disease resemble other, more common disorders. Consequently, patients with Fabry disease are frequently not diagnosed until long after the onset of symptoms. In 2007, the average time from symptom onset to diagnosis among Fabry Registry patients was 14 years for men and 16 years for women (Wilcox et al. 2008). This is concerning, because Fabry disease is treatable and there is considerable evidence that ERT is most effective when it is initiated early, before the onset of substantial organ damage (Banikazemi et al. 2007; Germain et al. 2007; Warnock et al. 2011). Increased awareness of Fabry disease among the general medical community will facilitate prompt diagnosis and reduce delays in treatment, both in LATAM and in the ROW. In addition, physicians should be encouraged to obtain a thorough medical history for all patients and to screen potentially affected family members.

Various limitations are associated with analyzing observational data from a disease registry, particularly for rare diseases with small numbers of patients. For example, not all patients have reported all types of clinical data to the Fabry Registry. While a schedule of recommended clinical assessments is provided, treating physicians determine the actual type and frequency of assessments according to individual patients' needs. In addition, some patients report data to the Fabry Outcome Survey, a smaller registry that tracks patients with Fabry disease; the baseline clinical characteristics of patients enrolled in the two registries may differ.

In summary, enrollment in the Fabry Registry within LATAM has increased substantially over the past several years, which may indicate increased awareness of Fabry disease in the LATAM region and increased interest in contributing data to this important database. Over time, as the Fabry Registry continues to collect clinical data from patients in LATAM, we will gain a better understanding of the nature of Fabry disease and treatment outcomes in this patient population.

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Synopsis

The Fabry Registry has collected data from 333 Latin American patients with Fabry disease; demographic and clinical characteristics of these patients were compared to Fabry Registry patients in the rest of the world.

Author Contributions

Name	Design	Analysis and data interpretation	Write the article	Content Critical review	Final approval
J Villalobos	X	X	X	X	X
JM Politei				X	X
AM Martins				X	X
G Cabrera				X	X
H Amartino				X	X
R Lemay	X	X		X	X
S Ospina		X		X	X
S Suarez Ordoñez			X	X	X
C Varas				X	X

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Competing Interest Statement

The authors declare independence from the sponsors and declare that the content of the article has not been influenced by the sponsors.

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Ethics Approval

The Fabry Registry Program has the approval of ethics committees. A specific ethics approval for this paper was not required and these documents are available upon request.

Conflict of Interest Disclosures

JV, JMP, AMM, SO, GC, and CV are members of the Fabry Registry Board of Advisors. JV, JMP, AMM, and GC have received research funds or travel support from Genzyme as well as speaking fees from Genzyme. HA has received speaking fees from Genzyme. RL is a full-time employee of Genzyme and SSO works as a contractor for Genzyme.

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RESEARCH REPORT

Questioning the Pathogenic Role of the *GLA* p.Ala143Thr "Mutation" in Fabry Disease: Implications for Screening Studies and ERT

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Abstract Fabry disease is an X-linked inborn error of glycosphingolipid metabolism caused by quantitative or qualitative defects in the lysosomal enzyme alfa-Galactosidase A (aGAL A), ultimately resulting in vital organ dysfunction. Mainly the kidneys, the heart, and the central nervous system are involved. While the classical phenotype of Fabry disease is readily recognizable, screening studies have identified clinical variants. Here, we report the phenotype associated with the *GLA* p.Ala143Thr (c.427G>A) mutation in 12 patients aged 42–83 years. None of the patients had classical Fabry signs or symptoms as angiokeratoma, hypohidrosis, acroparesthesia, or cornea verticillata. Possible Fabry manifestations were renal failure (5/12), stroke (7/12), and left ventricular hypertrophy (5/12), but these were not necessarily attributable to the p.Ala143Thr mutation, as a cardiac biopsy in one female and left ventricular hypertrophy and kidney biopsies in two males with renal failure and microalbuminuria lacked Gb-3 deposits. The literature data on this mutation as well as data collected in the Fabry Outcome Survey (FOS) database confirm these findings. The association of renal failure, stroke, and left ventricular hypertrophy with this mutation could be the result of selection bias, as most patients were detected in screening studies.

We conclude that care should be taken with attribution of vital organ dysfunction to *GLA* sequence alterations. In case of the p.Ala143Thr mutation, and possibly also other mutations associated with an attenuated phenotype, diagnostic tools such as biopsy and imaging should critically evaluate the relation of end-organ failure with Fabry disease, as this has important consequences for enzyme replacement therapy.

Introduction

Fabry disease (FD, MIM ID #301500) is an X-linked inborn error of glycosphingolipid metabolism caused by quantitative or qualitative defects in the lysosomal enzyme alfa-Galactosidase A (aGAL A). As a result, glycosphingolipids, mainly globotriaosylceramide (Gb-3), accumulate in different cells throughout the body, ultimately resulting in organ failure (Kint 1970; Brady et al. 1967). Classical FD has been described as a multisystem disease predominantly presenting in males with angiokeratoma, hypohidrosis, and acroparesthesia in childhood, followed by renal failure, left ventricular hypertrophy, stroke, and premature death in the fourth and fifth decade of life. Besides these cases, attenuated forms have been described with a less severe phenotype and a later onset. In males, a specific mutation, associated with a significant residual enzyme activity, can result in a less severe phenotype that presents later in life (e.g., the "cardiac variants") (NAKAO et al. 1995; Scheidt von W et al. 1991). In females, residual enzyme activity can be the consequence of skewed X-chromosome inactivation. In the present paper, we present clinical and pathological data on a series of 12 patients with the p.Ala143Thr mutation and compare these data with literature data (9 patients) and data from the Fabry Outcome Survey (FOS) (20 patients).

Patients and Methods

We retrospectively reviewed the charts of patients with the p.Ala143Thr mutation diagnosed in our different screening studies and in the subsequent pedigree analyses (Terryn et al. 2008; De Schoenmakere et al. 2008). These studies were conducted according the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human and were approved by the Ethics Institution Review Boards of participating centers. All patients gave written informed consent.

Measurement of aGAL A activity was based on a technique involving a dried blood spot sampled on filter paper (DBS) as described by Chamoles et al. (2001). To validate this technique in our laboratory setting, we performed an analysis of 50 control samples (non-nephrology, non-ICU, non-hematology, non-pediatric). In case of low aGAL A activity, DBS was repeated in a new blood sample.

In a second part, previously published cases of the p.Ala143Thr mutation were identified through a PubMed search from 1966 to September 6, 2011, entering "p.Ala143Thr", "A143T," AND "Fabry Disease" as MESH terms.

A third part of this study consists of the analysis of FOS data. FOS – the Fabry Outcome Survey – is a European outcomes database for patients with Fabry disease who are receiving, or are candidates for, Enzyme Replacement Therapy (ERT) with agalsidase alfa. Data from all consenting patients are entered into the database following a structured clinical assessment by a physician or a specialized nurse. FOS has been approved by the Ethics Institution Review Boards of participating centers and all patients gave written informed consent. All measurements performed routinely in clinical practice are entered into the database. Anonymous data are submitted electronically by participating physicians to the central FOS database. We summarized baseline available data on all adult (18+) patients with the p.Ala143Thr mutation in this database.

Kidney Biopsies

If kidney biopsies were available, they were reviewed by a local pathologist and by a renal pathologist with expertise in FD (AF). Sections were stained with H & E, PAS, Jones, Congo Red, toluidine blue, and trichrome.

Results

In total, 41 patients with the *GLA* p.Ala143Thr mutation were identified.

Twelve patients (three males and nine females) were detected through our screening studies and subsequent pedigree analysis (Table 1). "Classical" symptoms of FD (angiokeratoma, acroparesthesia, cornea verticillata, and hypohydrosis) were absent. A significant number of patients had left ventricular hypertrophy ($N = 5$) or a history of stroke ($N = 7$), but this could be due to a selection bias, as most of these patients were detected as a result of screening studies in populations with left ventricular hypertrophy or stroke (Terryn et al. 2008, accepted in the International Journal of Cardiology). Of note, residual enzyme function could be demonstrated in all patients. Patient 1, 2, and 5 had a kidney biopsy showing no signs of Fabry nephropathy. Proteinuria and renal failure in patient 1 could be attributed to diabetic nephropathy. Patient 2 was detected as a result of pedigree analysis and despite his low aGAL A he had no signs of FD and was asymptomatic besides intermittent paresthesias in both arms, that were aspecific according to an expert neurologist.

One female patient with pronounced left ventricular hypertrophy (LVH) and heart failure (patient 7) had a myocardial biopsy showing AL amyloid and no typical Gb-3 deposition. In our screening studies in high-risk

Table 1 Patients with p.Ala143Thr: own database

Patient	1	2	3	4	5	6	7	8	9	10	11	12
Age/Gender	48/m	46/m	42/m	74/f	53/f	74/f	78/f	70/f	83/f	54/f	48/f	48/f
Origin of the patient	Index patient – diagnosis as result of screening in LVH	Pedigree of pt 6	Pedigree of pt 6	Pedigree of pt 1	Pedigree of pt 1	Index patient – diagnosis as result of screening in hemodialysis	Index patient – diagnosis as result of screening in LVH	Index patient – diagnosis as result of screening in LVH	Index patient – diagnosis as result of screening in hemodialysis	Index patient – diagnosis as result of screening in stroke	Index patient – diagnosis as result of screening in stroke	Index patient – diagnosis as result of screening in stroke
Angiokeratoma	Absent	Absent	Absent	NA	Absent	Absent	NA	NA	Absent	Absent	Absent	Absent
Acroparesthesia	Absent	Absent	Absent	NA	Absent	Absent	NA	NA	Absent	Absent	Absent	Absent
Hypohydrosis	Absent	Absent	Absent	NA	Absent	Absent	NA	NA	Absent	Absent	NA	Absent
LVH	Yes	Absent	Absent	Absent	Absent	Yes	Yes	Yes	Yes	Absent	Absent	Absent
Septum/posterior wall thickness (maximal, mm)	14	Normal	Normal	Normal	10.5	15	23	16	20	Normal	Normal	11
Renal involvement	Renal failure, proteinuria	Micro-albuminuria	Absent	ESRD	Absent	ESRD Neprotic range proteinuria	Renal failure/ proteinuria	Absent	eGFR 38 mL/min	Normal	Normal	eGFR 68 mL/min
CNS involvement	Stroke	Absent	Absent	Stroke	NA	Stroke	NA	Absent	Stroke	Carotis dissection	Stroke	Stroke
α-aGAL A activity in DBS[a]	Undetectable, second measurement 0.24, third 011	Undetectable (second measurement NA)	0.04 s measurement 0.14	1.21	0.42	0.19	NA	0.25	0.48	0.25	0.89	0.25
Kidney biopsy	Kimmelstiel Wilson/no Gb3	Normal/no Gb3	NA	NA	Normal / no Gb3	NA	Heart: AL amyloid	NA	NA	NA	NA	NA

NA Not available, *LVH* Left Ventricular hypertrophy

[a] Normal values: 0.64–3.86 μmol/L/h

Table 2 Screening in high-risk groups in Flanders

	Hemodialysis[a]	Kidney transplantation[b]	Left ventricular hypertrophy[c]	Total high-risk population
Female (N)	742	395	178	178
Mutation in females (N) (%)	2 (0.27%)	0	3 (1.7%)	5 (0.38%)
Male (N)	180	278	362	820
Mutation in females (N) (%)	1 (0.56%)	1 (0.36%)	2 (0.55%)	4 (0.49%)
Total patients (N)	**922**	**673**	**540**	**2135**
Mutations (N) (%)	3 (0.33%)	1 (0.15%)	5 (0.93%)	9 (0.42%)
GLA p.Ala143Thr (c. 427G>A)	2	1	4	7 (0.33%)
GLA p.Trp236Arg (c.706T>C)	1	0	0	1 (0.046%)
GLA p.Ala5Glu (c.44C>A)	0	0	1	1 (0.046%)

[a] Wim Terryn et al. (2008)

[b] De Schoenmakere et al. (2008)

[c] Terryn W et al. (2012) Prevalence of Fabry disease in a predominantly hypertensive population with left ventricular hypertrophy. Accepted (June 2012) by the "International Journal of Cardiology"

populations (Table 2), *GLA* mutations were detected in nine apparently unrelated patients, with seven having the *GLA* p.Ala143Thr mutation.

The cases with p.Ala143Thr from literature are summarized in Table 3. Only two patients were diagnosed as the result of symptoms and signs (patient 4 and 7). One patient (patient 7) had a single angiokeratome. A second patient (patient 4) had a cramp-fasciculation syndrome. The other seven patients were diagnosed as the consequence of screening studies and had no typical Fabry symptoms. One male (patient 4) had a kidney biopsy. Typical Fabry inclusions were only noted in a few collecting ducts and distal tubules but not in podocytes or in the endothelium. Another male (patient 9) had a nephrectomy after transplantation because of bilateral renal cell carcinoma. Histological examination of the nonmalignant renal tissue showed chronic glomerulonephritis, hyalinization, and severe arteriosclerosis, but no lesions typical for Fabry disease.

The FOS data are summarized in Table 4. Among 1933 registered Fabry patients, 20 adults (12 females and 8 males) from the United Kingdom, Germany, France and Belgium had the p.Ala143Thr mutation. The median baseline eGFR (MDRD) in female patients was 83 mL/min/1.73 m^2 at a mean age of 39. In males, this was 74 mL/min for a median age of 45.

Only limited data were available on the subsequent evolution of kidney function in these patients before ERT was started. In females, median delta eGFR (mL/min/1.73 m^2/year) was −3.3, which is comparable with literature data on Fabry nephropathy. In males, however, median delta eGFR (mL/min/year) was +1.35 mL/min/1.73 m^2/year, which is in contradiction with expected kidney function deterioration in Fabry males which is up to −12.2 mL/min/1.73 m^2/year (Branton et al. 2002).

Many patients (male and female) had micro-albuminuria. Only three had macro-albuminuria (> 300 mg/24 h). The cause of albuminuria was not clear, as only two patients had been biopsied. The male (patient 16) did not show Fabry nephropathy but lupus nephritis, and was successfully treated with immunosuppressive therapy. Stroke was mentioned in only one 80-year-old female (patient 8); at this age, stroke cannot simply be attributed to FD alone.

Discussion

The p.Ala143Thr mutation is a previously reported missense mutation: resulting from a G to A transition at nucleotide position 247 in exon 3, leading to an Alanine to Threonine substitution and has been reported as being pathogenic (Eng et al. 1997).

The p.Ala143Thr mutation was first reported in 1997 (Eng et al. 1997). The propositus was a 1-month-old male infant serendipitously found to have deficient aGAL A activity with no family history of FD. It was concluded in the same paper that the phenotype associated with this mutation was unknown. In 2002, this mutation was detected in patients as a result of screening in dialysis patients (Spada 2002 JIMD abstract). In a second abstract (Spada 2003), the same author considered this mutation to be related to late-onset end-stage renal disease. From that time, we read in literature this is a "known pathogenic mutation," but it was not supported with biopsy data as proof of its pathogenicity. The association of this mutation with renal failure, as in our screening studies (Table 2) in renal failure or left ventricular hypertrophy, might thus be the result of selection bias.

In vitro expression of this mutant allele in COS 7 cells has been studied (Spada et al. 2006). There is 36 % of

Table 3 Patients with p.Ala143Thr from literature

Patient	1	2	3	4	5	6	7	8	9
Origin of the patients (reference)	Screening in dialysis (Ref. 1)	Screening in stroke (Ref. 2)	Screening in left ventricular hypertrophy (Ref. 3)	Clinical diagnosis (Ref. 4)	Screening in stroke (Ref. 5)	Screening in stroke (Ref. 5)	Clinical diagnosis (Ref. 6)	Screening in left ventricular hypertrophy (Ref. 7)	Screening in kidney transplant recipients (Ref. 8)
Age/sex	84/f	NA/m	74/f	34/m	66/f	43/f	39/m	56/f	67/m
Angiokeratoma	NA	NA	NA	Absent	NA	NA	One lesion	Absent	Absent
Acroparesthesia	NA	NA	NA	Absent	NA	NA	Absent	Absent	Absent
Hypohydrosis	NA	NA	NA	Absent	NA	NA	Absent	Absent	Absent
LVH	Absent	NA	Yes	Absent	NA	NA	Absent	Yes	Yes
Septum/ posterior wall thickness (mm)	Normal	NA	21	Normal	NA	NA	Normal	Asymm, max 15 mm	
Renal involvement	ESRD	NA	Normal	ClCr 160 ml/min Prot 0.1 g/24 h	NA	NA	GFR 70 mL/min	ClCr 58 mL/min	ESRD
CNS involvement	NA	Cryptogenic stroke	NA	Normal	TIA/white matter lesions	Stroke Carotis dissection	Absent	NA	Stroke
α-aGAL A activity	NA	2.08 (nl: 15.6 ± 6.2 nmol/h/mL)	25% of normal mean	1.9 (nl: 21.6 +/ 6.4 U/L)	NA	NA	1.5 μmol/L (nl: 3-20)	35.1 (nl: 22–56 microcat/g protein)	"low"
Kidney biopsy	NA	NA	NA	Minimal deposits	NA	NA	NA	NA	Endstage kidney, bilateral RCC, no Gb3

NA Not available, *ESDR* Endstage renal disease

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Table 4 FOS data on adult patients with the p.Ala143Thr mutation

Patient; code FOS	Sex	Age at baseline	aGAL A (nmol/h/mL)[a]	eGFR before start of ERT (MDRD, mL/min/1.73 m^2)	Proteinuria before start of ERT (mean) (mg/24 h)	Follow up before ERT (months)	Delta eGFR (mL/min/1. 73 m2/year)	Kidney biopsy	Stroke	ERT
1	F	32	2.7[a]	107	165	30	−3,3	No	No	Yes
2	F	55	4.9[a]	98	230	5	+16.1	No	No	Yes
3	F	21	0,48[b]	108	61	51	−6.1	No	No	Yes
4	F	29	0,22[b]	83	102	10	+7.0	No	No	Yes
5	F	33	0,17[b]	71	107	11	−7.6	No	No	Yes
6	F	38	0,79[b]	82	110	22	−1.6	No	No	No
7	F	47	NA	71	1802	NA	NA	No	No	Yes
8	F	80	NA	27	NA	30	−5.6	Yes	Yes	No
9	F	51	NA	85	NA	NA	NA	No	No	No
10	F	64	0,69[b]	67	140	8	−11.5	No	No	Yes
11	F	24	NA	NA	NA	NA	NA	NA	NA	Yes
12	F	40	12[a]	109	NA	1	+69.5	No	No	No
Median	***F***	***39***		***83***			***−3.3***			
13	M	26	0.5[c]	177	NA	6	−2.0	No	No	Yes
14	M	62	0,14[a]	67	303	21	+5.6	No	No	Yes
15	M	52	NA	74	120	10	+4.7	No	No	Yes
16	M	40	0,25[b]	15	2755	2	+91.3	Yes	No	NA
17	M	68	0,15[b]	43	185	13	−7.2	No	No	Yes
18	M	44	22[d]	112	137	NA	−8,0	No	No	Yes
19	M	46	13[d]	130	128	NA	NA	No	No	Yes
20	M	45	2.4	NA	41[e]	NA	NA	No	No	Yes
Median	***M***	***45***		***74***			***+1.35***			

NA Not available, *MDRD* Modification of diet in renal disease, *ERT* Enzyme replacement therapy

[a] (nmol/h/mL) normal values 3.4–13

[b] (nU/mg) normal values 0.36–0.84 mU/mg

[c] Measured shortly at birth

[d] nmol/MU/mg protein normal >33

[e] mg/gr creatinine

expressed aGAL A wild-type activity which is in agreement with residual enzyme function in our patients. The finding of a low aGAL A activity however is not directly related to FD. We found that the p.Ala143Thr mutation indeed is associated with a low aGAL A activity, but its contribution to the phenotype of our patients (stroke, renal failure, left ventricular hypertrophy) is unclear. We performed three kidney biopsies, all lacking typical Gb-3 deposits which are universally present in Fabry patients (Noël et al. 2012). Moreover, among the remaining patients described in this study, we could not find one patient with this mutation and renal failure in whom significant renal Fabry disease was proven by kidney biopsy. In the sphingolipidosis, the ratio of substrate influx into the lysosome and the capacity of the degrading system determines the storage and as such the course and severity of the disease. This is treated in quantitative terms by the so-called threshold theory (Kolter 2011). Only the decrease of enzyme activity below the critical threshold value causes storage of the corresponding lipid substrate. Decrease of enzyme activity to the calculated threshold value does not influence the turnover rate of the substrate (as above this threshold, there is no (linear) relation between enzyme activity and turnover) and pathological storage occurs only below this level. With the exception of acid ceramidase, a decrease of enzyme activity to values of 20 % of normal cells, a typical range for heterozygote carriers of inherited diseases, has no impact on the turnover rate (Kolter 2011).

Our findings corroborate these findings, as we found no deposition of Gb-3 in the lysosomes of the cells of our patients with the p. Ala143Thr mutation. On the basis of the "threshold theory" and the in vitro studies of Spada et al. (2006),

this could be predicted, as the in vitro expression of aGAL A in this genotype was 36 % of the wild type expression, which is well over the 20 % mentioned by Kolter 2011.

In FOS, kidney function in patients with p.Ala143Thr remains well preserved in males until their 40s, which is in contradiction with studies on natural history (Branton et al. 2002). Unfortunately, we have no biopsy data in all of these patients, so we cannot ascertain or exclude renal FD in many patients.

Attenuated Fabry phenotypes lacking the classical FD symptoms have been described as a consequence of residual aGAL A activity. Some mutations result in residual aGAL A activity. This has been described to result in "cardiac variants" that present later in life, with predominantly cardiac manifestations (Scheidt von W et al. 1991). Most of the female patients in this study could be regarded as "variants"; they have significant residual enzyme function, no classical FD symptoms, and mostly cardiac and neurological symptoms.

