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RESEARCH REPORT

A Homozygous Splice Site Mutation in *SLC25A42*, Encoding the Mitochondrial Transporter of Coenzyme A, Causes Metabolic Crises and Epileptic Encephalopathy

Arcangela Iuso • Bader Alhaddad • Corina Weigel • Urania Kotzaeridou • Elisa Mastantuono • Thomas Schwarzmayr • Elisabeth Graf • Caterina Terrile • Holger Prokisch • Tim M. Strom • Georg F. Hoffmann • Thomas Meitinger • Tobias B. Haack

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Abstract SLC25A42 is an inner mitochondrial membrane protein which has been shown to transport coenzyme A through a lipid bilayer in vitro. A homozygous missense variant in this gene has been recently reported in 13 subjects of Arab descent presenting with mitochondriopathy with variable clinical manifestations. By exome sequencing, we identified two additional individuals carrying rare variants in this gene. One subject was found to carry the previously reported missense variant in homozygous state, while the second subject carried a homozygous canonical splice site variant resulting in a splice defect. With the identification of two additional cases, we corroborate the association between rare variants in *SLC25A42* and a clinical presentation characterized by myopathy, developmental delay, lactic acidosis, and encephalopathy. Furthermore, we highlight the biochemical consequences of the splice defect by measuring a mild decrease of coenzyme A content in *SLC25A42*-mutant fibroblasts.

Arcangela Iuso and Bader Alhaddad contributed equally with all other contributors.

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Introduction

The mitochondrial matrix hosts a variety of vital enzymatic functions including fatty acid and pyruvate oxidation and the citric acid cycle. As the matrix is surrounded by a double membrane, mitochondrial carriers are required for an efficient exchange with the cytosol of metabolites, nucleotides, and cofactors. To date 53 members of the solute carrier family 25 (SLC25) have been identified (Palmieri and Monne 2016; Palmieri 2004, 2013, 2014). In vitro experiments have suggested that SLC25A42 is able to transport adenine nucleotides, adenosine 3', 5'-diphosphate (PAP), and to a lesser extent coenzyme A (CoA) and dephosphoCoA (dPCoA) through a phospholipid bilayer (Fiermonte et al. 2009). It has been proposed that SLC25A42 could import cytosolic CoA or dPCoA in mitochondria in counter exchange with PAP or adenine

nucleotides (Fiermonte et al. 2009), allowing mitochondria and cytosol to keep their CoA pools separated (Leonardi et al. 2005). However, the identification of coenzyme A synthase (COASY) in the mitochondrial matrix converting 4-phosphopantetheine to CoA made this hypothesis questionable (Dusi et al. 2014). Based on the finding that CG4241, the *Drosophila* ortholog of *SLC25A42*, transports dPCoA into mitochondria (Vozza et al. 2017), it has been proposed that SLC25A42 might exchange mitochondrial dPCoA with cytosolic ADP, allowing CoA to be produced both in the matrix by COASY and in the cytosol by the monofunctional dPCoA kinase. CoA plays an essential role in various anabolic and catabolic pathways as well as regulation of cellular processes via allosteric interactions and gene expression. These include the biosynthesis of fatty acids, ketone bodies, and cholesterol, amino acid metabolism, fatty acid oxidation, biosynthesis neurotransmitter acetylcholine, and acetylation of histones and regulation of gene expression. Defects within three of the five enzymatic steps involved in CoA biosynthesis have been linked both to childhood-onset forms of neurodegeneration with brain iron accumulation (PANK2, COASY) (Dusi et al. 2014; Bosveld et al. 2008) and to dilated cardiomyopathy with no neurodegeneration (PPCS) (Iuso et al. 2018).

In 2016, Shamseldin et al. identified a missense mutation in a highly conserved amino acid of SLC25A42 in a subject presenting with mitochondrial myopathy characterized by muscle weakness, chronic fatigue, and dysarthria (Shamseldin et al. 2016). The same group reported that *SLC25A42* knockdown in zebra fish caused morphological anomalies and motor defects which are rescued upon injection of the human wild-type *SLC25A42* mRNA, but not the mRNA carrying the subject's variant (Shamseldin et al. 2016). Recently, Almannai et al. identified the same founder mutation in 12 additional individuals presenting with variable manifestations ranging from asymptomatic lactic acidosis to a severe phenotype characterized by developmental regression and encephalopathy (Almannai et al. 2018). In the current manuscript, we report on the identification of a novel homozygous canonical splice site variant and the previously described missense homozygous variant in *SLC25A42* in individuals with a metabolic encephalopathy thereby confirming the role of *SLC25A42* mutations in human disease. Moreover, we provide tentative evidence that *SLC25A42* loss of function (LOF) mutations can cause indeed decreased cellular coenzyme A.