On the other hand, as FD remains the subject of screening studies in high-risk populations including patients with renal failure and/or stroke, there is a danger of misdiagnosis as a result of selection bias, especially as the p.Ala143Thr mutation was not only detected in screening studies in Belgium (Terryn et al. 2008; De Schoenmakere et al. 2008; Brouns et al. 2010) but also in newborn screenings in Italy (Spada et al. 2006), Taiwan (Lin et al. 2009) Austria (Mechtler et al. 2012), and in other screening studies (Monserrat et al. 2007; Elliott et al. 2011).

The prevalence of the p.Ala143Thr mutation in our high-risk populations (0.33 %, Table 3) is almost 20 times higher than in a European newborn population (0.017 %, Mechtler et al. 2012). Low α-aGAL A activity could be one cofactor contributing to endothelial stress, provoking stroke, renal failure, or other signs, and symptoms classically associated with FD. The lack of Gb-3 deposits on electron microscopy does not preclude high intracellullar (lyso)-Gb3 levels that could be pathogenic and cause endothelial cell dysfunction (Namdar et al. 2012), though this should be confirmed with further studies.

Despite the coexistence of renal failure, proteinuria, and low aGAL A activity in patient 1 from our database (Table 1), the diagnosis of Fabry nephropathy was offset by the biopsy that showed a typical case of diabetic nephropathy. Proteinuria and renal failure in patient 16 in the FOS database (Table 4) was secondary to lupus nephritis. The cardiac biopsy in patient 7 from our own database (Table 1) with LVH and heart failure showed AL amyloid. These examples prove that before accepting the diagnosis of FD, confirmation of a mutation and diminished enzyme activity are needed, as well as comprehensive clinical and pathological workup of the patients, where biopsies of the involved organ, next to other diagnostics tools as MRI in left ventricular hypertrophy, should confirm the diagnosis.

Only two patients with the p.Ala143Thr in FOS were reported to have had a kidney biopsy, in spite of a larger number of patients with renal failure. Nevertheless, most of the included patients were treated with ERT. This expensive treatment is possibly not warranted in these patients.

In stroke, establishing a diagnosis of FD is even more difficult as is the case also in several of our own cases, as biopsy of the affected organ is impossible. Diagnosis is especially difficult when other typical features of FD are lacking, as is the case for the p.Ala143Thr mutation.

It has been proposed by expert panels to start treatment in all adult (>16 years) male Fabry patients, and in all patients, pediatric, male, or female, "as soon as clinical signs and symptoms are observed" (Eng et al. 2006). In our cases, however, we have no knowledge on the contribution of the enzymatic defect to the patients' morbidity and no reliable prognostic data are available on the evolution in case of an "atypical" variant like the p.Ala143Thr. It is even questionable if these patients have Fabry disease at all; it has been suggested to call such mutations, biochemically true positive but clinically false positive, as "fringe mutations" (Houge et al. 2011). Moreover, ERT has been studied in the classical phenotype, but there are no studies on the effects in the atypical variants.

The inclusion of patients with the p.Ala143Thr or other mutations associated with an attenuated phenotype (e.g., N215S or p.Asn215Ser, Branton et al. 2002) in studies on the effectiveness of ERT could confound results and should be studied separately. The place of ERT in patients with the p.Ala143Thr mutation is still unclear and should be the subject of close study. The currently available databases such as the industry-sponsored FOS cannot answer this question. An independent international database with mandatory data collection could provide quality data for further study.

Based on our data, we conclude that the expressivity of the p.Ala143Thr mutation is extremely variable. The presence of this mutation is not to be directly associated with pathology, and we have no compelling data that label this mutation as "pathogenic." At most, it is "possibly" pathogenic. As a consequence, biopsy and clinical data should be collected in order to be able to understand the natural evolution and to decide on the need for ERT.

Conflict of Interest

WT received grants for research from Genzyme Belgium and Shire Belgium. DH received a research grant from Genzyme Belgium. RVH received grants from Genzyme Belgium. BW received funding from Genzyme Belgium. The other authors declare no conflict of interest.

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RESEARCH REPORT

A Systematic Review of BH4 (Sapropterin) for the Adjuvant Treatment of Phenylketonuria

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Abstract *Context*: Dietary management is the mainstay of effective treatment in PKU, but dietary restriction is difficult and additional treatment options are needed.

Objective: To systematically review evidence regarding sapropterin (BH4) use as an adjunct to dietary restriction in individuals with PKU.

Data Sources: Five databases including MEDLINE up to August 2011.

Study Selection: Two reviewers independently assessed studies against predetermined inclusion/exclusion criteria.

Data Extraction: Two reviewers independently extracted data regarding participant and intervention characteristics and outcomes and assigned overall quality and strength of evidence ratings based on predetermined criteria.

Results: BH4 research includes two randomized controlled trials (RCTs) and three uncontrolled open-label trials. Phenylalanine (Phe) levels were reduced by at least 30 % in up to half of treated participants (32–50 %). In one RCT comparing placebo on likelihood of a 30 % reduction in Phe, 9 % of those on placebo achieved this effect, compared with 44 % of the treated group after 6 weeks. Phe tolerance and variability were improved in treated participants in studies assessing those outcomes. No comparative studies assessed long-term outcomes including cognitive effects, nutritional status, or quality of life.

Conclusions: Adjuvant pharmacologic therapy has the potential to support individuals in achieving optimal Phe levels. BH4 has been shown to reduce Phe levels in some individuals, with significantly greater reductions seen in treated versus placebo groups. The strength of the evidence is moderate for short-term effects on reducing Phe in a subset of initially BH4-responsive individuals, moderate for a lack of significant harms, low for longer-term effects on cognition, and insufficient for all other outcomes.

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Introduction

Approximately 1 in 13,500–19,000 infants in the United States is born with phenylketonuria (PKU) (Hegge et al. 2009; National Institutes of Health 2001). Individuals with PKU have defective phenylalanine hydroxylase activity, leading to a toxic accumulation of phenylalanine (Phe) in the blood and multiple tissues (Webster and Wildgoose 2010) and potentially to intellectual disability, delayed speech, seizures, and behavior abnormalities (Erbe and Levy 2002; Wilcox and Cederbaum 2002; Fisch and Stassart 2004).

The mainstay for treatment of PKU is a diet that restricts the intake of Phe to control the Phe concentration in the blood. In general, the usual treatment goal is a blood Phe level of 120 to 360 μmol/L (National Institutes of Health 2001; Poustie and Wildgoose 2010). In addition to the low-Phe diet, patients replace nutrients that are absent in their restricted diet with Phe-free medical foods (Giovannini et al. 2007). With adherence to a Phe-restricted diet, poor outcomes can be mitigated. While advances in dietary management are improving outcomes for individuals with PKU, management of PKU can be difficult and onerous, leading to interest in identifying new ways of managing this lifelong condition. (Demirkol et al. 2011; Harding and Blau 2010; Blau et al. 2010; Giovannini et al. 2012)

In 2007, the United States Food and Drug Administration (FDA) approved sapropterin dihydrochloride (Kuvan®, formerly known as Phenoptin), the first pharmacologic treatment for PKU, under the stipulation that additional studies be conducted on efficacy and long-term safety. The goal of treatment with sapropterin dihydrochloride (hereafter, BH4) is to control blood Phe concentrations. Although treatment with BH4 would potentially allow a relaxation of the low-Phe diet, it is not intended to serve as a complete substitute for dietary intervention (Somaraju and Merrin 2010).

The mechanism of action of BH4 is as a cofactor of the phenylalanine hydroxylase (PAH) enzyme, increasing the activity level of the enzyme and increasing the amount of Phe that can be converted to tyrosine. Hypothetically, it should be more effective in individuals with residual PAH activity than in individuals with negligible to no enzyme activity. Typically, a BH4 loading test is performed to identify potential candidates who are most likely to benefit from treatment. Loading tests used in practice and in research vary in terms of target reduction (e.g., 30 % reduction in blood Phe level) and timeframe, and none has been established as optimal for identifying those patients who respond to treatment.

As part of a systematic review of adjuvant therapies for individuals with PKU, we reviewed the literature on BH4 use in individuals with PKU. The full review is available at http://www.effectivehealthcare.ahrq.gov.

Methods

Data Sources and Search Strategy

We searched the MEDLINE® (via the PubMed interface), PsycINFO, EMBASE, Cumulative Index of Nursing and Allied Health Literature, and the National Agricultural Library (AGRICOLA) databases. Our search strategies used a combination of subject heading terms appropriate for each database and key words relevant to PKU (e.g., phenylketonuria, pharmaceutical preparations, phenylalanine). We limited searches to the English language but did not set a date limit. We also manually searched the reference lists of included studies and of recent narrative and systematic reviews and meta-analyses addressing PKU.

Study Selection

We developed study inclusion and exclusion criteria in consultation with an expert panel of clinicians and researchers involved in treating or studying PKU. We included all study designs excepting individual case reports and required that studies include at least 10 individuals with PKU receiving BH4 for therapy after having shown positive results on a loading test.

Data Extraction and Synthesis

Using standardized forms, two investigators independently extracted data regarding study design; study populations; intervention and comparison groups; and baseline and outcome data, as well as data about harms or adverse effects. Principal outcomes of interest included cognitive and nutritional outcomes, quality of life, and ability to liberalize the diet while maintaining acceptable Phe levels. We also extracted data on the intermediate outcomes of Phe level, Phe tolerance (total Phe intake by weight an individual can tolerate while maintaining acceptable blood Phe concentration), and blood Phe variability. We present a descriptive analysis of the data given the low number of studies identified and variability in outcomes assessed, which precluded meta-analysis.

Quality Assessment

Two investigators independently assessed each study using quality assessment questions as appropriate for each study design. We adapted questions from quality tools including the Cochrane Risk of Bias tool. We evaluated the overall strength of the evidence (degree of confidence that the observed effect of an intervention is unlikely to change) for the primary outcomes. We used the approach to determine strength of evidence as described in the Agency for Healthcare Research and Quality Effective Health Care Program's *Methods Guide for Effectiveness and Comparative Effectiveness Reviews* (Agency for Healthcare Research and Quality 2008). We assessed the strength of evidence for key outcomes identified as the most clinically important: cognitive outcomes including IQ and executive function, nutritional outcomes, quality of life, and liberalization of diet. Secondary outcomes included changes in blood Phe levels, Phe variability, and Phe tolerance.

Table 1 Domains used to assess strength of evidence

Risk of bias: Reflects issues in study design and conduct that could result in biased estimates of effect
Consistency: Reflects similarity of effect sizes seen across studies. Consistency cannot be assessed when only one study is available
Directness: Reflects the relationship between the intervention and the ultimate health outcome of interest
Precision: Reflects the level of certainty around the effect observed

Assessments were based on consideration of the domains of risk of bias, consistency in direction of the effect, directness in measuring intended outcomes, and precision of effect (Table 1). We determined the strength of evidence separately for major intervention-outcome pairs using an approach described in more detail in the full review.

Results

Overview of Study Characteristics

Ten studies evaluated the effects of BH4 in participants with PKU (Table 2, Fig. 1) (Burton et al. 2010, 2011; Levy et al. 2007; Lee et al. 2008; Trefz et al. 2009, 2010; Vernon et al. 2010; Burlina and Blau 2009; Lambruschini et al. 2005; Humphrey et al. 2011). Although study populations overlap, the studies were conducted as separate studies and thus are presented as such in our analysis. Four of the studies are linked by common participants: two are multisite placebo-controlled randomized trials (RCTs) that contributed to FDA approval of BH4 (Levy et al. 2007; Trefz et al. 2009). One additional uncontrolled open-label trial was conducted separately from the family of studies (Vernon et al. 2010) as were one prospective cohort study (Humphrey et al. 2011), two retrospective case series (Burlina and Blau 2009; Burton et al. 2010), and two prospective case series (Lambruschini et al. 2005; Trefz et al. 2010).

The studies included up to 80 participants in the treatment arm, and the total number of individuals in all studies was 284, after accounting for duplication in participants across studies. The studies were performed in the United States, Canada, Australia, and Europe. Participants ranged in age from 3 to 58 years in the five trials and from birth to 34 years in the observational studies. Most participants had demonstrated responsiveness to BH4 in a loading study before being assessed for effectiveness response; however, the approach to assessing responsiveness varied by study, and the base populations tested for initial responsiveness were not consistent (Table 3).

BH4 was studied in doses ranging from 5 to 20 mg/kg/day. The follow-up period for the two RCTs was 10 weeks, and some participants in multiple studies (including the extension studies) were exposed to the drug for up to 2.6 years. One case series followed participants up to 7 years (Lambruschini et al. 2005), with an average follow-up of 3.5 years. The mean treatment duration among participants in another case series (Trefz et al. 2010) was 4 years and 8 months (range = 24–110 months). The degree to which participants were adherent to a restricted diet varied. One RCT (Levy et al. 2007) and its follow-on trial (Lee et al. 2008) included participants with PKU who were at least 8 years old with a mean age of 20 years, consuming a relaxed or unrestricted diet, and had baseline blood Phe level of >450/umol/L. The second RCT (Trefz et al. 2009) examined the effect of 20 mg/kg/day for 10 weeks in children ages 4–12 who were on a Phe-restricted diet with baseline blood Phe levels <480 umol/L. One uncontrolled open-label study examined a differential effect in those who maintained a restricted diet versus those who did not (Vernon et al. 2010).

All randomized trials (Table 4) and three case series evaluated reduction in blood Phe levels. Two trials and three case series reported on Phe tolerance (Trefz et al. 2009; Vernon et al. 2010; Burlina and Blau 2009; Lambruschini et al. 2005; Trefz et al. 2010), and one cohort study (Humphrey et al. 2011) and one case series reported on Phe variability (Burton et al. 2010). Only one case series (Lambruschini et al. 2005) assessed longer-term outcomes, including cognition and nutritional status. No study evaluated quality of life. BioMarin, the pharmaceutical company that holds the patent for sapropterin dihydrochloride, sponsored five studies (Burton et al. 2011; Levy et al. 2007; Lee et al. 2008; Trefz et al. 2009; Burton et al. 2010), including both RCTs (Levy et al. 2007; Trefz et al. 2009).

Key Study Results

One good-quality RCT (Levy et al. 2007) randomized 89 participants with PKU to receive either 10 mg/kg of BH4 ($N = 42$) or placebo ($N = 47$) once daily for 6 weeks. The primary outcome was the change in blood Phe from baseline to week 6. Participants' mean age was 21.5 years in the treatment group and 19.5 years in the placebo group.

Table 2 Overview of studies addressing BH4

Author, year Design Quality	Dosage, mg/kg/day	N	Age, years Mean and/or range	Biochemical Characteristics (Mean baseline Phe level, μmol/L)	Outcomes
Trefz et al. 2009	20	46	4–12	Treatment: 314 ± 107	• Phe level
RCT				Placebo: 303 ± 74	• Phe tolerance
Quality: fair					
Levy et al. 2007	10	89	20.4	Treatment: 842.7 ± 299.6	• Phe level
RCT			(8–49)	Placebo: 888.3 ± 323.1	
Quality: good					
Burton et al. 2011	5–20	90	4–50	613.1 ± 328.5	• Phe level
Uncontrolled open label[a]					
Quality: fair					
Vernon et al. 2010	10, 20	36	3–58	Restricted diet: 587.0	• Phe level
Uncontrolled open label				Unrestricted diet:1372.6	• Phe tolerance
Quality: good					
Lee et al. 2008	5, 10, 20	80	20.4	844 ± 398	• Phe level
Uncontrolled open label[b]			(8–49)		
Quality: good					
Humphrey et al. 2011	NR	34	Newborn –	NR	• Tyrosine level
Prospective Cohort			Roughly 8		• Phe/tyrosine ratio
Quality: poor			years		• Variability of Phe and tyrosine levels
Trefz et al. 2010	5–26	16	10 days −34 years	321 + 236 (responders)	• Phe level • Phe tolerance
Case series					
Quality: poor					
Burton et al. 2010	20	37	1.5–32 years	400.2	• Phe level
Case series					• Phe variability
Quality: poor					
Burlina and Blau 2009	10	12	2–16	433–1215 (range)	• Phe tolerance
Case series					
Quality: poor					
Lambruschini et al. 2005	5	14	2.4 months–12 years	382 ± 229	• Phe level
					• Phe tolerance
Case series					• Liberalization of diet
Quality: poor					• IQ, DQ
					• Micronutrient/plasma levels
					• Urine biopterin
					• Nutritional status

DQ Developmental quotient, *IQ* Intelligence quotient, *N* Number, *Phe* Phenylalanine

[a] Includes participants from Lee et al. 2008, Levy et al. 2007, and Trefz et al. 2009.

[b] Open-label continuation of Levy 2007; therefore participants are not unique

Adherence to treatment during the 6-week trial was reported as high, with 82 % of participants taking all doses correctly.

After 6 weeks of treatment, participants in the BH4 group had a significant decrease in mean blood Phe levels of −235.9 ± 257 μmol/L from baseline (843 μmol/L) compared with a 2.9 ± 239.5 μmol/L increase in mean Phe levels from baseline (888 μmol/L) in the control group ($p < 0.0001$). The mean blood Phe decreased in the BH4 group at 1 week and remained at that lower level until the 6-week end point, when the mean Phe level was 607 μmol/L. The estimated difference between treatment and placebo groups in the mean change in blood Phe at 6 weeks

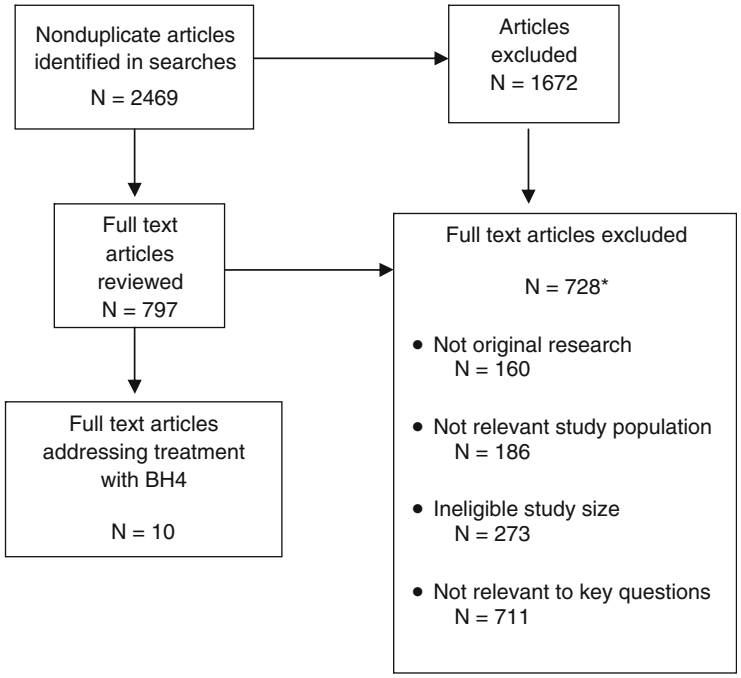

Fig. 1 Flow of studies identified for the review. *The total number of articles in the exclusion categories exceeds the number of articles excluded because most of the articles fit into multiple exclusion categories. *BH4* sapropterin, *N* number

Table 3 Variation in approach to assessing responsiveness to BH4

Study	Definition of BH4 Responsiveness	% Responders
Humphrey et al. 2011	Reduction in blood Phe of >30 % 15 h after BH4 loading at 20 mg/kg/day	NR[a]
Trefz et al. 2010	Reduction in blood Phe of >30 % after either a 20 mg/kg over 24 h loading test or 20 mg/kg/day over 8 days	94
Levy et al. 2007 Lee et al. 2008 Burton et al. 2011	Reduction in blood Phe of ≥30 % after 8 days of BH4 at 10 mg/kg/day	19.8[b]
Trefz et al. 2009	Reduction in blood Phe of ≥30 % after 8 days of BH4 at 20 mg/kg/day plus a blood Phe level ≤300 μmol/L on day 8	56
Vernon et al. 2010	Reduction of blood Phe level of at least 30 % or reduction to <360 μmol/L after day 7 on BH4 at 10/mg/kg/day or at 20 mg/kg/day for a total of 30 days	62 (classic PKU = 27, variant PKU = 100)
Lambruschini et al. 2005	Reduction in blood Phe of ≥30 % after 24 h of BH4 at 20 mg/kg/day	19.2
Burlina and Blau 2009	Reduction in blood Phe of >30 % after 24 h of BH4 at 20 mg/kg/day, and among those with Phe >450 μmol/L	76.63[c]
Burton et al. 2010	Reduction in blood Phe of ≥25 % after 2 weeks of BH4 at 20 mg/kg/day or among those with good control of Phe, an increase of Phe tolerance ≥200 mg/day by 4 weeks of Rx	NR

NR Not reported, *Phe* Phenylalanine, *PKU* Phenylketonuria

[a] Responsiveness described in Muntau et al. 2002

[b] Data on responsiveness for this study provided in Burton et al. 2007

[c] All participants had previously demonstrated responsiveness.

compared with baseline was −245 ($p < 0.0002$). A significantly higher proportion of participants receiving BH4 (44 %) had a 30 % or greater reduction in blood Phe levels compared with controls (9 %).

Almost all participants (16 of 17) for whom genotyping was performed had at least one mutation known to be associated with residual enzymatic activity. Responsiveness was not consistently linked to specific mutations. Despite

Table 4 Comparative studies and open-label trials of BH4 for the treatment of PKU

Author, year, Dosage Treated time Total *N*	Age, mean (years) ± SD	Key outcomes
Randomized Controlled Trials		
Trefz et al. 2009	G1: 7.7 ± 2.8	• Average blood Phe was lowered in the treatment group by 148.5 ± 134.2 μmol/L, compared to a decrease of 96.6 ± 243.6 μmol/L in the control group
20 mg/kg/day once daily compared to placebo	G2: 7.1 ± 2.0	• Blood Phe levels in the treated group were lower than in the placebo group by 135.2 μmol/L at week 3 ($p < 0.001$)
10 weeks N = 46		• Phe tolerance was increased to 20.9 ± 15.4 mg/kg/day (95 % CI: 15.4 to 26.4) in the treated group vs. 2.9 ± 4.0 mg/kg/day in the controls
Levy et al. 2007	G1: 21.5 ± 9.5	• Average blood Phe lowered in the treatment group by 235.9 ± 257 μmol/L vs. increase of 2.9 ± 239.5 μmol/L in controls ($p < 0.0001$)
10 mg/kg/day once daily compared to placebo	G2: 19.5 ± 9.8	• Estimated difference between groups in mean change in blood Phe was 245 ± 52.5, with a 95 % CI of −350 to − 141
6 weeks		• 44 % of the treated group had at least a 30 % Phe reduction Phe vs. 9 % of controls
N = 89		• 32 % of the treated group had at least a 50 % Phe reduction vs. 2 % of controls
Uncontrolled Open-Label Trials		
Burton et al. 2011(includes participants from Levy et al. 2007, Lee et al. 2008, Trefz et al. 2009)	G1: 16.4 ± 10.2	• Blood Phe concentrations were within target range for most subjects
		• In 50 % of participants with baseline blood Phe levels above treatment guidelines, levels were reduced to "within range" (not defined) during the study
5 – 20 mg/kg/day once daily 2.6 years		• Transitory low blood Phe levels (≤26 μmol/L) were observed in 4.5 % of subjects while 24 % had blood Phe levels ≤ 120 μmol/L that resolved without any intervention
N = 90		
Lee et al. 2008 (extension of Levy et al. 2007)	20.4 ± 9.6 (range 8–49)	• In Phase 1, all three doses (5, 10, 20 mg/kg/day) were associated with reduction in plasma Phe ($p \leq .01$)
Week 1–6 (Phase 1): forced dose-titration (5, 20, and 10 mg/kg/day for 2 weeks each)		• In Phase 2, 37 participants (46 %) showed a decrease in plasma Phe of at least 30 %, compared with week 0
		• In Phase 3, participants had a mean change in Phe from week 0 of −190.5 ± 355.7 μmol/L
Week 7–10 (Phase 2): 10 mg/kg/day Week 11–22 (Phase 3): 5, 10, or 20 mg/kg/day based on Phe concentration at week 2 and 6		• At week 22, at least 30 % reduction in Phe was seen by 46 % of participants overall (50% of those receiving 5 mg, 49% of those receiving 10 mg, and 42% of those receiving 20 mg saw at least 30% reduction in blood Phe)
22 weeks		
N = 80		
Vernon et al. 2010	23.4	• Nonresponders had a change in blood Phe level of 1422.3 to 1332.6 μmol/L
G1: Completed trial, 29	(range 3–58)	• Responders on a restricted diet had a reduction in blood Phe level from 484.9 to 226.1 μmol/L ($p < 0.001$)
G1a: Responders, 18 G1b: Nonresponders, 11		• Responders not on a restricted diet had a decrease in blood Phe level from 1049 to 553.7 μmol/L ($p = 0.035$)
Days 1–7: 10 mg/kg/day		• Nonresponders on a restricted diet had a change in Phe level from 1063.7 to 978.7 μmol/L
Days 8–37: 20 mg/kg/day for nonresponders		• Nonresponders not on a restricted diet had a mean change in blood Phe level from 1534.4 to 1465.4 μmol/L
37 days		• BH4 responders: 18 (62 %)

Table 4 (continued)

Author, year, Dosage Treated time Total *N*	Age, mean (years) ± SD	Key outcomes
N = 39		• Responders on a restricted diet achieved a Phe tolerance of 41 mg/kg/day compared to a starting tolerance of 21 mg/kg/day • Two individuals were able to liberalize from a restricted to an unrestricted diet
Prospective Cohort Studies		
Humphrey et al. 2011 G1: Responders G2: Nonresponders	Newborn to < 10 years	• Variation in Blood Phe greater in individuals nonresponsive to BH4 (responsive to BH4: median 338 μmol/L, 95 % CI: 329–346, mean: 358 μmol/L, 95 % CI 350–366; nonresponsive to BH4: median 338 μmol/L, 95 % CI 332–344, mean: 370 μmol/L, 95%CI 364–376)
Dosage NR		• Phe < 400 μmol/L: Responsive to BH4: 66.7 %, nonresponsive to BH4: 62 %
8 years		• Phe > 600 μmol/L: Responsive to BH4: 7.5 %, nonresponsive to BH4: 12.7 %
N = 34		• At Phe >600 μmol/L, median and mean tyrosine levels were higher among BH4-responsive individuals than those not responsive to BH4 • Variation in Phe/Tyr ratio greater in individuals nonresponsive to BH4 (mean = 6.12, 95%CI 5.9–6.3) vs. mean = 5.44 (95%CI: 5.3–5.6) in individuals responsive to BH4, particularly at Phe > 600 μmol/L

CI Confidence interval, *G* Group, *Phe* Phenylalanine, *PKU* Phenylketonuria, *Tyr* tyrosine

enrolling only those participants who had at least a 30 % reduction in blood Phe while taking BH4 in a 1-week loading test, not all participants were responsive to BH4 in the trial.

A 22-week uncontrolled, open-label trial (Lee et al. 2008) of good quality followed this RCT (Levy et al. 2007) and included three phases: dose titration, dose analysis, and a fixed dose phase. All participants enrolled in the previous RCT were eligible if they had taken at least 80 % of their scheduled dose in the trial and were willing to continue their current diet. The primary end point was mean plasma Phe levels at week 22 and mean changes from week 0. Of 87 participants who completed the previous RCT (Levy et al. 2007), 80 were enrolled in the extension trial (Lee et al. 2008), of whom 39 had previously received BH4 and 41 placebo. Participants' mean age was 20.4 years. High doses of BH4 (10 or 20 mg/kg/day were more effective at lowering Phe levels in the dose titration phase. By the end of the dose analysis phase with 10 weeks at 10 mg/kg/day, 46 % of participants had a decrease in plasma Phe of at least 30 % compared with week 0. During the fixed dose phase, most participants (92 %) received either 10 (46 %) or 20 mg/kg/day (46 %). By week 22, plasma Phe was reduced by 190.5 μmol/L compared to week 0. Mean plasma Phe decreased from 844 μmol/L at baseline to 645 μmol/L at week 10 and was maintained at a mean of 652 μmol/L at week 22. At week 22, 46 % of participants had achieved a 30 % reduction in plasma Phe concentration compared with week 0. The corresponding reductions for those receiving 5, 10, and 20 mg/kg/day were 50 %, 49 %, and 42 %, respectively.

Another RCT (Trefz et al. 2009) of fair quality enrolled children with PKU between 4 and 12 years of age who were on a Phe-restricted diet, had maintained blood Phe control (blood Phe level ≤480 μmol/L), and had an estimated Phe tolerance of ≤1,000 mg/day. The objective was to determine the safety and efficacy of BH4 at 20 mg/kg/day for 10 weeks in increasing Phe tolerance (defined as cumulative increase or decrease in Phe supplement at the last visit for which blood Phe level was ≤360 μmol/L) while maintaining blood Phe control. Participants maintained a stable, Phe-restricted diet, monitored by food diaries. Starting at the third week, a dietary Phe supplement was added or removed every 2 weeks based on Phe levels. Children with a blood Phe level of ≥1200 μmol/L in two consecutive weeks were withdrawn from study drug treatment and received dietary counseling.

Thirty-three children were randomized to 20 mg/kg/day of BH4 for 10 weeks, and 12 children received a placebo. After 10 weeks of treatment, the total mean ± SD of

Phe supplement tolerated by participants on BH4 increased significantly from 0 mg/kg/day at baseline to 20.9 ± 15.4 mg/kg/day. In contrast, the placebo group tolerated an increase of 2.9 mg/kg/day of Phe supplement. The adjusted mean difference between the groups in Phe tolerance was 17.7 ± 4.5 mg/kg/day ($p < 0.001$). Total Phe intake (dietary Phe intake plus total Phe supplement) also increased significantly from baseline in the BH4 group, approximately doubling to 43.8 mg/kg/day at 10 weeks compared to a slight increase in total Phe intake in the placebo group (16.3 mg/kg/day at baseline to 23.5 ± 12.6 at 10 weeks). Mean blood Phe levels decreased significantly in the BH4 group between baseline and the beginning of supplementation in week 3 (decrease of 148.5 ± 134.2 μmol/L). Some participants in the BH4 group had transient low blood Phe levels (<26 μmol/L) corrected with increased Phe supplementation.

An uncontrolled, open-label trial (Vernon et al. 2010) of good quality included participants with classic or variant PKU with any Phe level or diet. Eligible subjects received 7 days of open-label BH4 at 10 mg/kg/day with plasma Phe measurement on day 8 and weekly during a dietary modification period with dosage increased to 20 mg/kg/day until day 30 for nonresponders. Responders who were on a Phe-restricted diet underwent gradual liberalization of their diet to the maximum tolerated natural protein intake while still maintaining plasma levels in the range of 120 to 360 μmol/L.

Of the 29 individuals completing the study, 59 % were on some form of protein-restricted diet and had a mean baseline blood Phe of 587 μmol/L. Forty-one percent were not following protein-restricted diets and had a mean baseline blood Phe level of 1,372 μmol/L. Overall, 62 % were determined to be responders, with variable doses required for response. Four (27 %) of the classic PKU participants (defined as off-diet plasma Phe of >1,200 μmol/L) were responders, and 100 % of the variant PKU participants (>400 and <1,200 μmol/L) were responders. Of the 12 participants who were not on a Phe-restricted diet, 33 % were responders with a significantly decreased mean blood Phe level (554 μmol/L) compared with baseline (1049 μmol/L). Of the 17 participants who were on a Phe-restricted diet, 82 % were responders with significantly reduced mean blood Phe level of 226 μmol/L compared with baseline (485 μmol/L). Among individuals who were responders and on a Phe-restricted diet, the average Phe tolerance increased from 21 to 41 mg/kg/day. However, responders' Phe tolerance varied widely from an increase of 20 to 22 mg/kg/day to a non-protein-restricted diet in two participants.

One poor-quality prospective cohort study (Humphrey et al. 2011) assessed variability in blood Phe. Participants included nine children who were responsive to a 20 mg/kg BH4 loading test and 25 who were nonresponsive. Among those who were BH4 responsive, two were treated with BH4 alone and the rest also needed dietary modifications. Overall, there were no significant differences in mean and median blood Phe levels between the groups; however, above blood Phe levels of 600 μmol/L, confidence intervals around the mean were wider among BH4 nonresponsive participants. The authors equate these differences with variability in response.

Four poor-quality case series (Burlina and Blau 2009; Lambruschini et al. 2005; Burton et al. 2010; Trefz et al. 2010) evaluated dosages of BH4 ranging from 5 to 26 mg/kg/day for durations of 6 months to up to 9 years among BH4-responsive participants. All reported positive outcomes in terms of reduction in blood Phe and increased Phe tolerance. One case series (Lambruschini et al. 2005) also examined longer-term functional outcomes, including IQ and developmental quotient after 1 year of treatment, reporting no adverse effects as participants' Phe tolerance increased and the diet was liberalized. Nutritional status was unchanged with the exception of increases in selenium. In another case series (Burlina and Blau 2009), 12 participants were studied for up to 7 years on a dosage of 10 mg/kg twice a day. In this group, ranging in age from 2 to 16 years old, all participants eventually stopped Phe supplementation and relaxed dietary restrictions.

Another longer-term case series (Trefz et al. 2010) assessed Phe levels and increase in Phe tolerance in 16 individuals receiving BH4 for between 24 months to 9 years (mean = 56 months). The mean blood Phe level in responders ($n = 14$) was 321 ± 236 μmol/L, and the mean decrease in blood Phe was 54.6 % (range 28.4–85.6 %). Seven patients had stable Phe control without any dietary restriction. Of the remaining seven patients who were on dietary restrictions, six increased their Phe intake from a baseline of 200–300 mg/day to 800–1,000 mg/day. Psychomotor development among children 5–6 years of age was reported to be within normal range; however, results were not presented. One case series (Burton et al. 2010) provided data on Phe variability by measuring blood Phe at least six times before and after treatment initiation. Individual variability in Phe levels was lessened after treatment.

Harms Reported in Studies of BH4

Of the ten studies examining the effectiveness of BH4, four studies with overlapping participants (Burton et al. 2011; Levy et al. 2007; Lee et al. 2008; Trefz et al. 2009) reported any type of harm related to the intervention drug. Three studies (Burlina and Blau 2009; Lambruschini et al. 2005; Trefz et al. 2010) reported that no adverse events were observed during intervention, one study reported that BH4

was well tolerated with mild diarrhea occurring rarely (Humphrey et al. 2011), and there was no mention of harms in two studies (Vernon et al. 2010; Burton et al. 2010). The most common side effects reported during BH4 trials were headache, throat pain, upper respiratory infection, diarrhea, abdominal pain, and nausea and vomiting. Harms probably or possibly related to study treatment occurred at similar rates in both BH4 and placebo (23 vs. 20 % (Levy et al. 2007), 27 vs. 25 % (Trefz et al. 2009)) arms in RCTs.

Strength of Evidence

We rated the strength of the evidence (confidence that the current effect estimate will not change with future research) for the effects of BH4 on reducing blood Phe levels to clinically acceptable levels among BH4 responders in the short term (12 weeks or less) as moderate based on few studies. The strength of evidence for the indirect relationship of BH4 on IQ is low, based on current evidence and lack of direct measurement. Harms were noted to be rare and mild, and the strength of evidence for this observation is moderate. The strength of the evidence is insufficient for the direct effect of BH4 on improving all other outcomes (Phe tolerance and the ability to liberalize the diet, Phe variability, quality of life, and cognitive and nutritional outcomes).

Discussion

The treatment for PKU with dietary restriction of Phe in natural protein and use of Phe-free medical foods has been critical in reducing the incidence of irreversible neurocognitive impairment in individuals with PKU, and together with newborn screening has been a tremendous success story. However, families and patients report that, especially with the onset of adolescence and adulthood, dietary adherence and supplement use can be challenging. Little is known about rates of adherence to diet, and clinicians, patients, and families have lacked therapeutic options other than a lifetime of strict dietary management. Importantly, the ability to liberalize the diet has the potential to affect the quality of life of individuals with PKU who must be constantly vigilant about what they consume. Ideally, a therapeutic adjunct to dietary management would increase Phe tolerance, allowing for increased intake of dietary protein and micronutrients, and reducing (but likely not eliminating) the necessity for Phe-free medical foods.

All studies of BH4 evaluated intermediate outcomes (change in blood Phe levels and Phe tolerance). Almost no information is yet available, and none from RCTs studying longer-term outcomes, including cognitive impairment, quality of life, nutritional impact and status, and the ability to liberalize diet. In enriched populations (all participants had reduced Phe in initial loading tests) with varying levels of dietary and Phe control, up to half of the participants had Phe reductions of at least 30 % and the relationship of reductions in Phe to clinical outcomes was not assessed.

Further, a high proportion of treated participants who achieved a reduction in blood Phe of the study target of 30 % continued to have Phe levels above the clinical target. Nonetheless, an open-label extension trial demonstrated that reductions in Phe observed early in treatment could be maintained up to 22 weeks (Lee et al. 2008). On the other hand, the clinical goal for individuals maintaining dietary control could be to improve their quality of life by liberalizing their diet. In the trial targeting children with Phe <480 who were successfully maintaining a restricted diet, Phe tolerance was increased (Trefz et al. 2009). Even so, the impact on Phe tolerance was not uniform across the study population, and although many of the participants could modestly increase their protein intake, none could be on an unrestricted diet. Of note, a number of conditions may affect Phe tolerance, including illness, type of mutation, and degree of BH4 response among others; these are not assessed in the studies.

In all of the studies, compliance with BH4 was reported to be good over the short term. However, long-term sustainability of compliance with both BH4 and dietary therapy, especially given the variability in response, has not been evaluated, nor has durability of treatment effects. Authors from the uncontrolled open-label trial note that one responder reportedly discontinued BH4 after the trial as the small increases in Phe intake that BH4 allowed were not significant enough to warrant taking the medication. Certainly, observed increases in Phe tolerance were moderate at best in classic PKU in terms of allowing changes in diet, and the decision about trade-offs between reliance on medication and carefully titrating liberalization of the diet will need to be made by patients and their clinicians on an individual basis that balances available evidence with the individual's context.

Even though studies reporting harms consistently indicate that BH4 is well tolerated and without serious side effects, not all studies assessed and reported harms, and data are based on a small number of individuals, so ongoing registries will be important for supplementing these data.

Future Directions

Research on the use of BH4 as an adjuvant therapy in PKU management is relatively new and consists of small, tightly controlled multisite efficacy studies. The greatest research need in this area is thus for larger studies that include adequate numbers of participants. Given the known difficulty of accruing large numbers of participants, however, researchers should also use existing data sets and a consortium and multisite approach to gathering data.

Ideally, studies will be conducted in both tightly controlled and nonadherent populations, and among different age groups, with appropriate design and power for subgroup analyses. Research should continue to include RCTs, but prospective cohort studies that may have the potential to provide additional effectiveness data – including outside of a controlled clinical setting – adherence, and longer-term evidence would also be helpful to support understanding of the role of BH4 in clinical care. These studies should provide substantially more details on the range of benefits and harms associated with treatment.

Data are not currently available to understand potential modifiers of treatment effectiveness in order to select the best populations for targeting further research and treatment. Moreover, the significant variability in responsiveness to BH4 is unexplained, and subpopulations that have a unique response to this medication have not been well characterized. Causes of variability may be multifactorial and likely include individual patient and genotype differences, drug dose, and individual patient behavior such as dietary adherence. It is unclear, in particular, why a high proportion of individuals who have an initial response in loading studies do not have a durable response even over a few weeks in efficacy trials, even while those who do have a response demonstrate a significant effect. The degree to which this observed variation may be associated with suboptimal adherence should be assessed both in clinical trials and other types of studies.

Another area of potential research that could be explored in combination with studies of BH4 is the use of adherence supports for both drug and diet to optimize potentially positive outcomes. Long-term efficacy outcomes beyond 22 weeks and safety outcomes beyond 3 years are currently unavailable, as are measures of behavioral change and cognition and patient-reported outcomes including quality of life. Better markers of treatment effectiveness are needed; in addition, responsiveness to BH4 with reduction in blood Phe and improvement in Phe tolerance may not translate into responsiveness in cognitive outcomes. The degree to which reductions in blood Phe are associated with measurable cognitive outcomes or even patient perception of increased mental clarity is unknown; foundational research should be done to identify target outcomes for additional studies. Furthermore, explicit assessment of the potential for liberalization of the diet, and the subsequent nutritional effects on cognition has yet to be conducted.

Larger studies are also necessary to determine whether pharmacologic intervention is more advantageous in certain age groups or among individuals of varying dietary control of Phe, genotype, or severity of disease. A number of studies are reportedly under way to address gaps in the current literature. These include a long-term study of the effect of BH4 on neurocognitive function in young children, a study of the effect in adolescent patients with attention deficit hyperactivity disorder, and a registry that includes pregnant women (PKUMOMS).

Conclusions

Dietary management remains the mainstay of treatment for PKU, and maintaining control over the lifetime is an appropriate goal. Nonetheless, there is potential for supporting patients in achieving their clinical goals and possibly liberalizing their diet with adjuvant therapy. BH4 has been shown in two RCTs and three open-label trials to reduce Phe levels in some patients, with significantly greater reductions seen in treated versus placebo groups. We do not yet have the ability to reliably predict which patients are most likely to be responders, as all participants in the trials were initially responsive in screening tests, but not necessarily so in the efficacy studies.

One RCT also demonstrated increased Phe tolerance using BH4 among children on restricted diets. Overall, harms associated with the drug were minor and did not occur more frequently in the treatment group than in placebo arms. To date, there are no data to directly establish the potential effects of BH4 on longer-term clinically important outcomes, including cognition, executive function, and quality of life. Significant gaps in the evidence remain, including effectiveness of the drug in a range of patients outside of the clinical trial setting. Thus, while the strength of evidence is moderate for a large, positive effect of BH4 on reducing Phe levels over the short term in groups of patients showing initial responsiveness, evidence for the effect of BH4 on longer-term clinical outcomes is low and based on indirect associations.

Disclaimer

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Synopsis

BH4 is effective in reducing blood phenylalanine levels in some individuals with PKU; however, understanding of who will benefit and effects on longer-term outcomes such as cognition and quality of life is lacking.

Contributors' Statement

We acknowledge that all authors of this manuscript meet authorship criteria and have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; have participated in drafting the article or revising it critically for important intellectual content; and have given final approval of the version to be published.

Article Guarantor

Mary Lou Lindegren, M.D., accepts full responsibility for the work/conduct of the study, had access to the data, and controlled the decision to publish.

Competing Interest Statement

None of the authors has any competing interests.

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Ethics Approval

Ethics approval was not required for this study.