Subjects and Methods

Subjects

Case 1 is a 6-year-old Saudi boy born preterm at 34 weeks of gestation after a normal pregnancy. His consanguineous parents (second-degree relatives) as well as the two older sisters are healthy. After birth, the patient had delayed respiratory adaptation which required the admission in a neonatal intensive care unit and sepsis. He showed repetitive pulmonary infections during the first year of life and recurrent episodes of gastroesophageal reflux. He started crawling at 24 months of age, but currently he is not able to sit or walk. Diagnostic follow-up with electromyography, nerve conduction study, and metabolic investigations (VLCFA, organic acids, guanidino metabolites) did not reveal any specific diagnosis. MRI scan at the age of 1 year was normal, but at the age of 3 years showed symmetric hyperintensities in the putamen (Fig. 1). The boy developed dystonia, profound muscular hypotonia, and severe cognitive impairment with absent speech. His workup included elevated levels of lactic acid. Clinical features are summarized in Supplementary Table 1.

Case 2 is a 9-year-old boy born at term to healthy unrelated German parents with normal birth measurements (birth weight, 2,650 g; APGAR scores, 10/10; cord blood pH. not known). At the age of 1 year, he had an upper airway infection with fever and loss of appetite for 4 days and was admitted with a metabolic crisis at the fifth day in a comatose state. His metabolic workup showed rhabdomyolysis with elevated creatine kinase activity (12,000 U/L), hypoglycemia (30 mg/dL), hyperammonemia (300 μmol/L), and severe metabolic acidosis with elevated liver enzymes (GOT 346 U/L, GTP 78 U/L, LDH 540 U/L). Lactate levels were persistently elevated. Abdominal sonography showed an enlarged liver without structural changes, and an echocardiography was unremarkable. Brain CT scans were normal, but the EEG showed clear increase of delta waves. His clinical condition stabilized, but 1 month later during another febrile episode, he developed tonic-clonic seizure. These crises of fever and seizures repeatedly occurred over the years necessitating anti-epileptic medication. Bayley scale testing showed that his psychomotor development was delayed throughout the disease course. Specifically, the patient showed mild linguistic impairment, behavioral disorder (with impulsivity and aggressiveness), and mild generalized muscular hypotonia. Testing for very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD) showed a slightly reduction of palmitoyl-CoA oxidation. Biochemical analysis of a muscle biopsy at the age of 2 years showed normal activities of respiratory chain complexes and PDHc, and histological examination was normal. Testing glycosylation patterns were unremarkable. At age of 3 years, his TSH level was slightly elevated (4.25 μU/mL), and he was treated with iodine. His EEG follow-up through his growth was unremarkable. MRI scan at age of 9 years showed abnormal bilateral signal alterations in the caudate nucleus (Fig. 1). Clinical features are summarized in Supplementary Table 1.

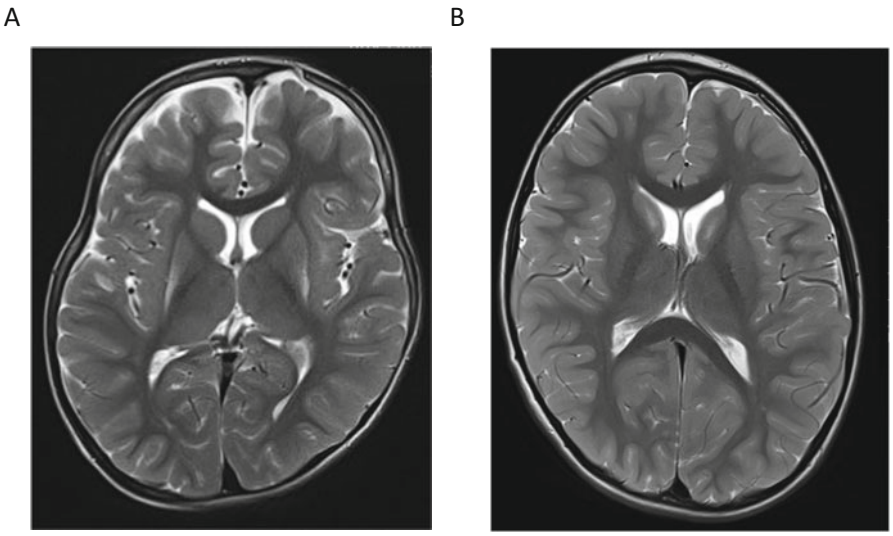

Fig. 1 MRI scans in cases with *SLC25A42* mutations. (**a**) The MRI of Case 1 taken at the age of 4 revealed bilateral putamen hyperintensities. (**b**) The MRI of Case 2 taken at the age of 9 revealed bilateral signal alterations in the caudate nucleus

Genetic Studies

Exome sequencing and variant filtering were performed as described previously (Haack et al. 2010). Briefly, coding DNA fragments were enriched with the SureSelect Human All Exon 50 Mb V5 Kit (Agilent, Santa Clara, CA, USA), and sequencing was performed on a HiSeq2500 system (Illumina, San Diego, CA, USA). Reads were aligned to the human genome assembly hg19 (UCSC Genome Browser) with Burrows-Wheeler Aligner (version 0.7.5), and detection of genetic variation was performed using SAMtools (version 0.1.19), Pindel (version 0.2.5a7), and ExomeDepth (version 1.0.0). 97.6% (Case 1) and 97.9% (Case 2) of the target were covered at least 20-fold.

RNA Sequencing and RT-PCR

RNA sequencing was performed as described (Haack et al. 2013). RNA was isolated from whole cell lysates using the AllPrep RNA Kit (Qiagen), and RNA integrity number (RIN) was determined with the Agilent 2100 Bioanalyzer (RNA 6000 Nano Kit, Agilent). For library preparation, 1 μg of RNA was poly(A) selected, fragmented, and reverse transcribed with the Elute, Prime, Fragment Mix (Illumina). End repair, A-tailing, adaptor ligation, and library enrichment were performed as described in the low-throughput protocol of the TruSeq RNA Sample Prep Guide (Illumina). RNA libraries were assessed for quality and quantity with the Agilent 2100 Bioanalyzer and the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). RNA libraries were sequenced as 100 bp paired-end runs on an Illumina HiSeq2500 platform.

For the RT-PCR, 1 μg of RNA was reverse transcribed with M-MLV reverse transcriptase (Promega, GmbH), and oligo dT. *SLC25A42* (NM_178526.4) exons were amplified with Thermo-Start *Taq* DNA Polymerase (ABgene, Epsom, UK) and primers listed in Supplementary Fig. 1a, applying the conditions described in Supplementary Fig. 1b, c.

Measure of Mitochondrial Content of Coenzyme A

One million cells were collected and spun down, and the pellet was resuspended in PBS in the presence of 0.5% NP40. The cellular membranes were broken by passing the suspension through a 1 mL insulin syringe ten times. Unbroken cells were removed by centrifugation at 1,500 rpm for 5 min. The cleared supernatant was recovered and used for the CoA measure according the manufacturer instructions (Abcam, ab138889). The kit is based on an ultrasensitive (detection limit of 40 nM) fluorometric detection of –SH group in CoA. It contains a proprietary fluorogenic acetyl-CoA green indicator dye that becomes strongly fluorescent upon reacting with –SH. For each measure, 50 μL of cleared cellular supernatant was used. Citation 3 was used for measuring fluorescence (BioTek).

Results

Identification of Rare Variants in *SLC25A42*

Exome data detected about 290 Mb regions of homozygosity in Case 1 confirming the consanguinity within the family. Based on that, a search for rare homozygous

variants with minor allele frequency (MAF) <0.1% in our in-house database comprising ~12,000 control exomes (04/2018) was performed. This search resulted in homozygous variants in 29 genes, which have been afterward filtered for previously reported genes in OMIM (phenotype key 3), HGMD, or ClinVar databases. This filtering step prioritized variants in three genes (*SLC25A42*, *UPB1*, and *HEATR2*) associated with autosomal recessive diseases. We considered *SLC25A42*, harboring the homozygous missense variant c.871A>G, p.Asn291Asp, as the best candidate gene based on the fact that the variant c.871A>G was previously reported as clinically relevant in individuals with overlapping clinical features (Almannai et al. 2018; Shamseldin et al. 2016). Furthermore, the variant is predicted to be damaging by in silico prediction programs (pph2, Sift, CADD). The variants in the other two genes (*UPB1* and *HEATR2*) did not fit or explain the phenotype of Case 1.

In Case 2, assuming a recessive mode of inheritance, a search for homozygous, potentially compound heterozygous, or X-chromosomal non-synonymous rare variants (MAF <0.1%) in our in-house database prioritized only one LOF variant, the homozygous splice change c.380+2T>A, p.(?) in *SLC25A42*. This variant is predicted to affect canonical splice donor site of intron 5 (Fig. 2A). The variant is present only once in heterozygous state in GnomAD database.

Sanger sequencing was used to confirm the identified mutations and test the carrier status of unaffected parents. Oligonucleotide sequences and PCR conditions are available upon request.

The c.380+2T>A Variant Abolishes the Canonical Splicing of *SLC25A42*

We investigated the possibility of alternative transcripts by analyzing RNA sequencing data generated from Case 2 and from control fibroblasts. Figure 2B summarizes in Sashimi plots all detected RNA splice variants. In control fibroblasts the only transcribed RNA was the canonical *SLC25A42* transcript (NM_178526.4). In Case 2 the canonical transcript was not expressed; instead three alternative transcripts were present. These transcripts differed from each other in the region encompassing exons 5 and 6. Two of these splice variants were produced by the usage of splice donor sites internal to exon 5, while a third variant was produced by retaining intron 5 in the transcript. In order to validate the presence of alternative transcripts, we performed an RT-PCR experiment with primers spanning exons 2–7 and 3–7. In Case 2 we observed three bands instead of one with all primer combinations. As control PCR we amplified the region spanning between exons 6 and 7, and we got only one band of the expected size both in the control and in Case 2 (Fig. 2C). Bands were cloned and sequenced. One fragment corresponded to an alternative transcript of *SLC25A42* missing 89 bp of exon 5, the other corresponded to a variant missing 46 bp of exon 5, and the last corresponded to a band having additional 541 bp and corresponded to the variant retaining the intron 5–6 (Fig. 2D). All three alternative splice variants might potentially encode for proteins of reduced molecular weight compared to the reference protein and share with it only the N-terminus region (https://www.ncbi.nlm.nih.gov/orffinder/). The variant with the 89 bp deletion might encode a protein of 19.6 kDa and the variant with the 46 bp deletion a protein of 14.6 kDa, and the variant with the intron retained a protein of 17.6 kDa (Fig. 2D). Immunoblot analysis with three different antibodies directed versus the N-terminus, C-terminus, or whole sequence of SLC25A42 was performed in order to verify the presence of alternative protein products in the fibroblast extract of Case 2. Unfortunately, all tested antibodies failed to detect the alternative forms in Case 2 fibroblasts, as well as the canonical SLC25A42 protein not only in Case 2, as expected, but also in control fibroblasts (not shown). Probably, the low expression level of *SLC25A42* in fibroblasts compared to other tissues (Supplementary Fig. 2) could explain why SLC25A42 is undetectable in total fibroblasts extracts.