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RESEARCH REPORT

Neonatal Bone Marrow Transplantation in MPS IIIA Mice

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Abstract Patients with some neurological lysosomal storage disorders (LSD) exhibit improved clinical signs following bone marrow transplantation (BMT). The failure of mucopolysaccharidosis (MPS) type IIIA patients and adult mice with the condition to respond to this treatment may relate to factors such as impaired migration of donor-derived cells into the brain, insufficient enzyme production and/or secretion by the donor-derived microglial cells, or the age at which treatment is initiated. To explore these possibilities, we treated neonatal MPS IIIA mice with whole unfractionated bone marrow and observed that nucleated blood cell reconstitution occurred to a similar degree in MPS IIIA mice receiving green fluorescent protein (GFP)-expressing normal (treatment group) or MPS IIIA-GFP marrow (control group) and normal mice receiving normal-GFP marrow (control group). Further, similar distribution patterns of GFP^+ normal or MPS IIIA donor–derived cells were observed throughout the MPS IIIA mouse brain. We demonstrate that *N*-sulfoglucosamine sulfohydrolase (SGSH), the enzyme deficient in MPS IIIA, is produced and secreted in a manner proportional to that of other lysosomal enzymes. However, despite this, overall brain SGSH activity was unchanged in MPS IIIA mice treated with normal marrow and the lysosomal storage burden in whole brain homogenates did not decrease, most likely due to donor-derived cells comprising <0.24% of total recipient brain cells in all groups. This suggests that the failure of MPS IIIA patients and mice to respond to BMT may occur as a result of insufficient donor-derived enzyme production and/or uptake by host brain cells.

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Introduction

Lysosomal storage disorders (LSD) are a group of inherited conditions that most commonly result from the absence of a catabolic enzyme, causing the accumulation of partially degraded substrates. In Sanfilippo syndrome, or mucopolysaccharidosis (MPS) types IIIA, B, C or D, degradation of the glycosaminoglycan heparan sulfate is incomplete and as a result heparan sulfate–derived oligosaccharide fragments accumulate within the lysosome. Patients afflicted with Sanfilippo syndrome are generally diagnosed between 2–6 years of age and show a gradual deterioration of central nervous system function, increased aggression, hyperactivity, and sleep disturbance (Neufeld and Muenzer 2001). Several naturally occurring animal models of MPS IIIA have been

identified, including a mouse model resulting from a missense mutation that causes an amino acid change from an aspartic acid to an asparagine at position 31 of the lysosomal enzyme *N*-sulfoglucosamine sulfohydrolase (SGSH; EC 3.10.1.1) (Bhaumik et al. 1999; Bhattacharyya et al. 2001). Congenic C57BL/6 MPS IIIA mice display reduced SGSH activity resulting in accumulation of heparan sulfate–derived disaccharides, GM2 and GM3 gangliosides and unesterified cholesterol, as well as behavioral changes such as reduced learning ability in the Morris Water Maze test, changes in open field activity, and altered anxiety-related behaviors (Crawley et al. 2006; Fraldi et al. 2007; Lau et al. 2008, 2010).

Allogeneic hematopoietic stem/precursor cell transplantation (BMT) halts disease progression in several neurological LSD, such as MPS type I and metachromatic leukodystrophy, provided that a suitable donor match can be identified and that the transplant is undertaken early in the disease course (Shapiro et al. 1995). Despite the successful engraftment of donor cells, it appears that BMT in MPS IIIA patients is unable to improve or stabilize cognitive function (Klein et al. 1995; Sivakumur and Wraith 1999). Umbilical cord blood stem cell transplants have also been undertaken in Sanfilippo patients, with a 5-year probability of survival of 56% (Prasad et al. 2008). Two of the children treated at less than 2 years of age who survived the transplant may have had a modest improvement in cognitive skills, though were still developmentally delayed overall (Prasad et al. 2008). Pre-symptomatic MPS IIIA mice transplanted at 4 weeks of age show complete engraftment of donor cells in peripheral blood as well as high levels of SGSH activity and reduced heparan sulfate–derived oligosaccharide storage in the bone marrow compartment, measured using the disaccharide marker GlcNS-UA (Lau et al. 2010). While there was a reduction in brain GlcNS-UA of 27%, this was insufficient to mediate a clinical improvement in the behavioral phenotype of the transplanted mice.

The basis of the differential response of patients with MPS IIIA and MPS I or metachromatic leukodystrophy to BMT is not known. One possibility is that the high concentration of undegraded heparan sulfate–derived fragments in host MPS IIIA tissues reduces the mobilization of donor bone marrow–derived cells into circulation, thereby resulting in fewer engrafted donor cells within the affected brain. Heparan sulfate proteoglycans modulate a wide variety of biological interactions, including the binding of several key molecules involved with hematopoietic stem cell homing and mobilization. For example, heparin, a highly sulfated form of heparan sulfate, forms tight complexes with neutrophil elastase and cathepsin G which render these proteases 2–5 times less active than their free counterparts toward natural and synthetic substrates (Frommherz et al. 1991). Considering that neutrophil elastase and cathepsin G proteolysis mobilizes hematopoietic precursor/progenitor cells into circulation (Levesque et al. 2001), reduced activity of these enzymes due to interactions with heparan sulfate could potentially result in inefficient repopulation of donor-derived cells in blood and organs following BMT. Heparan sulfate–derived GlcNS-UA is elevated 15-fold in newborn MPS IIIA mouse brain, progressively increasing to maximal levels exceeding 250-fold by 13 weeks of age (Crawley et al. 2006). Therefore, transplanting mice in the neonatal period, when GlcNS-UA is less abundant, may improve donor-derived cell mobilization and the efficacy of BMT. In the murine MPS VII model, BMT is more effective when delivered to neonatal compared to adult mice (Birkenmeier et al. 1991; Sands et al. 1993). Also, the clinical efficacy of cell-mediated therapies, such as BMT, is impacted by the ability of both the transplanted cells to produce and secrete enzyme containing mannose-6-phosphate moieties and the affected host cells to efficiently internalize this enzyme and target it toward the lysosome via the mannose-6-phosphate pathway (reviewed in Kornfeld 1992). In this study, we have examined several factors that may influence the outcome of BMT in MPS IIIA mice and the effect of neonatal transplantation on neuropathological storage.

Materials and Methods

Mice and Genotyping

All procedures were approved by the institutional Animal Ethics Committee and were conducted as per the guidelines of the National Health and Medical Research Council of Australia. MPS IIIA B6.Cg-Sgsh^{mps3a} ("MPS IIIA") and unaffected (wild-type or heterozygous; subsequently referred to as "normal") mice were obtained from a breeding colony maintained at the Women's and Children's Hospital (Crawley et al. 2006). The genotypes of the mice were determined from a toe clip collected at 5–7 days of age by extracting genomic DNA by overnight incubation at 37 °C in 50 μL of 50 mM Tris, pH 8.0, 2 mM NaCl, 1 mM EDTA, 0.5% (v/v) Tween20, and 0.5% (v/v) Triton X-100 supplemented with 1.2 mg/mL proteinase K. After heating at 95 °C for 10 min, 1–2 μL of clarified lysate was amplified with forward primer 5′-NNT CTG TCT TCC TCA GCG-3′, reverse primer 5′-GAT AAG GCT GTG GCG GGA CAG GG-3′ (final concentration of 4 ng/μL of each primer), 0.2 mM dNTP (Roche, Mannheim, Germany), and 1.25 U HotStarTaq DNA Polymerase (Qiagen, Doncaster, Australia) in a 50 μL reaction by denaturing at 94°C for 15 min followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 40 s, and a 5 min final extension at 72°C. After visualization of the 105 bp PCR product, amplified DNA was digested with 5 U

*Aci*I (New England Biolabs, MA, USA) at 37 °C before electrophoresis through a 4.5% (w/v) agarose gel. As the G91A mutation abolishes an *Aci*I site, wild-type mice display bands at 74, 16, and 15 bp while MPS IIIA mice display bands at 90 and 15 bp. Heterozygote mice display bands at 90, 74, 16, and 15 bp.

Breeding pairs of C57BL/6-Tg(UBC-GFP)30Scha/J mice (Schaefer et al. 2001) were purchased from the Jackson Laboratory (ME, USA) and a pedigreed breeding colony was maintained at the Women's and Children's Hospital. These mice express green fluorescent protein (GFP) under the control of the human ubiquitin C promoter and wild-type levels of SGSH and are subsequently referred to as "normal-GFP" mice. Mice were confirmed as GFP-positive by viewing a toe clip under ultraviolet light.

A normal-GFP dam ($SGSH^{+/+}GFP^{+/+}$) was mated with an MPS IIIA stud ($SGSH^{-/-}GFP^{-/-}$) to generate offspring heterozygous for the *SGSH* and *GFP* genes. The F1 animals were inter-crossed and the resultant F2 offspring were assessed for GFP expression (see "Flow Cytometry" section) and normal, carrier, or affected *SGSH* gene status. A brother/sister founder pair of MPS IIIA mice homozygous for the GFP allele ("MPS IIIA-GFP"; $SGSH^{-/-}GFP^{+/+}$) were used to establish a pedigreed colony.

Neutrophil Elastase and Cathepsin G Activity

Following CO_2-mediated euthanasia, bone marrow extracellular fluid was extracted from 6-week-old normal and MPS IIIA mice by flushing the hind-leg bones with 0.5 mL ice-cold phosphate-buffered saline (PBS) using a 21 G needle ($n = 9$–10 mice per group). For neutrophil elastase activity, 10 µL of sample was diluted with 90 µL of 0.1 M Tris–HCl, pH 7.5, 0.5 M sodium chloride, and 0.01% (v/v) sodium azide and then mixed with an equal volume of 800 µM MeOSuc-Ala-Ala-Pro-Val-pNA substrate (Calbiochem, Australia). For cathepsin G activity, 10 µL of sample was diluted in 90 µL of 0.1 M Tris–HCl, pH 8.3, 0.01% (v/v) sodium azide and mixed with 100 µL of 800 µM Suc-Ala-Ala-Pro-Phe-pNA (Calbiochem, Australia). Standard curves were prepared using recombinant neutrophil elastase (Calbiochem, Australia; 0–128 mU/mL) or cathepsin G (Calbiochem, Australia; 0–20 mU/mL). Following a 3-h incubation at 37 °C, the absorbance was measured on a Victor3™ multilabel reader (Perkin Elmer) at 405 nm to quantitate the amount of cleaved pNA. Protease activity was normalized to total protein content as determined by bicinchoninic acid assay (Pierce, IL).

Transplantation and Necropsy

Donor normal-GFP or MPS IIIA-GFP mice (8–10 weeks old) were treated with 5-fluorouracil (150 mg/kg intraperitoneal; Sigma, MO, USA). Three days later, the mice were euthanized and donor bone marrow cells were harvested by flushing the hind-leg bones with Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS) and 50 U penicillin/0.05 mg/mL streptomycin (Sigma, MO, USA) using a 23 G needle (Lau et al. 2010). The cells were passed through a 100 µm cell strainer and red blood cells were lysed with Gey solution (155 mM ammonium chloride, 10 mM potassium hydrogen carbonate) at 37 °C for 10 min. Viable cells were counted with trypan blue dye (Sigma, MO, USA) and resuspended in PBS containing 30 U/mL heparin. Normal and MPS IIIA recipients (3 days old) were placed into a polypropylene container subdivided into 2.5 cm × 2.5 cm compartments and irradiated with 4 Gy using a megavoltage linear accelerator at a dose rate of 2 Gy/min. The pups then received 1–2 × 10^6 donor cells (in 30 µL total volume) intravenously via the superficial temporal vein. In the pilot study, 3-day-old mice were irradiated with 6 Gy prior to transplantation.

At 8 weeks post-transplant, the recipient mice were overdosed with CO_2 and whole blood collected via cardiac puncture and transferred to an EDTA-coated vacuette. The brain was removed, divided along the midline and one hemisphere was further sectioned in the coronal plane into five 2-mm thick slices and frozen at −20 °C. The other hemisphere was post-fixed in 4% paraformaldehyde in PBS at 4 °C for 24 h, cryo-protected in 30% (w/v) sucrose in PBS (Sigma, MO, USA) and embedded in Tissue-Tek OCT compound (Tokyo, Japan).

Flow Cytometry

The −/−, −/+, or +/+ status of the *GFP* gene during the generation of the MPS IIIA-GFP strain was determined in up to 20 µL of blood collected from the saphenous vein using 4% (w/v) EDTA-treated capillary tubes. The percentage of donor cell reconstitution in leukocytes was determined in duplicate samples of 50 µL whole blood taken at euthanasia (Lau et al. 2010). Erythrocytes were lysed in 2 mL of FACS lysing solution (BD Biosciences, NJ, USA). The leukocytes were blocked with IntraGam®P (CSL Ltd, Parkville, Australia), labeled with PE-Cy5-conjugated anti-CD45 (1:10 dilution; BD Biosciences, NJ, USA) and then washed with 0.5% (w/v) bovine serum albumin (Sigma, MO, USA) in IsoFlow Sheath Flow (Beckman Coulter, CA, USA). The cells were then analyzed on a FACSCalibur flow cytometer (Beckton Dickson, NJ, USA) equipped with CellQuest software (version 3.1).

SGSH Activity and GlcNS-UA Measurement in Tissue Homogenates

Livers, spleens, and brain tissues (slice 2) were homogenized in 500 µL of 20 mM Tris, 500 mM sodium chloride, pH 7.4, and sonicated twice for 30 s each. Samples for SGSH activity

measurement were dialyzed overnight in 200 mM sodium acetate, pH 5.2, and incubated with 400 pmol of a tritiated, heparin-derived tetrasaccharide substrate (Hopwood and Elliott 1982) at 60 °C. The amount of substrate and product were separated and quantified by high-performance liquid chromatography and normalized to total protein content (MicroBCA kit; Pierce, IL, USA).

The relative amount of a disaccharide marker (GlcNS-UA) of heparan sulfate storage was determined in brain samples from experimental mice or from untreated MPS IIIA brain as an internal control (50 μg total homogenate per sample). The tissues were derivatized with 1-phenyl-3-methyl-5-pyrazolone (Sigma, MO, USA) and assessed by liquid chromatography electrospray ionization tandem mass spectrometry analysis using a PE Sciex API 4000 QTRAP triple quadrupole mass spectrometer with a turbo spray source, as previously described (Hemsley et al. 2009). The intra-assay coefficient of variation of the quality control brain homogenate was 4.9%.

Quantitative Real-Time PCR

Genomic DNA was extracted from brain slices 3 and 5 according to published methods (Joshi et al. 2008), except that DNA was precipitated with 0.1x volume of 3 M sodium acetate and 2× volumes of 100% ethanol. The purity and concentration of DNA was determined at 260 nm using a Nanodrop (ND-1000, version 3.7.0; Thermo Scientific, Scoresby, Australia).

Primer Express Software (version 3; Applied Biosystems, CA, USA) was used to design EGFP forward (5′-GACGACGGCAACTACAAGAC-3′) and reverse (5′-GTCCTCCTTGAAGTCGATGC-3′) primers and hypoxanthine guanine phosphoribosyl transferase (HPRT) forward (5′-GTGGGAATGCGCAATCACT-3′) and reverse (5’-TCCACTCTTCAGGTGGAAAATAGG-3′) primers. The efficiency (E) of each primer set was determined using 10-fold dilutions of normal-GFP genomic DNA (0.05 to 500 ng) and was calculated from the slope of the standard curve (cycle threshold (Ct) against log genomic DNA concentration) using the formula $E = -1 + 10^{(-1/\text{slope})}$.

Real-time quantitative PCR reactions were carried out with an Applied Biosystems 7300 Real-Time PCR System in 96-well clear optical reaction plates with optical adhesive covers (Applied Biosystems, CA, USA). Reactions (30 μL) were performed in triplicate, with 100 ng genomic DNA, SYBR® Green PCR master mix (Applied Biosystems, CA, USA) and either EGFP (3 μM) or HPRT primers (10 μM). The cycling conditions employed were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Ct values were determined using the 7000 Sequence Detection software (v1.3; Applied Biosystems, CA, USA). The relative number of EGFP copies versus HPRT copies was determined using the method of Pfaffl (2001) and expressed as a percentage of GFP-expressing cells compared to a reference untreated normal-GFP mouse brain (100% GFP-positive).

GM3 Ganglioside Immunohistochemistry and GFP Visualization

Tissues were processed, imaged, and assessed in a single batch by an experimenter without knowledge of the genotype/treatment status of the samples. Frozen brain sections (6 μm thick) were cut in the sagittal plane and collected onto Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). GM3 ganglioside secondary storage was quantitated by staining with a monoclonal anti-GM3 antibody (Seikagaku Biobusiness Corporation, Tokyo, Japan; 1:750) using published methods (Lau et al. 2010). For GFP imaging, cryo-sections were rinsed in PBS and mounted with Vectashield mounting media containing 4’,6-diamidino-2-phenylindole (DAPI) to label all nuclei (Vector Laboratories, CA, USA). Images were captured using an Olympus Colorview Soft Imaging System and an Olympus BX41 (GM3 ganglioside) or BX61 (GFP) microscope, and the percentage of immunostained area was determined with AnalySIS Lifescience software (version 2.8, Build1235, Olympus Soft Imaging Solutions).

Cell Culture

All cells were derived from neonatal mice euthanized by decapitation at 0–4 days of age. Skin fibroblast cultures (Villegas and McPhaul 2005) were grown in DMEM/Nutrient Mixture F-12 Ham (Sigma, MO, USA) with 20% FCS (ThermoElectron Corporation, Melbourne, Australia), 2 mM glutamine (SAFC Biosciences, KS, USA) and 50 U penicillin/0.05 mg/mL streptomycin (Sigma, MO, USA). Mixed neural cells derived from whole brain (Sutherland et al. 2008) were maintained in DMEM (Sigma, MO, USA) with 5% FCS (ThermoElectron Corporation, Melbourne, Australia), 5% horse serum (Institute of Medical and Veterinary Science, Adelaide, Australia), 2 mM glutamine (SAFC Biosciences, KS, USA), and 50 U penicillin/0.05 mg/mL streptomycin (Sigma, MO, USA). Cells were cultured at 37 °C with 5% CO_2.

Lysosomal Enzyme Activity Assays

Neural cells and fibroblasts were cultured until confluence. The cells were then given a media change and cultured for 1 week without further media changes. Conditioned media was collected and stored frozen and control unconditioned media samples were also included. The cells were detached with 10% trypsin (SAFC Biosciences, KS, USA) in PBS

(Sigma, MO, USA), centrifuged at 6,240*g* for 5 min and resuspended in 300 μL of 20 mM Tris, 500 mM sodium chloride, and pH 7.4. Samples were subjected to six cycles of freezing/thawing in a slurry of dry ice and ethanol.

SGSH activity was determined by mixing 8 μL of sample with 3 μL of 200 mM sodium acetate, pH 5.2, and 1 μL of tritiated, heparin-derived tetrasaccharide substrate (400 pmol) and incubating for 16 h at 60 °C. The conversion of substrate into product was then measured as described for brain homogenates. For all other enzyme assays, 10 μL of sample was mixed with 40 μL of 0.9% NaCl and 50 μL of the appropriate substrate and incubated at 37 °C. After 1 h (α-mannosidase and α-fucosidase), 2 h (α-iduronidase; IDUA), or 4 h (α-*N*-acetylglucosaminidase; NAGLU), the reaction was quenched with 1.5 mL glycine buffer (200 mM glycine, 125 mM sodium carbonate, pH 10.7) and the amount of free 4-methylumbelliferone (4MU) was measured on a Perkin Elmer LS-50B spectrofluorometer. The substrates used were 4MU-2-acetomido-2-deoxy-α-D-glucopyranoside (NAGLU; 2 mM in 200 mM sodium acetate, pH 4.3; Toronto Research Chemicals, North York, Canada), 4MU-α-L-idopyranosiduronic acid (IDUA; 2 mM in formate buffer, pH 3.6; Toronto Research Chemicals, North York, Canada), 4MU-α-mannopyranoside (α-mannosidase; 4 mM in acetate buffer, pH 4.0; Melford Laboratories, Chelsworth, UK), and 4MU-α-L-fucopyranoside (α-fucosidase; 1 M in citrate phosphate buffer, pH 5.0; Melford Laboratories, Chelsworth, UK).

The activity in unconditioned media controls was subtracted from activity measured in the conditioned media samples to determine the secreted activity in the media. The calculated activity in each sample was then corrected for total protein content to give a total amount of intracellular or extracellular activity. Secreted enzyme activity was calculated as the percentage of extracellular activity as a proportion of the sum of intracellular and extracellular activity.

GlcNS-UA Determination in Co-cultured Normal and MPS IIIA Cells

Normal-GFP and MPS-GFP fibroblasts (2 weeks post-confluent) were plated into 6-well trays at various ratios of normal and MPS IIIA cells, with each well containing 5×10^6 cells in total, based on a previous report that the number of microglia in the brain is thought to remain constant in adulthood (Lawson et al. 1992) and therefore in a transplantation setting the donor microglia would replace recipient microglia rather than supplement microglial numbers. To prevent clonal dominance of normal-GFP cells in the co-cultured populations, the cells were detached after 48 h with 10% trypsin (SAFC Biosciences, KS, USA) in PBS (Sigma, MO, USA), centrifuged at 6,240*g* for 5 min and resuspended in 150 μL of 20 mM Tris, 500 mM sodium chloride, pH 7.4. The cells were sonicated twice for 30 s and the total protein content was quantitated using a MicroBCA kit (Pierce, IL, USA). The relative amount of GlcNS-UA was determined in 100 μg of cell extract, as described for brain homogenates. The intra-assay coefficient of variation of an internal quality control sample of cultured cells was 2.6%.

Statistics

Data are expressed as the mean ± SEM. Data were analyzed using one-way ANOVA with GraphPad Prism software (v.502) with post hoc comparisons made using the Bonferroni correction factor or with unpaired t-tests. Statistical significance was considered to be $P < 0.05$.