Loss of *SLC25A42* Is Associated with Reduced Amounts of Coenzyme A in Fibroblasts

We measured total cellular CoA in fibroblasts from Case 2 and a healthy control. We found that the total cellular CoA was reduced of a 20% in Case 2 compared to control fibroblasts. Although mild, the CoA deficiency was consistently found in all independent measurements (Fig. 3).

Discussion

The homozygous missense mutation c.871A>G, p.Asn291Asp in *SLC25A42* was proposed as a molecular cause of mitochondrial myopathy initially in a single case of Arab descent (Shamseldin et al. 2016). Clinical features included elevated lactate levels in plasma, motor developmental delay, and proximal muscle weakness. Histological examination performed on a skeletal muscle biopsy showed ragged red-like fibers with enhanced subsarcolemmal oxidative enzyme activity as well as cytochrome c oxidase-negative fibers. Additional clinical findings included dysarthria, scoliosis, and nonprogressive myopia. Creatine kinase activity, nerve conduction velocities, brain MRI, and echocardiography were reported normal. The boy had normal cognitive function and the disease course was

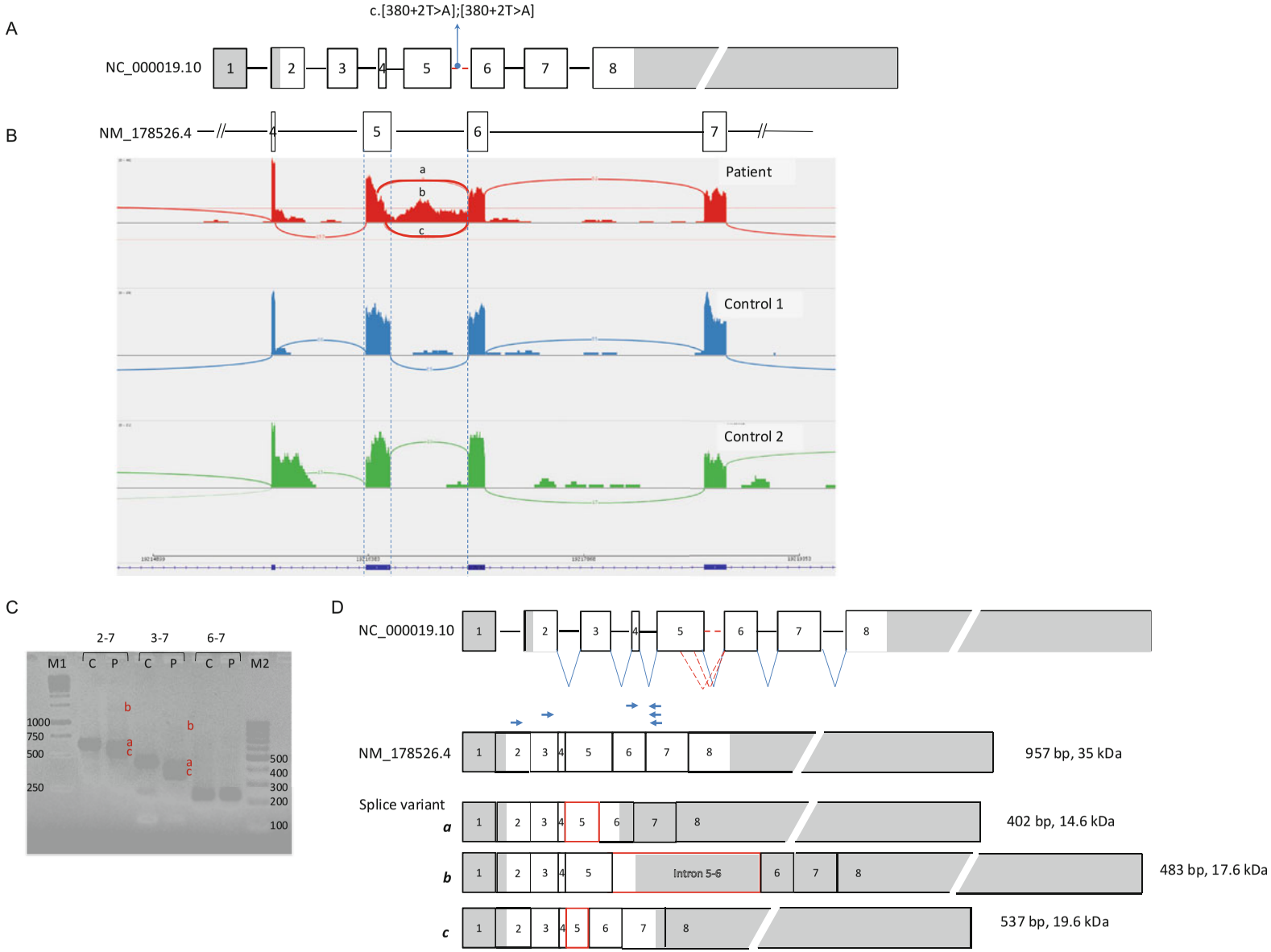

Fig. 2 *SLC25A42* gene and transcripts. (**A**) Schematic representation of the *SLC25A42* gene (NC_000019.10), and localization of the variant c. 380+2T>A in Case 2. The same length has been used for all introns. The mutation nomenclature is based on the splice variant NM_178526.4. (**B**) Sashimi plots summarizing the analysis of transcriptome data for *SLC25A42* in Case 2 and two unrelated control fibroblasts (Controls 1 and 2). The canonical transcript of *SLC25A42* is drawn at the top (NM_178526.4), while the three alternative transcripts are indicated in lowercase (a, b, c) directly on the Case 2 plot. (**C**) PCR products of cDNA from a control (C) and Case 2 (P) using primers spanning regions: exon 2–exon 7 (2–7), exon 3–exon 7 (3–7), and exon 6–exon 7 (6–7). M1 and M2 indicate 100 bp and 1 kb ladders, respectively. (**D**) Schematic representation of the canonical splicing (blue lines) producing the canonical transcript NM_178526.4, and the aberrant splicing (dashed red lines) producing alternative transcripts. Primer pairs used for the RT-PCR are indicated on the top of the canonical transcript

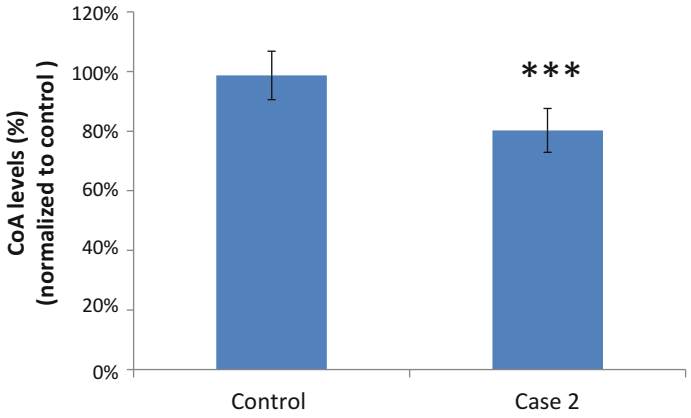

Fig. 3 CoA measurement. Total cellular CoA has been measured in control and in *SLC25A42*-mutant fibroblasts. Values were normalized to the median of the control in each independent experiment ($n = 2$). *p*-values were calculated with an independent sample *t*-test. All *p*-values were two-sided with a significance level of 0.05

stable. Subsequently, 12 additional cases of Arab descent carrying the same founder mutation and presenting with a broader phenotypic spectrum characterized by the presence of developmental delay and lactic acidosis as key clinical features were reported (Almannai et al. 2018). The severity of the clinical manifestations was highly variable even within affected individuals of the same family, ranging from asymptomatic lactic acidosis to severe intellectual disability, metabolic crisis, and multiorgan involvement. In four individuals was also noticed the appearance of a movement disorder, mainly dystonia. For the first time, *SLC25A42* mutations were associated with encephalopathy in several individuals.

In the current manuscript, we provide additional evidence for the implication of biallelic *SLC25A42* mutations in mitochondrial diseases, as we diagnosed two additional cases with pathogenic variants in *SLC25A42*. Case 1, of Arab descent, carried the already described missense change c.871A>G, p.Asn291Asp, while Case 2, of European descent, carried the homozygous splice change c.380+2T>A, p.(?) as a novel disease-causing mutation. Both individuals presented with lactic acidosis, muscular hypotonia, and developmental delay as observed in the case described in Shamseldin et al. (2016) and some cases from Almannai et al. (2018), although the muscular hypotonia and cognitive impairment were more severe in Case 1 than Case 2.

Moreover, Case 1 developed also dystonia, confirming the association of *SLC25A42* with movement disorders (Almannai et al. 2018). In both individuals brain MRI showed abnormalities, similarly to what observed in individuals with neurological symptoms described in Almannai et al. (2018). Our observations remark that specific mutations in SLC25A42 are likely to be identified in different ethnic populations, consistent with founder effects, and that SLC25A42 deficiency manifests not only with isolated myopathy plus lactic acidosis but includes often severe presentations with encephalomyopathy.