Results

Donor Leukocyte Engraftment Following Myeloreduction

Three-day-old normal and MPS IIIA pups were transplanted with syngeneic GFP-expressing mononuclear bone marrow cells following irradiation with 4 Gy. The three treatment groups were (1) normal recipients receiving normal-GFP donor cells ($n = 9$ mice; 8 male, 1 female), (2) MPS IIIA recipients receiving MPS IIIA donor cells ($n = 5$ mice; 4 male, 1 female), and (3) MPS IIIA recipients receiving normal-GFP donor cells ($n = 7$ mice; 3 male, 4 female). The body weights of the three cohorts were indistinguishable at 2 months post-transplantation ($P > 0.05$; Fig. 1a). Quantitation of donor-derived peripheral blood leukocyte reconstitution revealed no significant differences in mobilization between the transplanted groups of mice, with an average of 25%, 24% and 21% $CD45^+GFP^+$ donor cells measured at 8 weeks post-transplantation in control normal mice, control MPS IIIA mice, and treated MPS IIIA mice, respectively (Fig. 1b). Further, the catalytic activity of two serine proteases involved in the mobilization of hematopoietic precursor/progenitor cells was determined in bone marrow extracellular fluid from 6-week-old mice. Although there was a trend toward an increase in neutrophil elastase activity in MPS IIIA mice, statistically significant differences were not measured ($P > 0.05$; Fig. 1c). Likewise, no significant differences in cathepsin G activity were measured between genotype groups ($P > 0.05$; Fig. 1c).

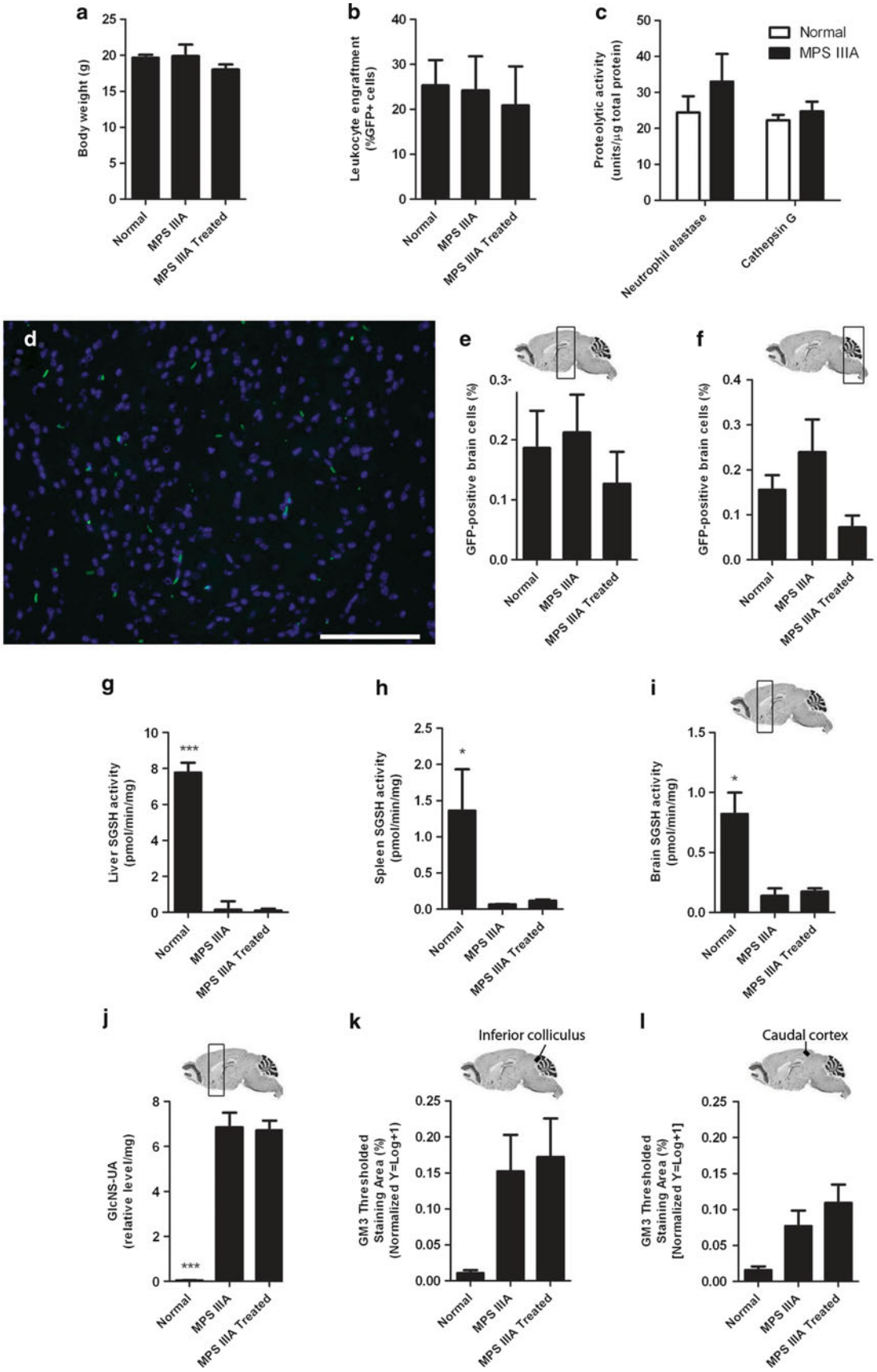


Fig. 1 Effect of transplantation of neonatal MPS IIIA mice preconditioned with 4 Gy. (**a**) The weight of MPS IIIA recipient mice transplanted with normal-GFP donor bone marrow ("MPS IIIA Treated"; $n = 7$ mice) and transplanted control groups of normal

GFP-Expressing Donor Cells Repopulate Throughout the Brain, but at Low Levels

To verify that donor-derived cells had successfully migrated and engrafted within the central nervous system, GFP-expressing cells were visualized in brain tissue sections. GFP-positive cells were widely distributed throughout the brains of all groups of transplanted mice, including regions such as the cerebellum, cerebral cortex, hippocampus, thalamus, inferior colliculus, superior colliculus, and brainstem (Fig. 1d and Supplementary Figure 1). To obtain a quantitative measure of the number of donor-derived cells in the brain, the relative GFP transgene copy number (compared to the HPRT gene as an endogenous control) was determined in hemi-coronal brain slices by quantitative real-time PCR and compared to untreated transgenic mouse brains that expressed GFP in all nucleated cells (i.e., all cells except for red blood cells). Initial validation experiments showed that the amplification efficiency was similar for the GFP and HPRT primer sets (101.6% and 95.4%, respectively). In addition, dissociation curve analysis demonstrated the formation of a single peak for each primer set, with a melting temperature of 84 °C and 80 °C for GFP and HPRT primers, respectively.

The relative number of GFP transgene copies was examined in brain slices 3 and 5 of transplanted mice by real-time quantitative PCR. In brain slice 3, the relative level of GFP repopulation was, on average, 0.1–0.2%, with no difference detected between treatment/genotype groups (Fig. 1e). The maximum percentage of GFP engraftment measured in the brain was 0.52% in a normal transplanted control mouse. A similar low level of donor cell repopulation was measured in brain slice 5 (0.1–0.2% on average; Fig. 1f).

Effect of Transplantation on SGSH Activity and Substrate Accumulation

To determine the effect of donor cell transplantation, SGSH activity was measured in liver, spleen, and brain homogenates. In the liver, MPS IIIA mice transplanted with MPS IIIA bone marrow displayed 1.8% of the SGSH activity measured in normal mice transplanted with normal bone marrow (Fig. 1g). Transplantation of normal-GFP bone marrow cells in MPS IIIA mice had no impact on liver SGSH activity (1.3% of wild-type SGSH activity). Similar trends were observed in the spleen (Fig. 1h) and brain parenchyma (Fig. 1i), with transplantation of normal-GFP cells into MPS IIIA recipients yielding no significant differences in SGSH activity compared to control-transplanted MPS IIIA mice.

Heparan sulfate–derived GlcNS-UA was considerably elevated in the brain of control transplanted MPS IIIA mice receiving MPS IIIA-GFP bone marrow relative to normal mice transplanted with normal-GFP donor cells (165-fold normal; $P < 0.0001$; Fig. 1j). Transplantation of normal-GFP bone marrow did not significantly reduce the relative level of GlcNS-UA in MPS IIIA recipient mouse brain (162-fold normal).

The effect of treatment on secondary storage products was also examined at 8 weeks post-transplant. In both the inferior colliculus (Fig. 1k) and caudal cortex (Fig. 1l), numerous GM3 ganglioside-immunoreactive puncta were observed in MPS IIIA mice transplanted with MPS IIIA-GFP bone marrow. In contrast, GM3 ganglioside immunoreactivity was infrequently observed in the transplanted normal control mice. Delivery of normal-GFP donor bone marrow to MPS IIIA recipient mice had no observable treatment effect on the amount of GM3 ganglioside immunoreactivity (Fig. 1l-k).

The Percentage of Extracellular SGSH Activity is Similar to Other Lysosomal Enzymes

We next examined whether the proportion of secreted SGSH and NAGLU (EC 3.2.1.50; deficient in MPS IIIB) in wild-type cells was altered compared to the hydrolases deficient in MPS I, α-mannosidosis, and α-fucosidosis (i.e., neurodegenerative LSD that are successfully treated by allogeneic BMT). In skin fibroblast cultures, SGSH activity

Fig. 1 (continued) mice receiving normal-GFP cells ("Normal"; $n = 9$ mice) and MPS IIIA mice receiving MPS IIIA cells ('MPS IIIA'; $n = 5$ mice) was recorded at 8 weeks post-treatment. (**b**) The percentage of donor-derived GFP^+CD45^+ leukocytes was measured by flow cytometry. (**c**) The catalytic activity of two proteolytic enzymes that are involved with the mobilization of bone marrow cells into circulation was measured using chromogenic substrates in bone marrow extracellular fluid harvested from untreated normal (*open bars*) and MPS IIIA (*filled bars*) mice (n = 9-10 mice per group). (**d**) Cryo-sections were examined for the presence of donor-derived GFP-expressing cells (*green*) compared to the total number of DAPI-stained cell nuclei (*blue*) in the cerebellum. Scale bar is 100 μm. The relative GFP transgene copy number (compare to the HPRT gene as an endogenous control) was determined via quantitative real-time PCR in transplanted mouse (**e**) brain slice 3 and (**f**) slice 5. An untreated transgenic normal-GFP mouse brain was used as a calibrating sample (100%). (**g**) Liver, (**h**) spleen, and (**i**) brain SGSH activity was measured in tissue homogenates using a radiolabeled tetrasaccharide substrate and HPLC separation. (**j**) The relative amount of GlcNS-UA disaccharide was determined by tandem mass spectrometry as a measure of the amount of primary storage material in the transplanted mouse brain. The effect of treatment on GM3 ganglioside storage in the brain was quantitated in the (**k**) inferior colliculus and (**l**) caudal cortex using immunohistological methods and AnalySIS Lifescience software. All data are expressed as the mean ± SEM. $*P < 0.05$, $***P < 0.001$ versus the control-transplanted MPS IIIA control group

was reduced in cell extracts compared to all other assayed lysosomal enzymes (Fig. 2a). Similar trends were observed in neural cells and in media samples of both cell types, although not all differences reached statistical significance (Fig. 2b–d). The proportion of extracellular SGSH activity was comparable to the percentage of extracellular NAGLU and α-iduronidase (EC 3.2.1.76) activity in both cell populations and was significantly higher than that of α-fucosidase (EC 3.2.1.51; Fig. 2e–f). α-Mannosidase (EC 3.2.1.24) was the only lysosomal enzyme that was measured at a significantly higher percentage of extracellular activity compared to SGSH (Fig. 2e–f).

Preconditioning with Higher Irradiation Doses Improves Donor Cell Engraftment but Induces Acute Radiation Syndrome Symptoms

In an attempt to increase the degree of donor-derived cell engraftment in affected organs, including the brain, 3-day-old normal mouse pups were preconditioned with a higher myeloablative dose of 6 Gy before normal-GFP donor cell infusion ($n = 4$ mice) and compared to a second cohort of normal mice irradiated with 4 Gy and transplanted with normal-GFP cells ($n = 5$ mice). At 4 weeks post-transplant, analysis of $CD45^+GFP^+$ peripheral blood leukocytes showed that the donor-type chimerism was $10 \pm 3\%$ in mice preconditioned with 4 Gy. Donor cell engraftment was significantly improved following preconditioning with 6 Gy, with $37 \pm 4\%$ $CD45^+GFP^+$ donor cells measured ($P < 0.001$). As observed in the earlier study, mice irradiated with 4 Gy showed no signs of acute radiation syndrome. In contrast, recipient pups that received 6 Gy displayed significant growth retardation at 4 weeks post-treatment ($P < 0.05$), weighing 28% less than mice irradiated with 4 Gy, and also developed ataxia and involuntary tremors. Due to the severity of the side effects of the preconditioning regime in the latter group of mice, the study was terminated and the mice euthanized at 4 weeks of age.

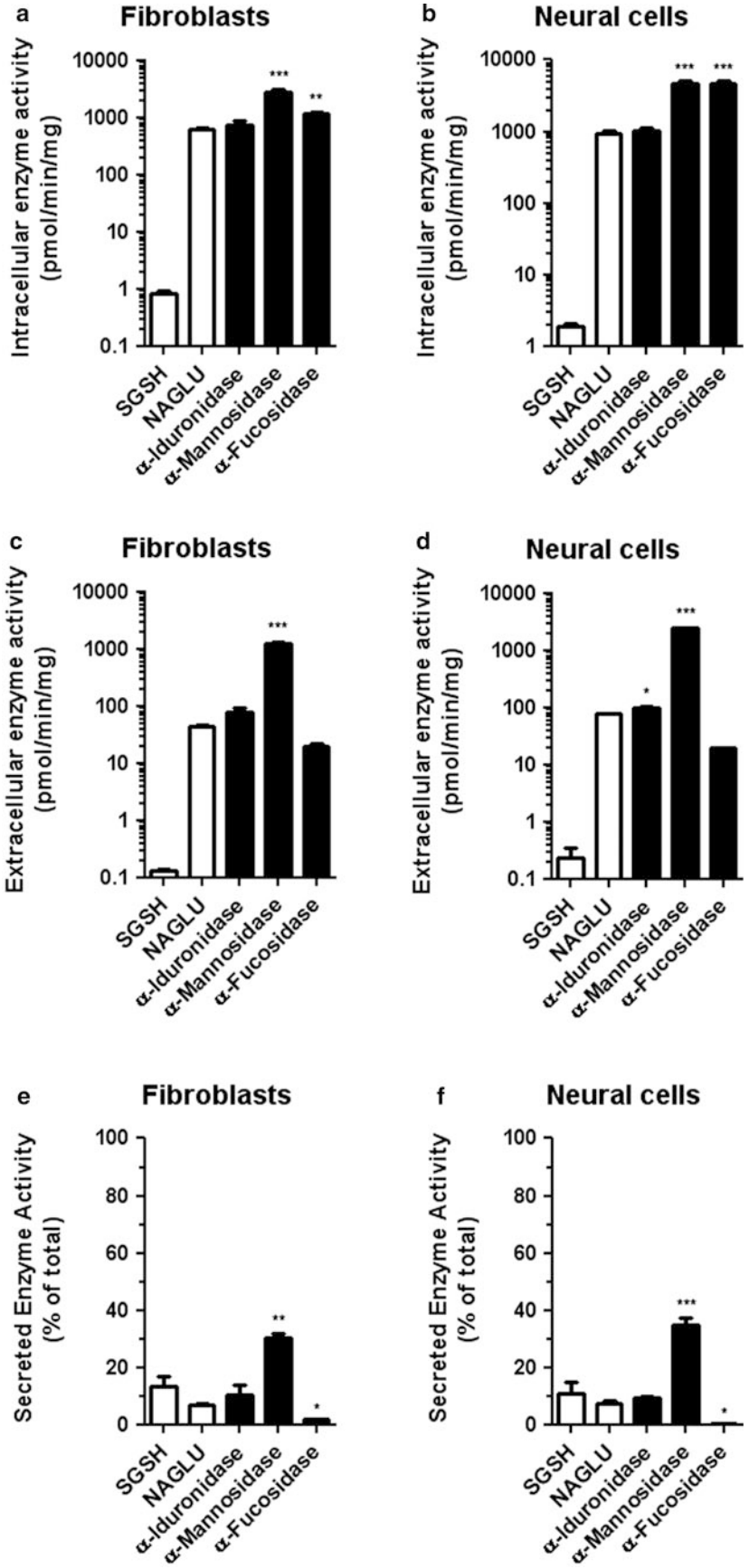
Reduction of Substrate in MPS IIIA Cultured Cells After Co-incubation with Normal Cells

Previously, we achieved a 27% reduction in brain GlcNS-UA when approximately 3% of the total brain cells were of donor origin (unpublished data and Lau et al. 2010). Cells of macrophage/microglial origin have been estimated to make up to 12% of total brain cells (Lawson et al. 1990), which raised the following question: if a BMT regime was able to replace all host microglia with donor cells, what level of amelioration of stored GlcNS-UA is achievable in the transplanted MPS IIIA mouse brain? We cultured MPS IIIA cells (0% normal cells) and determined the relative level of GlcNS-UA accumulating within them. Additional cultures were established so that they contained 90% MPS IIIA cells and 10% normal cells or 80% MPS IIIA cells and 20% normal cells, engraftment levels that may be feasible if all microglia were donor-derived. We anticipated that SGSH released from the normal cells would be internalized by MPS IIIA cells using mannose-6-phosphate receptor-based mechanisms, subsequently mediating a reduction in GlcNS-UA in the MPS IIIA cells.

We observed that co-culture of MPS IIIA cells with 10% normal cells mediated a statistically significant reduction of 14.5% in GlcNS-UA (Fig. 3). This consisted of a 5% reduction from cross-correction of the MPS IIIA cells by the input normal cells with the remainder due to a reduced amount of input GlcNS-UA by the addition of fewer MPS IIIA cells. Also, co-culture with 20% normal cells enabled a 37% total reduction within the timeframe of the experiment (21% derived from cross-correction of MPS IIIA cells). These data are consistent with our in vivo findings (Lau et al. 2010) and suggest that even if all MPS IIIA brain microglia/macrophages were able to be replaced with unaffected donor-derived cells, the level of stored heparan sulfate–derived substrates may not be reduced beyond approximately one-third.

Discussion

We evaluated the therapeutic efficacy of BMT in neonatal (3-day-old) MPS IIIA mice. This equates to approximately midway through the second trimester of a human fetus in terms of brain development (Clancy et al. 2001). A myeloreductive preconditioning regime (4 Gy, 1×10^6 donor cells delivered intravenously) enabled replacement of approximately 25% of $CD45^+$ leukocytes of donor origin and did not result in measurable increases in SGSH activity in visceral organs or the brain. Subsequently, there was no effect of treatment on primary or secondary storage compounds in the brain. Likewise, transplantation of MPS IIIB mice at 2–4 days of age after irradiation with 2 Gy did not result in a significant increase in brain NAGLU activity or ameliorate neuropathology (Heldermon et al. 2010). These data contrast with those from MPS VII mice, where transplantation resulted in ~ 4% of wild-type β-glucuronidase activity, focal reductions in lysosomal vacuolization in the brain and normalization of auditory-evoked brainstem responses (Sands et al. 1993, 1995). Given that this is a knockout model, all of the measured β-glucuronidase would be newly synthesized enzyme. Even greater reductions in lysosomal storage were observed when higher irradiation doses were utilized (Sands et al. 1993). Improvements in the accelerating rotor-rod test were also evident in Twitcher mice, a model of globoid-cell leukodystrophy, transplanted at 3 days of age (4 Gy; 1×10^6 donor cells),



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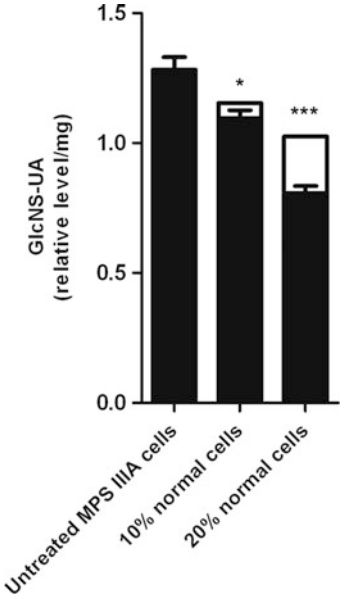

Fig. 3 GlcNS-UA storage in co-cultured normal and MPS IIIA fibroblasts. Normal-GFP and MPS-GFP cells (5×10^6 cells in total) were co-cultured for 48 h at different ratios. The relative amount of GlcNS-UA was then determined by tandem mass spectrometry and compared to the untreated group (100% MPS IIIA; filled bars). The calculated maximum amount of GlcNS-UA in the MPS IIIA cell portion of each treatment group due to dilution with the normal-GFP cells is denoted by the open bars (i.e., 90% or 80% of the relative levels of measured GlcNS-UA measured in the 100% MPS IIIA cultures). Data are expressed as the mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$ versus the untreated group

despite brain galactosylceramidase activity not being significantly increased following treatment (Lin et al. 2007). Twitcher mice transplanted at 8–11 days of age (9 Gy; $3–5 \times 10^7$ donor cells delivered intraperitoneally) generated up to 15% normal levels of galactosylceramidase activity in the brain, which was sufficient to prevent hind-limb paralysis (Hoogerbrugge et al. 1988). It is evident that MPS IIIA disease–related factors impair or reduce the capacity for clinical efficacy to be achieved with BMT in human MPS III types A, B, and C (Klein et al. 1995; Shapiro et al. 1995) and murine MPS III types A and B (Zheng et al. 2004; Heldermon et al. 2010; Lau et al. 2010), and the growing body of experimental data strongly suggest that BMT with unmodified cells should not be considered as a treatment for MPS III.

So why does BMT stabilize neurocognitive function in some LSD but is unable to resolve the disease pathology in other disorders? In our study, the SGSH activity generated under these transplantation conditions was insufficient to correct primary and secondary storage, which could potentially be explained by a number of reasons. First, inadequate migration of donor-derived cells into the brain following transplantation would reduce the number of healthy cells able to produce SGSH within the brain. It is well-established that bone marrow-derived cells are capable of migrating across the blood–brain barrier after myeloablative conditioning and then trans-differentiate into microglia, the resident macrophage population of the brain (e.g., Cogle et al. 2004; Simard and Rivest 2004). Microglia represent between 5% and 12% of total brain cells, dependent on the brain region (Lawson et al. 1990), and considering that proliferation of resident microglia also contributes up to half of the brain macrophage pool (Lawson et al. 1992), the absolute maximum proportion of donor cells that can migrate and repopulate the brain is less than 12%. Between 0.1–0.2% of recipient brain cells were found to be of donor origin in the brain of neonatally-transplanted MPS IIIA mice, and our attempt to raise the percentage of donor leukocytes in peripheral blood from 25% (and subsequently increase the proportion of donor cells within the brain) by increasing the irradiation preconditioning dose to 6 Gy resulted in acute radiation syndrome. Increased chimerism may be achieved using increased cell doses or alternate irradiation protocols (e.g., divided dose of 6 Gy). Also, the assessment of additional groups at longer times post-transplant could possibly have increased the number of donor-derived cells within the brain.

Quantitative real-time PCR analysis of brain tissue from adult MPS IIIA mice transplanted in a previous study (where 93% donor-type leukocyte chimerism was achieved) revealed that donor cells represented only 2.8 ± 1.2% of brain cells (unpublished data and Lau et al. 2010). BMT did not improve the behavioral phenotype of these treated MPS IIIA mice. The low level of donor cell repopulation in the MPS IIIA mouse brain is unlikely to be the result of a defect in mobilization of bone marrow–derived cells from the bone marrow compartment to peripheral blood, as the activity of two serine proteases involved in cellular mobilization was similar in normal and MPS IIIA mice. These proteases, cathepsin G and neutrophil elastase, have been implicated in altered bone marrow progenitor cell retention/mobilization in sialidosis mice (Yogalingam et al. 2008). Additionally, no differences in GFP-expressing donor leukocyte reconstitution

Fig. 2 (continued) Lysosomal enzyme activity in wild-type cells. (**a, c, e**) Murine skin fibroblasts or (**b, d, f**) mixed neural cell cultures were harvested at 7 days post-confluence. The amount of (**a, b**) intracellular enzyme activity in cell extracts and (**c, d**) extracellular enzyme activity in conditioned media samples was determined using fluorogenic or radiolabeled substrates for SGSH, NAGLU, α-iduronidase, α-mannosidase, and α-fucosidase. LSD that do not respond to BMT are depicted by open bars while LSD where BMT stabilizes neurocognitive decline are depicted by filled bars. (**e, f**) The percentage of extracellular activity as a percentage of the total measured activity for each enzyme is shown. All data are expressed as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus SGSH activity

or the percentage of GFP^+ brain cells were measured between normal and MPS IIIA recipients, suggesting that movement of donor cells from the vasculature to the brain was unimpaired in the disease state. This contrasts with the increased brain repopulation in transplanted metachromatic leukodystrophy mice (Biffi et al. 2004) and may be one of the factors contributing to the successful correction of neurological symptoms in this disorder.

It is possible that even if all microglia were normal in their enzyme content, this would still be insufficient to generate enough enzyme activity to mediate clinical improvements. This concept is supported by data generated in mouse models of Gaucher disease that either expressed glucosylceramidase activity in the microglia (and skin) or in skin fibroblasts only (Enquist et al. 2007). Both models showed neurological abnormalities, with only marginal delays in the onset of symptoms and minimal impact on life span. Further, we demonstrated that even co-culture of MPS IIIA cells with 20% normal cells (a level that cannot be achieved by transplantation alone) is unable to normalize GlcNS-UA storage. Co-incubation of approximately equal numbers of MPS I and MPS II cells (where the MPS I cells provide the corrective enzyme for the MPS II cells and vice versa) for 48 hours normalizes sulfated glycosaminoglycan storage (Neufeld and Cantz 1971). Further studies are warranted to elucidate whether this level of storage reduction can be achieved in MPS IIIA cells after co-incubation with higher doses of normal cells and the degree of primary storage reduction that is required for an impact on clinical symptoms.

Second, correction of MPS IIIA cells relies on the effective production and secretion of mannose-6-phosphorylated SGSH by the donor cells and subsequent cation-independent mannose-6-phosphate receptor (CI-MPR)-mediated uptake of SGSH by the neighboring host cells, and thus inadequate SGSH production, secretion, or endocytosis may also contribute to the lack of therapeutic benefit in transplanted MPS IIIA mice. SGSH exhibited the lowest activity in the intracellular and extracellular fractions of wild-type cells and this may relate to differences in assay sensitivity (natural substrate for SGSH versus 4MU-conjugated substrates) or the natural abundance of the enzymes. However, as each ratio was measured under identical assay conditions, our calculation of the ratio of intracellular versus secreted enzyme remains valid. No relationship was evident between the percentage of secreted lysosomal enzyme and the efficacy of BMT, with α-mannosidase and α-fucosidase (enzymes deficient in LSD that respond to BMT) displaying the highest and lowest percentage of extracellular enzyme activity, respectively. This suggests that pathological changes in the relative proportion of secreted SGSH are not the primary cause for the lack of correction in MPS IIIA. It remains to be ascertained whether the CI-MPR is mis-localized in host MPS IIIA cells, thus reducing the efficiency of exogenous SGSH uptake by affected cells. Pompe disease fibroblasts showed less efficient uptake of recombinant α-glucosidase enzyme due to reduced CI-MPR bioavailability at the plasma membrane, with the extent of abnormalities closely correlating with disease severity (Cardone et al. 2008). Increasing the amount of SGSH produced via ex vivo modification of bone marrow–derived cells with a gene therapy vector may overcome this issue (Zheng et al. 2004; Langford-Smith et al. 2012).

In conclusion, BMT was ineffective at ameliorating primary and secondary storage in the MPS IIIA mouse brain despite transplantation occurring in the neonatal period. Further studies are required to elucidate the mechanism(s) that result in differential responses to transplantation amongst LSD to direct the design of an effective BMT-mediated treatment for MPS IIIA.

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Synopsis

Despite intervention in the neonatal period and demonstration of unimpaired mobilization, engraftment, and secretion of lysosomal *N*-sulfoglucosamine sulfohydrolase enzyme by normal cells, bone marrow transplantation does not improve neuropathology in the murine MPS IIIA brain.

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CASE REPORT

Therapeutic Efficacy of Magnesium Valproate in Succinic Semialdehyde Dehydrogenase Deficiency

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Abstract Succinic semialdehyde dehydrogenase deficiency (SSADHD), a disorder of γ-aminobutyric acid (GABA) metabolism, manifests typically as a nonprogressive neurodevelopmental disorder with cognitive deficiency, neuropsychiatric morbidity and epilepsy. Therapy targets symptomatic seizures and neurobehavioral disturbances. We report an adolescent female with SSADHD whose unresponsiveness to a broad spectrum of antiepileptics was circumvented with magnesium valproate (MgVPA). Epilepsy remains well controlled in our patient, with concomitant improvements in behavioral symptoms and an absence of adverse symptoms. MgVPA intervention may have utility in SSADHD.

Introduction

The clinical, metabolic, and molecular phenotypes of SSADHD have been reviewed (Pearl et al. 2003a, b). The metabolic lesion leads to accumulation of two neuromodulators, gamma-hydroxybutyric acid (GHB) and GABA (Wong et al. 2004). The phenotype of SSADHD is primarily neuropsychiatric, with onset in childhood with hypotonia and developmental delay. Expressive language impairment and sleep anomalies are also encountered. Atypical absence, myoclonic and generalized tonic-clonic seizures may be observed, as well as photosensitivity, electrical status epilepticus of sleep and partial seizures (Pearl et al. 2011). Chronic high endogenous levels of GABA likely elicit down-regulation of GABA receptors, a hypothesis supported by data derived from the corresponding murine model (Cortez et al. 2004; Buzzi et al. 2006; Wu et al. 2006) and clinical PET and transcranial magnetic stimulation studies (Pearl et al. 2009, 2011).

Neuropsychiatric morbidity represents an ongoing challenge in adolescents and adults with SSADHD (Knerr et al. 2008). Behavioral abnormalities include hyperactivity, aggression, inattention, obsessive-compulsive symptoms and sleep disturbances, and present treatment challenges especially when compounded with seizures. Vigabatrin (VGB), an irreversible inhibitor of GABA transaminase, is predicted to decrease GHB production (Ergezinger et al. 2003; Gropman 2003; Gibson et al. 1995). Clinical outcomes with VGB, however, have been inconsistent, and progressive retinopathy with consequent peripheral vision loss represents an undesirable long-term adverse effect (Chiron and Dulac 2011). Moreover, additional augmentation of GABA levels during VGB intervention may also be undesirable. Other antiepileptics, including carbamazepine, lamotrigine and ethosuximide have been variably effective, and benzodiazepines may be efficacious for moderating agitation and aggression, but adversely exacerbate ataxia and hypotonia. Additional pharmaceuticals (e.g., methylphenidate, risperidone, fluoxetine and fluvoxamine) have been employed in selected cases to treat behavioral symptoms (Pearl et al. 2003a, b).

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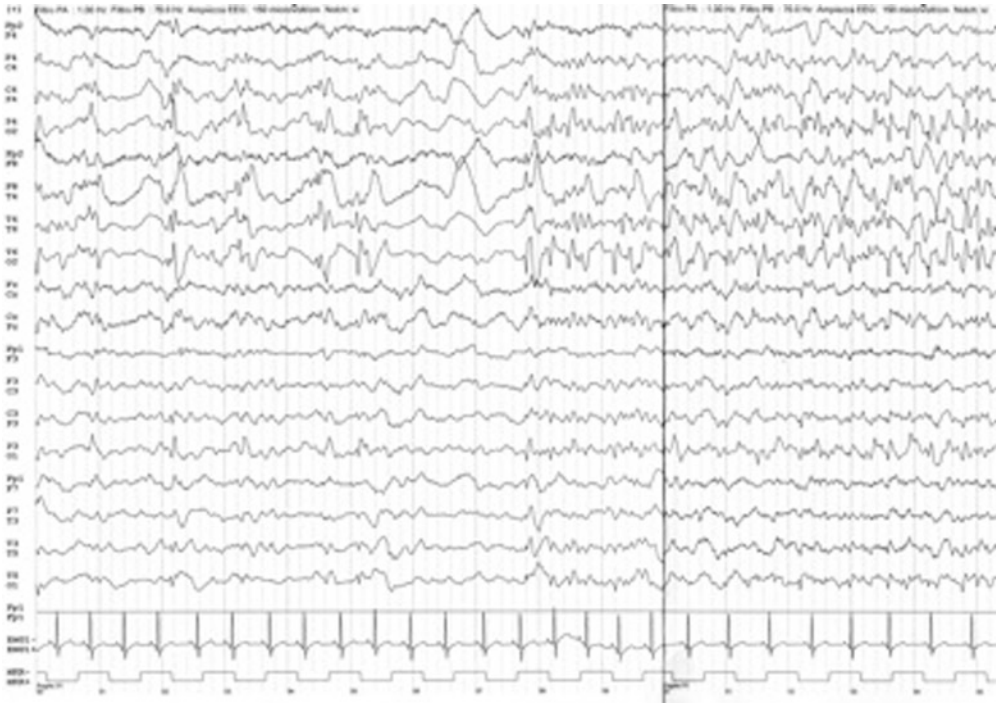

Fig. 1 EEG (age 14) showing right periodic lateralized epileptiform discharges (PLEDS) evolving into rhythmic sharp and slow wave ictal activity maximally from the right posterior temporal region

Valproic acid has not been broadly employed in SSADHD, primarily due to its ability to inhibit any residual SSADH activity (Simler et al. 1981; Shinka et al 2003). Anecdotal reports, however, have reported improved seizure control, without improvements in neurobehavioral symptoms. We present an adolescent with SSADHD in whom MgVPA showed both efficacy in seizure control and an improved behavioral phenotype.

Case Report

The second of two sibs, our patient was born following a full-term pregnancy with a normal birth and perinatal course. She presented in infancy with developmental delay and diffuse hypotonia. Associated manifestations included hyporeflexia, delayed speech, ataxia, hyperkinesia, short attention span, stereotyped movements, and EEG abnormalities at 3 years of age.

Diagnostic studies at 1 year of age (basic metabolic panel, cranial magnetic resonance imaging) were normal. Until 7 years of age no pharmacological interventions were employed. MgVPA therapy (20 mg/kg/day) was introduced at 7 years based upon behavioral difficulties and EEG alterations, without observable adverse effects. At age 13, MgVPA was stopped because a reasonable level of performance had been achieved and the parents requested treatment termination.

One year following cessation of MgVPA administration, the patient displayed a febrile episode with gradual decay of her state of vigilance and new onset of complex partial seizures. The latter were characterized by altered interpersonal interactions, fluctuating vigilance, disorientation, bewildered appearance, expression of fear, dysarthria and clonus involving primarily the left arm.

At this time (age 14 years), EEG recordings revealed disorganization of the basic background rhythm with slow waves of high voltage predominantly in the right hemisphere associated with a pseudo-periodic sharp-wave discharges mainly localized on the right hemisphere. In addition, the EEG showed intermixed sharp-wave discharges maximally emanating from the right posterior temporal regions and rhythmic sequences of theta/delta activity localized on the same regions (Fig. 1).

Magnetic resonance imaging at this time showed signal alterations characterized by T2/ FLAIR hyperintense signal, marked T1 hypointensity, and restricted diffusion of the bilateral parasagittal frontal lobes and right insula and temporal lobe (Fig. 2).

There were no changes following gadolinium administration, and there was a normal appearance of the circle of Willis with magnetic resonance angiography.

Additional laboratory evaluations were normal for ammonia, lactate, hematological panels, and hormonal assays, as were virological studies other than the evidence of prior EBV infection. Cerebrospinal fluid showed

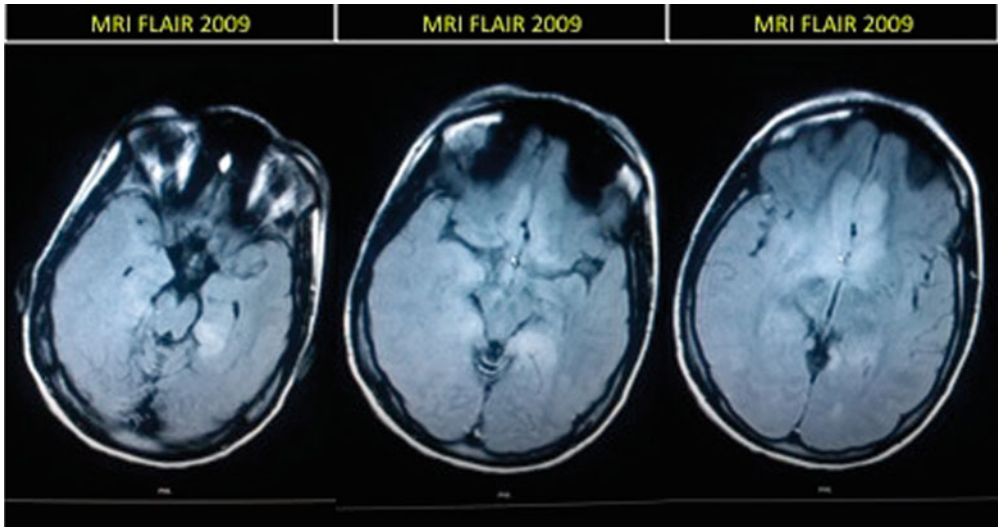

Fig. 2 T2/FLAIR MRI showing extensive abnormalities of the bilateral parasagittal frontal lobes and right insula and temporal lobe

normal parameters and no detection of oligoclonal bands, CMV, EBV, HHV6, HSV1,2, VZV, or Mycoplasma DNA.

Urine organic acid analysis revealed moderate elevation of GHB with subtle elevations of 4,5 dihydroxyhexanoic acid lactone and threo 4,5 hydroxy hexanoic acid. Confirmation of SSADHD was established via molecular analysis that identified a homozygous deletion of two nucleotides in exon 1 (c.160-161delCT) (as previously described by Akaboshi et al. 2003). This allele was confirmed as heterozygous in both parents.

The patient was treated with acyclovir, ceftriaxone, dexamethasone and barbiturates intravenously, in addition to a number of enteral antiepileptics (carbamazepine, oxcarbazepine, levetiracetam, phenobarbital). Intervention with antiepileptics was subsequently stopped due to adverse effects. Olanzapine was prescribed for ensuing psychiatric symptoms, without evidence of clinical benefit.

At age 15 years, MgVPA (15 mg/kg/day) was reintroduced due to ongoing seizure activity and deteriorating behavior resulting in a gradual improvement in behavior performances and seizure control. Blood parameters (liver functions, ammonia, erythrocyte, white cell, and platelet counts) were cautiously monitored with normal results. One year following MgVPA reintroduction, our patient remained seizure-free with marked behavioral improvements. Significantly, there was an improvement in disinhibited behavior including nonrecognition of danger, aggression and coprolalia. The EEG demonstrated improved background organization (Fig. 3).

MRI, repeated at 16 years of age, revealed evolution of the previously documented acute changes (Fig. 4).

Discussion

Our patient with confirmed SSADH deficiency presented with neurological deterioration characterized by complex partial seizures and possible limbic encephalitis at 14 years of age. Although there are concerns about a relative contraindication to using valproate in this patient population, our patient had no adverse effects during its use (MgVPA 20 mg/kg/day) in childhood when selected to treat a combination of neuropsychiatric deficits and then appeared to benefit from this agent after presenting with the encephalitic illness.

Magnesium and MgVPA have been successfully employed in other epileptic and learning disorders (Dósa et al. 2010; Zou et al. 2010; Porras-Kattz et al. 2011). For example, Mg has been used to mitigate refractory status epilepticus in patients with POLG-1 mutations (Pandey et al. 2010; Visser et al. 2011). With the recent clinical emphasis on avoiding valproate in children with POLG-1 mutations because of potential precipitation of epilepsia partialis continuans (Saneto et al. 2010), it is possible that magnesium had an important therapeutic role in our patient despite the general clinical tendency to avoid valproate in SSADH deficiency due to potential inhibition of residual enzymatic activity (Shinka et al. 2003). Overall, it appears that valproate was beneficial toward seizure amelioration and improved behavioral features for our patient's condition, and there may have been a therapeutic role for magnesium, which will require further characterization.

In conclusion, we present therapeutic efficacy of MgVPA in the behavioral and epileptic phenotype in a patient with SSADH deficiency. Valproate is not necessarily contraindicated in SSADH deficiency, and magnesium may have an important role.

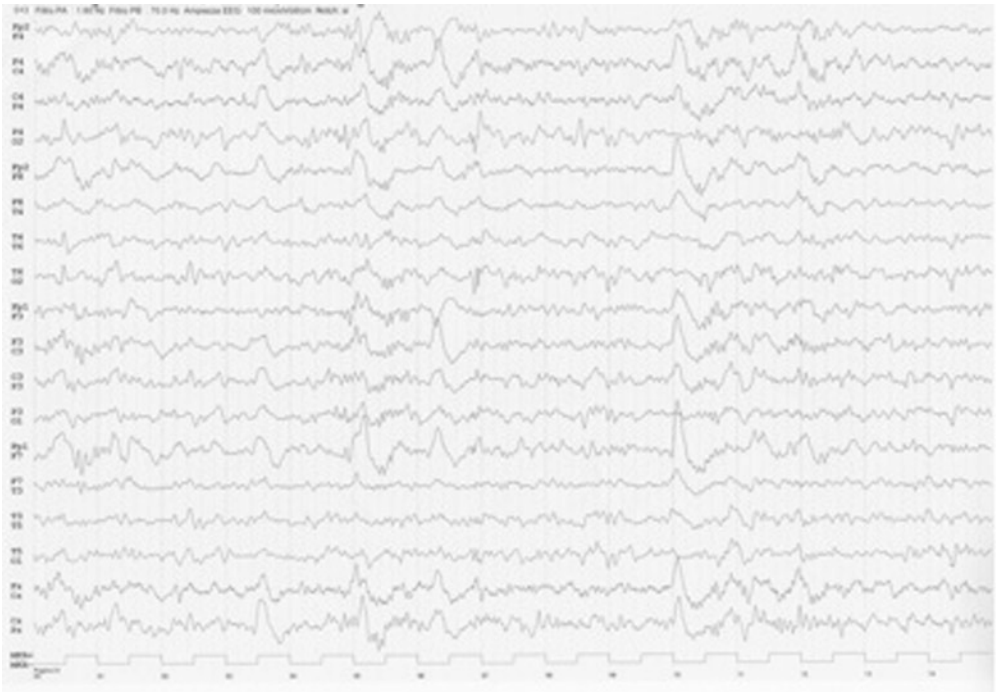

Fig. 3 Follow-up EEG (age 16 years) showing improvement with more background symmetry and reduction in the right hemispheric epileptiform activity

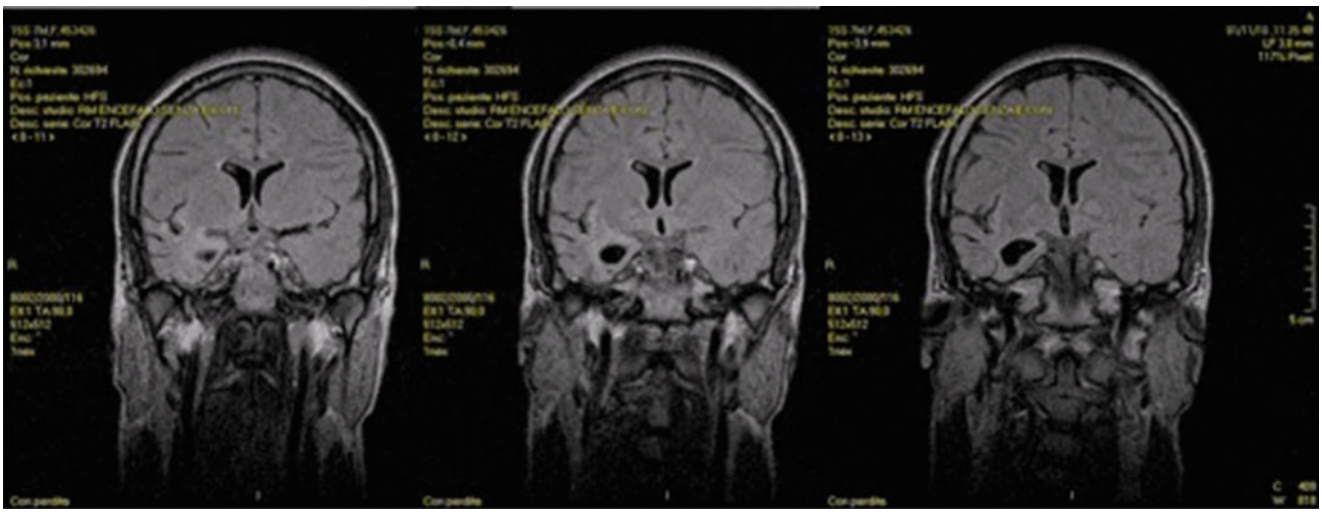

Fig. 4 Follow-up MRI after 2 year interval. FLAIR sequences show evolution of the lesion with loss of parenchyma in the right temporal and insular areas

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RESEARCH REPORT

Barriers to Transplantation in Adults with Inborn Errors of Metabolism

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Abstract *Background:* Transplantation in patients with inborn errors of metabolism (IEM) may be used as rescue therapy for acute decompensation, organ replacement, or disease-modifying therapy. We sought to quantify the use of transplantation in adults with IEM.