To further investigate the suggested role of SLC25A42 in CoA metabolism, we took advantage of available fibroblasts cell line (Case 2 only). Indeed, we observed a mild but significant reduction in the amount of total cellular CoA. We speculated that the defect might be more pronounced and functionally relevant in tissues containing more mitochondria and high *SLC25A42* expression levels (brain, muscle, liver, heart, and kidney). Considering that the concentration of CoA is almost 100-fold higher in mitochondria than in the cytosol, the reduction of total cellular CoA detected in our experiments might possibly reflect the decrease in mitochondrial CoA content. How SLC25A42 impairment leads to reduced cellular CoA remains unclear, and additional experiments are required to elucidate the mechanism leading to the defect. However, the decrease in cellular CoA suggests pantethine as possible treatment for *SLC25S42*-affected individuals. In fact, pantethine supplementation replenished CoA levels and improved the phenotype in a *Drosophila* model of PKAN (*PANK2*-associated neurodegeneration) presenting with reduced levels of CoA and neurodegeneration (Rana et al. 2010) and rescued the viability in a *Drosophila* model of *PPCS* deficiency (Iuso et al. 2018).

In conclusion, with the identification of two additional cases carrying mutations in *SLC25A42*, we corroborate the association between pathogenic mutations in *SLC25A42* and a heterogeneous clinical spectrum including myopathy, developmental delay, lactic acidosis, and encephalopathy. Furthermore, we highlight the functional consequences of the novel homozygous variant c.380+2T>A, suggesting a possible therapeutic approach for *SLC25A42*-affected individuals.

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Synopsis

Mutations in *SLC25A45* cause epileptic encephalopathy.

Details of the Contributions of Individual Authors

Conceived and designed the work: AI, BA, HP, and TBH. Performed the experiments: AI, CT, and EG. Analyzed and interpreted the clinical and genetic data: CW, UK, EM, TS, TMS, GFH, TM, and TBH. Drafted the article: AI, BA, and TBH. Critically revised the draft: all authors.

Compliance with Ethics Guidelines

Conflict of Interest

A. Iuso, B. Alhaddad, C. Weigel, U. Kotzaeridou, E. Mastantuono, T. Schwarzmayr, E. Graf, C. Terrile, H. Prokisch, T. M. Strom, G. F. Hoffmann, T. Meitinger, and T. B. Haack declare no conflict of interest.

Details of Ethics Approval

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

A Patient Consent Statement

Written informed consent was obtained from all individuals or caregivers.

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RESEARCH REPORT

Apparent Acetaminophen Toxicity in a Patient with Transaldolase Deficiency

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Abstract Transaldolase deficiency (MIM#: 606003) is a rare autosomal recessive defect in the pentose phosphate pathway. Affected individuals are at risk for progressive liver failure and hepatocarcinoma. In the transaldolase-deficient mouse model (*Taldo1*$^{-/-}$), these hepatic complications are accentuated by oxidative stress related to acetaminophen administration. We report a 13-month-old transaldolase-deficient male who developed mild liver failure after receiving standard doses of acetaminophen during a febrile respiratory syncytial virus infection. He was admitted for respiratory distress with neutropenia and thrombocytopenia, but developed an enlarged nodular liver with accompanying splenomegaly and rising alpha-fetoprotein which peaked 2 weeks after acetaminophen exposure. Whole exome sequencing revealed compound heterozygous variants c.512_514delCCT (p.Ser171del) and c.931G > T (p.Gly311Trp) in *TALDO1* (HGNC:11559), which encodes transaldolase (EC 2.2.1.2), a key enzyme in ribose metabolism. Urine polyols and plasma metabolomics confirmed the diagnosis of transaldolase deficiency. Studies on the *Taldo1*$^{-/-}$ mouse model demonstrate acetaminophen-induced liver failure can be prevented by administration of the antioxidant *N*-acetylcysteine. Moreover, a published report showed treatment of a transaldolase-deficient patient with *N*-acetylcysteine was associated with a decrease in alpha-fetoprotein levels. After discontinuation of acetaminophen and prior to initiation of *N*-acetylcysteine treatment, our patient demonstrated resolving alpha-fetoprotein levels suggesting acetaminophen incited the liver failure.

Conclusion: Our observations support the conclusion from mouse model studies that transaldolase-deficient patients are uniquely sensitive to acetaminophen and should avoid this antipyretic. Recognition of this individualized toxicity and avoidance of acetaminophen are essential for management of these patients.