Methods: A 10-question online survey was sent through the email list of adult IEM physicians maintained by the Society for the Study of Inborn Errors of Metabolism and posted on the website of the Society of Inherited Metabolic Diseases.

Results: Thirteen centers from five continents responded. These centers, ranging in size from <50 adult patients (three centers) to >500 (two centers), reported 57 adult patients who had undergone transplantation. 29/57 (51 %) came from the two largest centers and 27/57(47 %) were renal transplants for Fabry disease (FD). Only seven transplants were identified as being done for acute decompensation. Eight of thirteen centers had not had patients with IEM passed over on the transplant list but four of these eight had not referred a patient for transplantation. 4/13 centers had patients passed over on the transplant list and reasons cited included: (a) transplant team not comfortable with underlying disease, (b) cognitive impairment in patient raised concerns about compliance, (c) multisystem disease makes single organ transplantation inappropriate, and (d) not at enough risk of life-threatening decompensation.

Conclusions: Excluding renal transplantation for FD, there is low use of transplantation in adults with IEM. Some barriers to transplantation reported by adult centers could be improved with development of educational and management modules for both transplant and metabolic programs.

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Introduction

Organ transplantation has been used as a therapeutic modality for many inborn errors of metabolism (IEM). Transplantation can be done to provide organ replacement therapy as in the case of patients with renal failure from Fabry disease (FD) (Weidemann et al. 2010) or methylmalonic aciduria (MMA) (McGuire et al. 2008). It can be a life-saving therapy for patients with acute metabolic decompensation from unstable conditions like maple syrup urine disease (Mazariegos et al. 2012) and urea cycle defects (UCD) (Morioka et al. 2005). Finally, it can be used to modify the disease course of progressive IEMs, particularly those with involvement of the central nervous system (such as Krabbe disease, metachromatic leukodystrophy, adrenoleukodystrophy, and mucopolysaccharidoses) where alternative treatment strategies are limited (Boelens et al. 2010). Recently, there has been a move to increase the availability of adult specialty clinics to allow the transition

of patients from the pediatric to the adult health care system. We sought to quantify the use of transplantation as a therapeutic modality in adults with IEM.

Methods

A 10-question online survey was sent out through an email list maintained by a representative of the Society for the Study of Inborn Errors of Metabolism (SSIEM) of physicians who identify their interest as the care of adults with IEM. The survey link was also posted on the Society for Inherited Metabolic Disorders (SIMD) website. The survey asked for details of center size (number of adult patients followed), location, and if the center had referred patients for transplantation (and, if so, indications for the procedure). Centers were also asked if they had referred a patient for transplantation who subsequently had been rejected by the transplant team or passed over while on the transplant waiting list (and, if so, indications for this decision). The survey was available for completion for 2.5 months before data were collected for analysis.

Results

Thirteen centers from five continents responded and details of their responses are shown in Table 1. These centers, ranging in size from <50 adult patients (three centers) to >500 (two centers), reported 57 adult patients who had undergone organ transplantation. 29/57 (51 %) of the transplant recipients were from the two largest centers and 27/57(47 %) of transplants were renal transplants for Fabry disease (FD). As expected, most transplants (38/57 or 67 %) were performed because of organ failure. Surprisingly few transplants were performed for acute metabolic decompensation (porphyria $N = 2$, UCD $N = 5$, four of which were at a single center), given that these centers collectively follow thousands of patients. A small number transplants were performed in patients with the intention of modification of disease course (metachromatic leukodystrophy (2), and Krabbe (1)) although, as the reasons for transplantation were not specified in all cases (adrenoleukodystrophy $N = 6$, GSD $N = 1$, and MNGIE $N = 1$), it is possible that some of the remaining cases were transplanted with the intention of modifying the disease course. One patient received a bone marrow transplant for myelodysplasia in the context of Gaucher disease.

Eight of 13 centers had not had patients with IEM passed over while on the transplant list but four of these eight had never referred a patient for transplantation so this may represent a referral bias. 4/13 centers had patients who were passed over while on the transplant list and reasons cited included: (a) transplant team not comfortable with underlying disease, (b) cognitive impairment in patient raised concerns about compliance with post-transplant care, (c) involvement of other organ systems by the IEM made single organ transplantation inappropriate, and (d) candidate considered to be not at enough risk of life-threatening decompensation to justify organ transplantation.

Discussion

Our study, the first to explore this area, reveals that transplantation for acute metabolic decompensation, or as a disease-modifying therapy, is infrequent in centers looking after adults with IEM. The majority of transplants were done for end-stage renal disease and such transplants (which are primarily organized by consultant nephrologist rather than the IEM center) are done for symptom management (such as end-stage renal disease regardless of cause) rather than to modify disease course. Only seven patients were reported to have received transplantation for acute metabolic decompensation (in relatively common IEM such as porphyria and UCD) despite the fact that the 13 centers who responded to our survey were caring for thousands of adults with IEM. In considering the barriers to transplantation in this patient population, they can be categorized into four groups: (a) patient-specific factors, (b) lack of evidence on treatment outcomes, (c) factors resulting from organ allocation policies, and (d) factors specific to the metabolic and transplant programs.

1. Patient-specific factors – when comparing pediatric patients with IEM with adults, it may be reasonable to assume that those patients who survive to adulthood have less severe disease and therefore are less likely to require a transplant for survival. While it is true that the survival rate of patients presenting over the age of 12 years with conditions like urea cycle defects and symptomatic hyperammonemia is much higher than those with earlier onset presentations (Enns et al. 2007), mortality rate is high in patients with severe symptoms of an IEM regardless of age. For example, 25 % of female OTC patients who present with coma (Enns et al. 2007) and 29 % of adults with acute presentations of MCAD (Lang 2009) will die. These publications (Enns et al. 2007, Lang 2009) reflect a publication bias in that they focus on symptomatic cases and many adult patients with IEM such as MCAD will be asymptomatic. However, it is clear from these publications that adults with severe symptoms of their IEM may have poor outcomes. Importantly, the IEM literature suggests that earlier transplantation results in improved neurologic outcomes in patients with urea cycle defects (McBride

Table 1 Summary of responses regarding organ transplantation from adult centers

	Location	Number of adults with IEM[a] followed at that center	Number of transplant procedures performed	Indications for transplantation				Number of patients with nonperformance[b] of transplantation
Centers with no patients receiving transplants	Europe	51–100	0	Not applicable				None reported
	Europe	51–100	0	Not applicable				None reported
	Europe	101–250	0	Not applicable				None reported
	Asia	101–250	0	Not applicable				None reported
	Australia	251–500	0	Not applicable				1
Organ				Kidney	Liver	HSCT[c]	Other	
Centers with patients who received transplants	Europe	<50	3	Fabry (2; combined with heart)		Gaucher with myelodysplasia (1)	Heart Fabry disease (2; combined with kidney)	None reported
	Europe	<50	2			MNGIE[d] (1)	Unknown (1)	None reported
	Europe	<50	2		GSD[e] (2)			None reported
	South America	51–100	10	Fabry (1); Porphyria (1)	UCD[f] (1); Porphyria (1)	ALD[g] (6)		2
	North America	251–500	8	Fabry (3); MMA[h] (1); cystinosis (1)	Porphyria (1)		Heart Fabry (2); GSD (1)	3
	Europe	251–500	3	Fabry (2)			Heart GSD (1)	3
	North America	>500	6	Fabry (1)	UCD (4)	Krabbe (1)		4
	Europe	>500	23	Fabry (19); MMA (1); Gaucher (1; combined with lung)		Metachromatic leukodsytrophy (2)	Lung Gaucher (1; combined with kidney)	None reported

[a] Inborn error of metabolism

[b] Nonperformance of transplantation refers to patients who are referred to the transplant service and rejected or are placed on the list and repeatedly bumped

[c] Hematopoetic stem cell transplantation

[d] Mitochondrial neurogastrointestinal encephalopathy

[e] Glycogen storage disease

[f] Urea cycle defect

[g] Adrenoleukodystrophy

[h] Methylmalonic aciduria

et al. 2004), maple syrup urine disease (Mazariegos et al. 2012), and some lysosomal storage diseases (Wynn et al. 2009). Finally, the burden of comorbid disease which may precipitate metabolic decompensation is higher in adults than in children (Summar et al. 2005; Lang 2009). These data suggest that severity of symptoms, rather than age of symptom onset, should be the main factor in considering transplantation.

2. Lack of evidence on therapy outcomes – there are no systematic studies available which assess the efficacy of transplantation in adults with IEM. Adults are included in many case series (for example, see Morioka et al. 2005; Summar et al. 2005; Mazariegos et al. 2012) and there is not a suggestion in these series that the outcomes are worse for the adults than for the children but data are not analyzed separately to make that determination. Such analysis is required as the risks of transplantation in adults (who have comorbid disease) will differ from those in children. Also, data on natural history of many IEM in adults are completely lacking making it difficult for clinicians to weigh the risks and benefits of transplantation. Finally, the lack of information on prognostic factors in adults makes it difficult to ascertain which patients are most likely to benefit from organ allocation. A registry which collects data on adults with IEM who have undergone transplantation might be one strategy by which evidence could be collected on the outcomes of transplantation in these patients.
3. Organ allocation policies – organs from cadaveric donors are a limited resource and existing organ allocation protocols, created to prioritize patients with end-stage organ failure, may disadvantage patients with IEM. If we consider liver transplantation, both the United Network for Organ Sharing (www.UNOS.org) and Eurotransplant (www.Eurotransplant.org) use the Model for End-Stage Liver Disease (MELD) scoring system to prioritize patients for liver transplant. The MELD score is based on INR, creatinine, and bilirubin so a patient without a defect in hepatic synthetic function, i.e., without end-stage cirrhosis, would have a low MELD score. It became clear that the MELD score does not adequately reflect the need for transplantation in some conditions including hepatocellular carcinoma, and IEM (Bernardi et al. 2011). To address this problem, a working group developed a short list of genetic conditions (including familial hyperoxaluria and familial amyloid polyneuropathy) to which exceptions to the MELD system are accepted (Freeman et al. 2006) and "unusual metabolic diseases" are included, although few are named as exceptions. A "Share-15" policy was adopted in the US (where 15 % of the organs were to be shared amongst patients with MELD scores below 15) but as programs used these organs for patients on their list with higher MELD scores (Washburn et al. 2011), this did not have the desired effect. In our study, some centers had experienced patients being withdrawn from the transplant list because they were not "sick enough" and this is understandable when one considers the limitations and restrictions of the current allocation process. Finally, as patients with IEM have a low incidence of comorbid disease, they might be expected to have better long-term survival than patients with other diseases like chronic hepatitis who are at risk of numerous extra-hepatic morbidities or recurrence of the primary liver disease (e.g., hepatitis C where graft recurrence is almost universal). Indeed, patient survival in large series patients with UCD undergoing liver transplantation was significantly higher than in patients undergoing liver transplantation for other reasons at that same center (Morioka et al. 2005). Age and need for life support are two powerful predictors of survival after liver transplantation (Dutkowski et al. 2011) and patients with IEM are likely to be younger, to be stable between episodes of decompensation, and to have fewer comorbid diseases than patients with end-stage liver disease or cancer and thus might be predicted to have improved survival after receiving liver transplantation relative to those with other diseases such as chronic hepatitis or hepatocellular carcinoma. Modifications to the allocation process could be made to adjust for these disparities and allow patients with IEM to be appropriately prioritized within the organ allocation ranking system. Such modifications could weight variables such as: (a) likelihood of recurrent acute decompensation, (b) risk of neurological injury with recurrent decompensation, and (c) expected life span after transplantation (to reflect age and comorbid diseases).
4. Program-specific factors – in our survey, 5/13 centers had not referred a patient for transplantation. Half of all the transplants reported in our survey were renal transplants for Fabry disease, a situation in which one could expect that the referral for transplantation would arise not from the metabolic center but from the nephrologist caring for the patient with end-stage renal disease. In centers that had referred patients for transplant assessment, patients were sometimes rejected because the transplant team was not familiar with the condition. This suggests that education of both metabolic and transplant centers about the use of transplantation in IEM, and ongoing discussion regarding referred patients and potential patient referrals, may increase the rate of referrals to transplant programs. Also, qualitative research which evaluated the functioning of transplant program committees at different centers (Volk et al. 2011) suggested subjective parameters such as the perception that the patient was "too

well" or concerns about psychosocial barriers including psychiatric disease and social supports affected decisions made by the program committee as to whether or not to list the patient for transplantation. In our survey, metabolic centers had experienced patients being rejected or passed over because they were perceived to be "too well" (even though they may be at risk of dying or neurologic compromise with the next disease exacerbation) or due to concerns about compliance with post-transplant immunosuppressive medications due to cognitive impairment. However, patients with IEM, even with cognitive impairment, may be capable of, and familiar with, following a very complex regime of tight nutritional control, use of metabolic formulae, and multiple medications and, in such patients, a transplant, by relaxing their dietary restrictions, may actually make it easier for them to comply with a treatment program. Thus, guidelines and education around the role that these subjective factors play in making a decision around listing a patient for transplantation may be helpful for transplant programs. Also, education, as well as a realization for the need for advocacy on behalf of these patients, would be helpful for metabolic centers to more effectively address these valid if subjective concerns which influence transplant program committee internal decisions.

Our study is limited by participation bias. We were interested in the perceptions of physicians caring for adults with IEM about transplantation and this study was not intended to be a survey of transplant outcomes. We recognize that not all centers caring for adults with IEM may have received the link to the survey and we have no way of ascertaining how many centers which did receive the link to the survey chose not to participate. Also, we cannot ensure that those centers who did respond included all patients under their care who had received transplants. Further, we cannot exclude that the study was free of the nonresponse or voluntary response bias that may have reflected a physicians experience or lack of experience with transplantation in their centers. However, despite these sources of potential bias inherent to any survey sampling, we believe that this study has highlighted some of the factors which IEM physicians feel may limit a patient's access to transplantation and may be an important starting point to further investigate barriers to the use of transplantation in the adult population with IEM.

Conclusions

The use of transplantation for acute metabolic decompensation or to modify the disease course in adults with IEM is uncommon. There is an urgent need to collect data on the outcomes of adult patients with IEM who undergo transplantation so as to allow clinicians to define prognostic factors and the risk:benefit ratio of considering organ transplantation for some indications. Some of the barriers to the use of transplantation may also be modified through the use of educational materials for transplant and metabolic programs and evaluation of guidelines which underlie organ allocation processes.

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Take Home Message

Barriers exist to the use of transplantation as disease-modifying therapy in adults with IEM.

Competing Interests

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No ethics approval was needed for this study.

Contributions

All authors contributed to the study design, data interpretation, and preparation of the manuscript. Dr. Sandra Sirrs serves as guarantor for this manuscript.

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CASE REPORT

Bone Dysplasia as a Key Feature in Three Patients with a Novel Congenital Disorder of Glycosylation (CDG) Type II Due to a Deep Intronic Splice Mutation in *TMEM165*

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Abstract Three patients belonging to two families presented with a psychomotor-dysmorphism syndrome including postnatal growth deficiency and major spondylo-, epi-, and metaphyseal skeletal involvement. Other features were muscular hypotrophy, fat excess, partial growth hormone deficiency, and, in two of the three patients, episodes of unexplained fever. Additional investigations showed mild to moderate increases of serum transaminases (particularly of aspartate transaminase (AST)), creatine kinase (CK), and lactate dehydrogenase (LDH), as well as decreased coagulation factors VIII, IX, XI, and protein C. Diagnostic work-up revealed a type 2 serum transferrin isoelectrofocusing (IEF) pattern and a cathodal shift on apolipoprotein C-III IEF pointing to a combined N- and O-glycosylation defect. Known glycosylation disorders with similar N-glycan structures lacking galactose and sialic acid were excluded. Through a combination of homozygosity mapping and expression profiling, a deep intronic homozygous mutation (c.792 + 182G>A) was found in *TMEM165* (*TPARL*) in the three patients. TMEM165 is a gene of unknown function, possibly involved in Golgi proton/calcium transport. Here we present a detailed clinical description of the three patients with this mutation. The TMEM165 deficiency represents a novel type of CDG (TMEM165-CDG). This disorder enlarges the group of CDG caused by deficiencies in proteins that are not specifically involved in glycosylation but that have functions in the organization and homeostasis of the intracellular compartments and the secretory pathway, like COG-CDG and ATP6V0A2-CDG.

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Abbreviations

ALT	Alanine transaminase
AST	Aspartate transaminase
CDG	Congenital disorder(s) of glycosylation
CK	Creatine kinase
IEF	Isoelectrofocusing
LDH	Lactate dehydrogenase

Introduction

Congenital disorders of glycosylation (CDG) are genetic defects of glycan synthesis and glycan attachment to proteins and lipids. Their number is steadily growing with some 50 CDG having been reported (Jaeken 2010, 2011; Rafiq et al. 2011; Saigoh et al. 2011; Baasanjar et al. 2011; Hennet 2012). Most of them are multisystem diseases, but skeletal abnormalities are an important/predominant clinical feature in only about 20 % of the known CDG. We present three patients, including a brother and sister, with a novel hypoglycosylation disorder characterized by a psychomotor retardation-dysmorphism syndrome with postnatal growth deficiency and major spondylo-, epi-, and metaphyseal skeletal involvement. This paper describes the clinical and laboratory findings in detail. The biochemical and molecular data are reported elsewhere (Foulquier et al. 2012).

Patients and Methods

Patients

Patient 1

This boy is the first child of unrelated Ashkenazi parents originating from Georgia. The second child is a healthy girl. The third child, a girl, was similarly affected and will be described in this paper as patient 2. Patient 1 was born at the postmenstrual age of 40 weeks after a normal pregnancy. Birth weight was 3,250 g (50th centile), length 50 cm (25th centile), and head circumference 36 cm (50th centile). He presented at the age of 5 weeks with feeding problems and failure to thrive due to cow's milk protein allergy. Growth delay was noted within months. At 19 months of age, length was 72 cm (3rd centile is 73 cm), weight 10.7 kg (10th centile), and head circumference 48.5 cm (50th centile). Fat excess, joint hyperlaxity, hypotonia, muscle weakness, and hepatosplenomegaly were noted during the first years. Psychomotor milestones were delayed: unsupported sitting at 9 months, independent walking at 2 years, and first words at 18 months. He was first seen in our hospital at the age of 11 years because of generalized epi-metaphyseal dysplasia and joint destruction tentatively diagnosed as Desbuquois syndrome. He presented with short stature (but normal head growth), generalized adiposity, and muscular hypotrophy. He had a waddling gait and was dysmorphic (midface hypoplasia, internal strabism of the right eye, moderately high arched palate, small teeth, low-set and stiff auricles, pectus carinatum, dorsolumbar kyphosis and severe sinistroconvex scoliosis, short distal phalanges, genua vara, pedes planovalgi (Fig. 1)). Additional clinical features included rapidly growing dense hair, long and dense eyelashes, delayed dentition, a hoarse voice, joint hyperlaxity (particularly of the interphalangeal joints) except for mild extension deficit of elbows and knees and hip abduction of 45°, joint "cracking" when walking, and generalized hyporeflexia and hypotonia.