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Introduction

Transaldolase deficiency (MIM#: 606003) is a rare inborn error of pentose metabolism first described in a Turkish family in 2001 (Verhoeven et al. 2001). Typical features include intrauterine growth restriction, triangular faces, loose wrinkly skin at birth, and development of progressive liver failure (Eyaid et al. 2013). Transaldolase (EC 2.2.1.2) is encoded by *TALDO1* (HGNC:11559) and catalyzes the rate-limiting reversible conversion of sedoheptulose-7-P and glyceraldehyde-3-P to erythrose-4-P and fructose-6-P, an essential step in the pentose phosphate pathway. Deficiency impairs

recycling of ribose-5-P through the non-oxidative branch of the pentose phosphate pathway and leads to reduced synthesis of antioxidants NADPH, NADH, and glutathione (Hanczko et al. 2009). As a result, these patients are predicted to be sensitive to oxidant stress. Consistent with this prediction, the transaldolase-deficient mouse model, *Taldo1*$^{-/-}$, demonstrates increased oxidative stress and sensitivity to acetaminophen toxicity (Hanczko et al. 2009; Perl et al. 2011). Here, we report on a 13-month-old transaldolase-deficient boy who developed nodular hepatosplenomegaly after receiving standard doses of acetaminophen during a respiratory syncytial virus infection. Our observations support the suggestion that these patients are uniquely sensitive to standard doses of acetaminophen and stress the importance of early diagnosis and informed management of this disorder.

Materials and Methods

High-Throughput Sequencing

The family was submitted to the Baylor-Hopkins Center for Mendelian Genomics. Genomic DNA was purified from fresh whole blood using the Gentra Puregene Kit (Qiagen Sciences, Germantown, MD). SureSelect Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA) was used for exon targeting and paired end 100 bp reads using the Illumina HiSeq2000 platform (Illumina, Inc., San Diego, CA). Read alignment to reference genome (NCBI human genome assembly build 36) (Hubbard et al. 2009) was performed using the Burrows-Wheeler Alignment tool (Li and Durbin 2009). We also performed local realignment and base call quality recalibration using GATK (McKenna et al. 2010). Using the PhenoDB Variant Analysis Tool (Sobreira et al. 2015), we analyzed the whole exome sequencing data by applying a filter designed to prioritize rare (Minor Allele Frequency $< 1\%$) functional variants (missense, nonsense, splice site variants, and indels) that were homozygous or compound heterozygous in the proband. Urine polyol and sugar analysis was performed by Baylor Genetics Laboratories by gas chromatography-mass spectrometry stable isotope dilution analysis. Plasma metabolomics were performed by Baylor Genetics Laboratories using a combination of liquid chromatography-mass spectrometry/mass spectrometry technologies.

Results

Our 13-month-old patient was the 2.3 kg (4th percentile) product of a 37.5-week gestation in a 39-year-old G4P1122 woman whose pregnancy was complicated only by onset of intrauterine growth restriction starting at approximately 30 weeks of gestation. Birth length was 47.6 cm (30th percentile), and head circumference was 31.5 cm (7th percentile). At birth, he had a bronzed, aged appearance with wrinkled skin, decreased subcutaneous fat, prominent subcutaneous veins, open sagittal suture, enlarged anterior fontanelle, and pointed nasal tip (Fig. 1a). Complete blood count revealed leukopenia (7.8×10^9/L), thrombocytopenia (100×10^9/L), and normal hemoglobin (151 g/L). He required a 4-day stay in the neonatal intensive care unit for hypothermia (35.4°C) and hypoglycemia (glucose nadir 1.89 mmol/L).

Over the first year of life, he continued to have a bronzed, aged appearance with minimal subcutaneous fat, prominent wrinkles in the skin of his hands, visible scalp veins, a large abdomen without reported hepatomegaly, and thin extremities (Fig. 1a). He was followed monthly by his pediatrician until the age of 7 months. His growth curve remained below the third percentile with length $Z = -3.5$ and weight $Z = -2.77$ (Fig. 1b). He had no prior hospitalizations and exhibited mild language delays with two words ("mama"/"dada") at 13 months.

At 13 months of age, he was admitted to the hospital with fever and stridor secondary to a respiratory syncytial virus positive illness of 4 days duration. He received three doses of acetaminophen at home over 3 days prior to admission and two doses in the hospital over the first day of admission (all 10–15 mg/kg/dose). By the second day of admission, he developed firm, nodular hepatosplenomegaly. Ultrasound and MR imaging of his liver on the third hospital day revealed a 9 cm liver (sagittal measurement) with numerous T1 hyperintense nodules throughout, the largest measuring 1.1 cm (Fig. 1c). Elastography stiffness measurement of 2.7 m/s correlated with moderate to advanced hepatic fibrosis. His spleen measured 8.8 cm in length with a volume of 115 cm, consistent with mild splenomegaly. The nodular hepatomegaly has remained stable (9–9.5 cm sagittal measurement on ultrasound) for 1 year since admission. His splenomegaly has since resolved.

Laboratory investigation during his hospitalization revealed elevated alpha-fetoprotein (AFP) (peak 319 μg/L), elevated alanine aminotransferase (ALT, 73 units/L), prolonged INR (1.3), leukopenia, and thrombocytopenia (white blood cell count 1.4×10^9/L, hemoglobin 105 g/L, platelets 53×10^9/L). Plasma amino acids were remarkable for a mildly elevated methionine (58 μmol/L, normal range 7–43) suggestive of hepatocellular dysfunction. Urine organic acids 12 days after acetaminophen dosing showed increased fumarate, glutarate and significantly increased excretion of 2-ketoglutarate suggestive of a disturbance of mitochondrial energy metabolism. The peak of 5-oxoproline (pyroglutamate) was within normal for age. He had normal results for total bilirubin, direct bilirubin, aspartate amino transferase (AST), and a mildly shortened prothrombin time (11.4 s) and activated partial thromboplastin time (29 s).