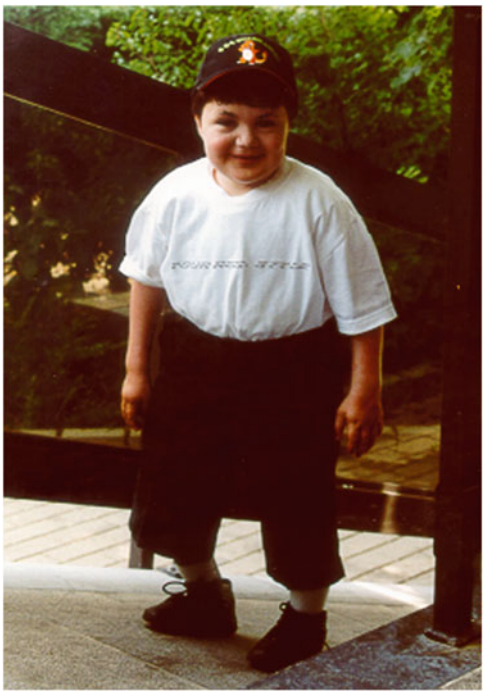

Fig. 1 Photograph of patient 1

Table 1 Abnormal results of serum parameters in the three patients

	Patient 1	Patient 2	Patient 3	Normal
AST (U/L)	195–357	229–528	224–459	<32
ALT (U/L)	43–54	32–59	32–104	<31
CK (U/L)	1,376–1,915	297–2,546	319–918	<145
LDH (U/L)	540–703	749–608	587–967	240–480
Factor VIII (%)	45	44	29	50–150
Factor IX (%)	63	42	30	70–130
Factor XI (%)	45	44	48	70–130
Protein C (%)	66	48	67	70–150

Laboratory investigations showed normal levels of serum alkaline phosphatases, calcium, phosphate, osteocalcine, 1,25-hydroxyvitamin D, immunoglobulin G, blood clotting factors II, V, X, XII, von Willebrand factor antigen, von Willebrand factor Ristocetine cofactor, and antithrombin, and increases of serum AST, ALT, CK, and LDH. There was a mild to moderate decrease of cholinesterase, and of clotting factors VIII, IX, XI, protein C, and protein S (Table 1). At age 16 years, there was an onset of adrenarche (circulating de-hydro-epi-androsterone-sulfate 52.4 μg/dL,

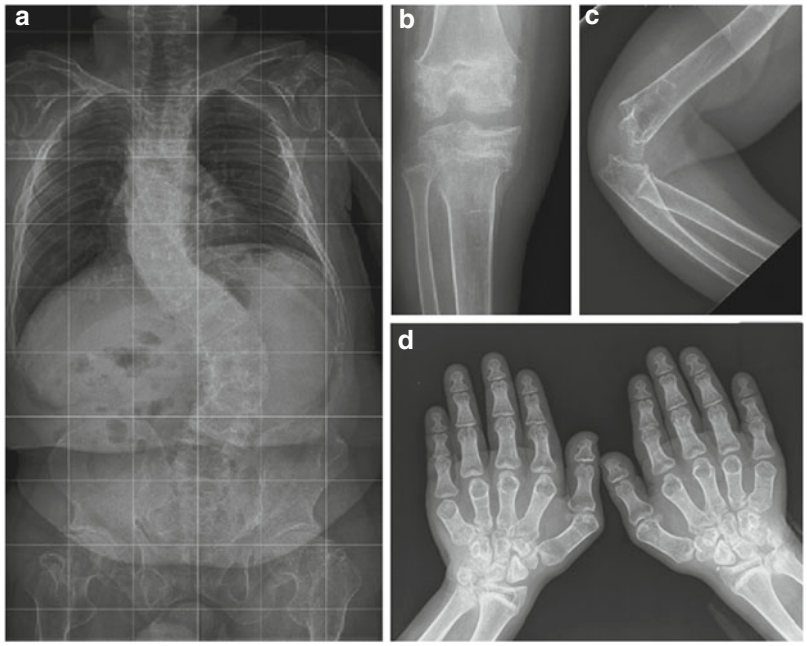

Fig. 2 Radiological presentation of the skeleton of patient 1: (**a**) full spine at 20 years; (**b**) knee at 14 years 4 months; (**c**) elbow at 15 years 6 months; (**d**) hands at 15 years 6 months

normal for age being 180–350 μg/dL) but not yet of puberty (circulating luteinizing hormone 0.4 IU/L (normal 1.7–8.6 IU/L), follicle stimulating hormone 0.3 IU/L (nl 1.2–7.7), total testosterone 25 ng/dL (nl 300–1,000), free testosterone 0.27 ng/dL (nl 5–20), sex hormone binding globuline 2.58 μg/dL (nl 0.7–1.6)).

Technical investigations: Imaging of the skeleton showed osteoporosis and important epi- and metaphyseal dysplasia with broad metaphyses, irregular epiphyses, and thin bone cortex. The vertebrae were flattened and beaked and the phalanges were broad (Fig. 2). On echocardiography heart morphology and function were normal. Ophthalmological examination revealed a right convergent strabism and "in fundo" temporal epithelial pigment alterations, which were more pronounced in the left eye. MRI of the brain showed some atrophy, enlarged ventricles, periventricular and subcortical white matter abnormalities, as well as relative hypoplasia of the anterior pituitary, and absence of the normal hyperintensity of the posterior pituitary. A skin biopsy was normal on histology as well as on electronmicroscopy.

Tentative treatment with growth hormone (28 μg/kg/day) in late prepuberty did normalize the circulating levels of IGF-I (from <75 μg/L to >200 μg/L) and did reduce total-body fat fraction from 28.5 % to 22.3 % within 1 year, but failed to elicit a detectable increment of growth velocity (possibly due to a concomitant aggravation of scoliosis) and was thus halted after 2 years. At age 21, the young man has a short stature (122.5 cm, Z-score −10) but a normal head circumference (55 cm, between 10th and 25th centiles) (Fig. 3).

Patient 2

This girl was the third child of unrelated Ashkenazi parents from Georgia and the sibling of patient 1. Pregnancy and delivery were uneventful. Hypotonia and hip dysplasia were noted in early infancy.

She was admitted at age 5 months because of psychomotor delay, hypotonia, vomiting, and an infection-related phase of respiratory insufficiency. The girl had ptosis of the right upper eyelid, torticollis toward the left, external strabism of the left eye, and had rather large lowly implanted ears, a long filtrum, a high arched palate, a short and broad neck, a broad thorax with increased nipple distance, a sacral dimple, absent second-toe nails, hypotonia, hyporeflexia, joint hyperlaxity, right hip dysplasia, and hepatosplenomegaly (respectively, 2 and 3 cm below the costal margin).

At 11 months she was readmitted because of recurrent, unexplained fever up to 40°C during 4 months. She was a rather obese baby with weight 8.81 kg (between 25th and 50th centiles), length 68 cm (between 3rd and 10th centiles), and head circumference 44.6 cm (25th centile). She could sit unsupported for a few moments and roll over from the side to the prone or supine position, but she was not babbling. She had a large fontanel. The abdomen was slightly enlarged with liver and spleen edge at 2 cm below the costal margin. No cause for the recurrent fever was identified.

At 14 months she was admitted with a pyelonephritis. Her weight was then 8.5 kg (10th centile), and her length and head circumference were virtually the same as at

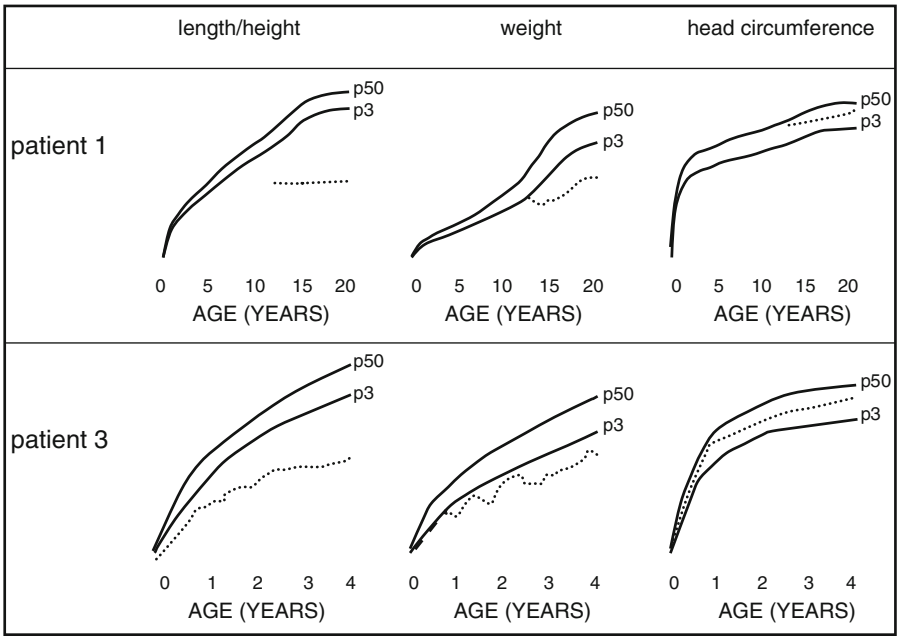

Fig. 3 Growth curves showing the evolution of length, weight, and head circumference in patients 1 and 3 (*dotted lines*); p3 is 3rd centile (*full line*), p50 is 50th centile (*full line*)

11 months (length Z-score −3.6, head circumference on 10th centile). One month later she died in an enterocolitis-related episode of shock.

Laboratory investigations showed normal results for the peripheral blood count, serum cholesterol (total, HDL-, and LDL-), lactate, albumin, amino acids, thyroxine, thyroid stimulating hormone, thyroxine-binding globuline, alkaline phosphatase, osteocalcine, arylsulfatase A, PT, APTT, clotting factors V, VII, X, XII, von Willebrand factor antigen and von Willebrand factor Ristocetine cofactor, protein S, platelet aggregation and platelet adhesion, as well as the immunoglobulins A, G, M, and D. Urinary amino acids, organic acids, and oligosaccharides were normal. Abnormal results included increases of serum AST, ALT, CK, LDH, and cerebrospinal fluid protein (566 mg/L; normal 120–450). There was a slight decrease of serum cholinesterase (6.3 kU/L; normal range 7–19) and of blood clotting factors VIII, IX, XI, antithrombin, and protein C (Table 1).

Technical investigations: Abdominal ultrasound examination was normal except for splenomegaly. Echocardiography was normal. Radiological examination of the skeleton showed generalized osteoporosis, discrete irregular metaphyses of the long bones, discrete plathyspondyly, broad iliac wings, horizontal acetabular roofs, and subluxation of the right femur. Ophthalmological investigation showed macular epithelial pigment alterations. Electrophysiological examination revealed normal sensory nerve conduction, whilst motor nerve conduction showed normal velocities but decreased amplitudes. On electromyography there were no active signs of denervation but sequelae suggestive of past denervation. Muscular biopsy showed normal histological and ultrastructural findings.

Patient 3

This boy was the fourth child of unrelated Ashkenazi parents from Georgia. This family was not related to the previously mentioned family. He was born after a normal 40 weeks pregnancy with birth weight of 3,360 g (25th centile) and length of 49 cm (between 3rd and 10th centiles). Hepatomegaly, a small open ductus Botalli and a small patent foramen ovale, were noted shortly after birth. The neonatal phase was complicated by blepharitis of the right eye, feeding difficulties (attributed to cow's milk intolerance), diarrhea, and irritability with opisthotonus. The boy presented craniofacial dysmorphism with relative macrocephaly, tongue protrusion, anti-mongoloid slanting of the eyes, a flat nose, and relatively large and posteriorly rotated ears. At age 3 months, he was admitted because of unexplained respiratory problems (restrictive lung pathology) for which long-term invasive ventilation via tracheostomy was started. Hypotonia, muscular weakness, body adiposity, joint hyperlaxity (with cracking noise on mobilization), and brisk tendon reflexes were noted. He had regularly bouts of fever up to 38.5 °C without evidence for infection. At 14 months he developed epilepsy, responding to levetiracetam. This could be be stopped at age 2 years 10/12. The mechanical ventilation could be stopped at age 3 and the tracheostomy was closed at age 4. At age 6 years,

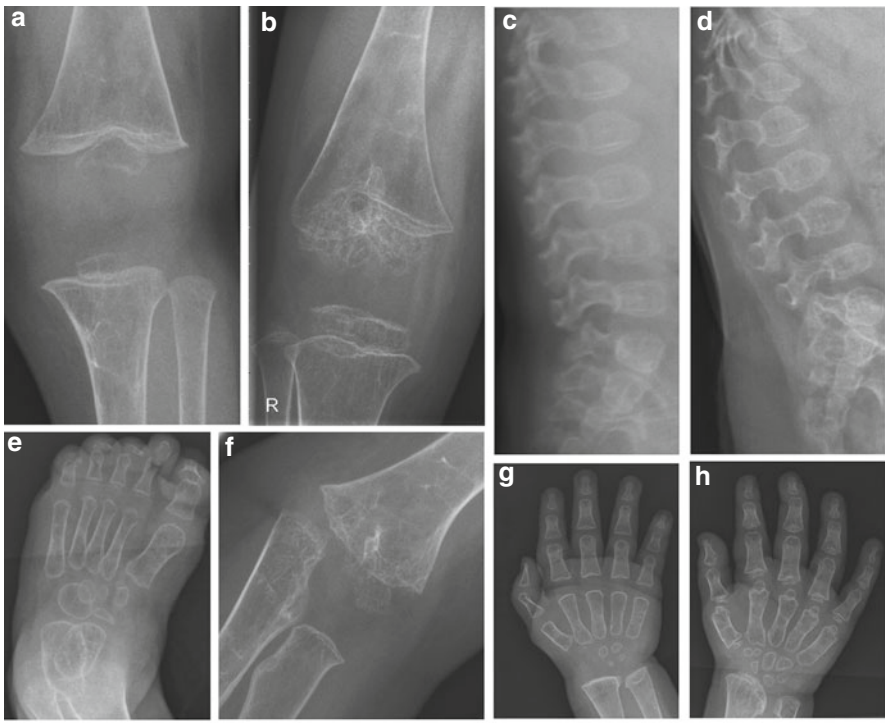

Fig. 4 Radiological presentation of the skeleton of patient 3: (**a**) knee at the age of 1 year; (**b**) knee at 5 years 7 months; (**c**) lumbar spine at 1 year; (**d**) lumbar spine at 4 years; (**e**) foot at 22 m; (**f**) elbow at 3 years 10 months; (**g**) hand and wrist at 1 year; (**h**) hand and wrist at 3 years 6 months. See text for description

he does not follow normally with his eyes, does not grasp for objects, and cannot sit unsupported; body length SDS is approximately −7 but head circumference is normal (49.9 cm, 25th centile) (Fig. 3). He has a distended, tympanic abdomen with liver edge at 3 cm and spleen at 2 cm below the costal margin.

Laboratory investigations showed normal serum calcium, phosphate, alkaline phosphatases, osteocalcine, arylsulfatase A, prolactin, TSH, free T4, PT, aPTT, and cholinesterase. Abnormal results included increases of serum AST, ALT, CK, LDH, and decreases of serum haptoglobin (<0.2 g/L; normal range 0.3–2), and of clotting factors VIII, IX, XI, protein C, and protein S (Table 1).

Tentative treatment with growth hormone (30 μg/kg/day, initiated at age 18 months) did normalize the circulating levels of IGF-I (from 35 μg/L to 59 μg/L) and did reduce total-body fat fraction from 27.4 % to 18.9 % within 5 months, but failed to elicit a detectable increment of growth velocity or a detectable improvement of respiratory status, and was thus halted.

Technical investigations: radiology of the skeleton at age 1 year revealed generalized osteopenia, a J-like sella turcica, hypoplasia of the skull base, mild anterior beaking of vertebrae D11, D12, L1, and L2, broad radial and ulnar metaphyses, strongly underdeveloped carpal bones, plump and broad phalanges, horizontal acetabula, very discrete opacification of the right proximal femur epiphysis, no ossification of the left proximal femur epiphysis, broad proximal femur metaphyses, broad metaphyses and strongly underdeveloped distal femoral and proximal tibial epiphyses (Fig. 4). Echocardiography at 14 months was normal except for a small pericardial effusion. Ophthalmological examination, electromyography (26 months), and motor and sensory nerve conduction velocities were normal. Brain MRI showed evidence for de- or dysmyelination and could not visualize the neural pituitary gland.

Methods

Isoelectrofocusing (IEF) of serum transferrin was performed as described (Jaeken et al. 1984).

IEF of serum apolipoprotein C-III was performed as described (Wopereis et al. 2003).

Transferrin N-glycan analysis by MALDI TOF MS was performed as described in Sturiale et al. 2008 and Papac et al. 1996 (data not shown).

Results

IEF of serum transferrin shows a type 2 pattern: decrease of 6-, 5-, and 4-sialotransferrin and increase of 3-, 2-, 1-, and 0-sialotransferrin (Fig. 5)

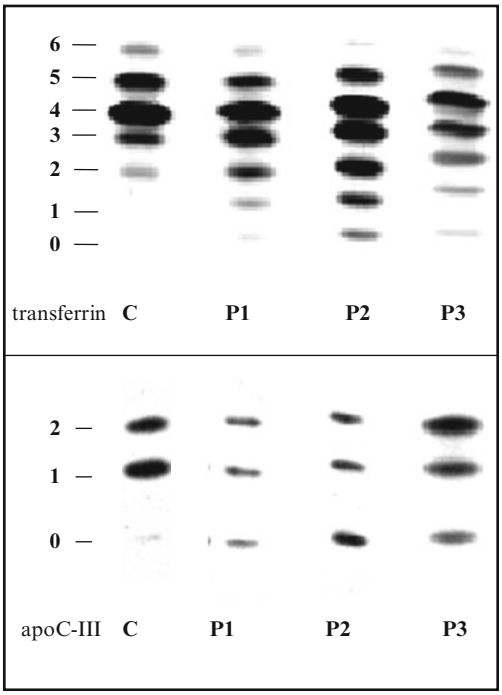

Fig. 5 Serum transferrin IEF and ApoC-III IEF in the three patients compared to a control. Sialo fractions (0 to 6 and 0 to 2, respectively) are indicated on the left. C is control; P1 is patient 1; P2 is patient 2, P3 is patient 3

IEF of apolipoprotein C-III shows a decrease of the monosialo-apo C-III and an increase of the asialo-apo C-III (Fig. 5)

Serum transferrin glycan analysis shows an increase of the biantennary glycan lacking sialic acid, and an abnormal biantennary glycan lacking sialic acid and galactose. In addition there is an increased fucosylation (data not shown)

Discussion

The described patients show a congenital disorder of glycosylation with a type 2 serum transferrin pattern and a cathodal shift of serum apoC-III pointing to a combined N- and O-protein glycosylation defect. The known glycosylation disorders with a type 2 serum transferrin IEF and similar N-glycan structures (such as the COG-CDG) were excluded by mutation analysis or linkage analysis.

Common clinical features include moderate psychomotor retardation, feeding problems (in infancy), facial dysmorphism with low-set ears, hypotonia, joint hyperlaxity, hepatosplenomegaly, osteoporosis, and spondylo-, epi- and metaphyseal dysplasia aggravating with age. After infancy (patients 1 and 3), midface hypoplasia, muscular hypotrophy, fat excess, growth retardation, and partial growth hormone deficiency completed this picture. An unusual symptom in patients 2 and 3 (seen also in some COG-CDG patients; Zeevaert et al. 2008) was recurrent fever without evidence for infection. Features only present in patient 3 (family 2) were respiratory problems due to unexplained restrictive lung pathology and transient epilepsy. It remains uncertain whether these features are secondary to the CDG.

The main biochemical findings in all three patients were an increase of serum AST (moderate), ALT (very mild), CK (moderate), and LDH (mild), as well as a decrease of coagulation factors VIII, IX, XI, and protein C. Ophthalmological examination in patients 1 and 2 showed macular epithelial pigment alterations, and was normal in patient 3. Common findings on brain MRI in patients 1 and 3 were white matter abnormalities and absent visualization of the neural pituitary gland.

Skeletal manifestations are an important/predominant part of the phenotype in only about 20 % of the known CDG: EXT1/EXT2-CDG (Jennes et al. 2009), B4GALT7 (Seidler et al. 2006), B3GAT3-CDG (Baasanjar et al. 2011), GALNT3-CDG (Chefetz and Sprecher 2009), SLC35D1-CDG (Hiraoka et al. 2007), LFNG-CDG (Sparrow et al. 2006), PIGV-CDG (Horn et al. 2011), autosomal recessive cerebrocostomandibular like syndrome due to COG1-CDG (Zeevaert et al. 2009), and ATP6V0A2-CDG (Guillard et al. 2009). The spectrum of these skeletal manifestations is very broad which can be explained by the fact that glycosylation is an important posttranslational modification of numerous proteins, including extracellular matrix

proteins and proteins involved in bone metabolism, for example, collagen I (Coman et al. 2007) and FGFR-3 (Winterpacht et al. 2000). The function of these proteins can be affected by a changed or absent glycosylation site. For example, a mutation affecting a putative glycosylation site of FGFR-3 leads to hypochondroplasia. Other hypotheses about the pathophysiology of bone abnormalities in CDG include lysosomal dysfunction (Garel et al. 1998) and polymorphisms in alternative pathways (Coman et al. 2008).

Thus, the bone anomalies in the three patients are unprecedented among CDG patients. Clinically, their skeletal phenotype can be classified in group 13 (among 40 groups) of the classification of genetic skeletal disorders (revision 2010), namely, the spondylo-epi-(meta)-physeal dysplasias (Warman et al. 2011).

Eventually, the molecular defect in these patients was identified through a combination of homozygosity mapping and expression profiling. The three patients are homozygous for a deep intronic splice mutation in *TMEM165 (TPARL)* c.792 + 182G>A (Foulquier et al. 2012). This mutation was not identified in 100 Jewish control alleles.

This disorder enlarges the group of CDG that results from deficiencies in proteins that are not specifically involved in glycosylation but that have functions in the organization and homeostasis of the intracellular compartments and the secretory pathway. Other examples of these are the COG-CDG (Foulquier 2009), ATP6V0A2-CDG (Guillard et al. 2009), and SEC23B-CDG (Bianchi et al. 2009; Schwarz et al. 2009). We propose to call this CDG subgroup "CDG plus".

Take Home Message

Bone dysplasia is the main clinical feature of three patients with a novel congenital disorder of glycosylation due to a mutation in *TMEM165*.

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