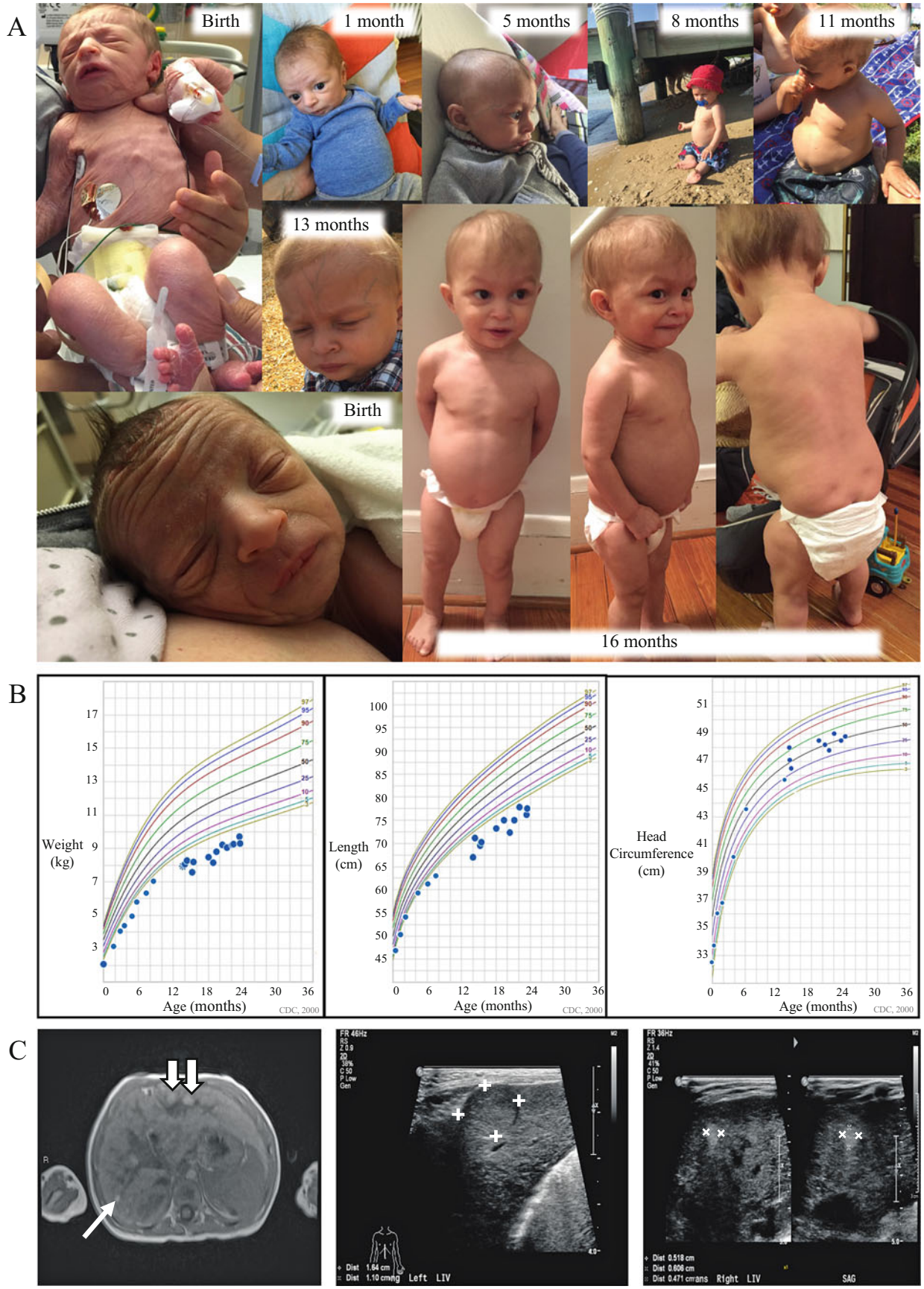

Fig. 1 Physical features. (**a**) Our patient was noted to have extensive skin wrinkling particularly at birth and decreased subcutaneous fat with easy visualization of vessels. (**b**) He continued to grow at less than the fifth percentile for both weight and height, with normal head

Whole exome sequencing performed through the Baylor-Hopkins Center for Mendelian Genomics at 15 months of age revealed compound heterozygous variants in the *TALDO1* gene: maternally inherited c.512_514delCCT (p.Ser171del) and paternally inherited c.931G > T (p.Gly311Trp). The p.Gly311Trp variant is not reported in gnomAD (Lek et al. 2016). Glycine-311 is part of a highly conserved hydrophobic cluster which contributes to β-strand packing (Thorell et al. 2000). An alternative variant in the same codon, p.Gly311Arg, was previously described in a nonconsanguineous Chinese child with transaldolase deficiency (Balasubramaniam et al. 2011). The second variant, deletion of Ser-171, causes inactivation and proteasome-mediated degradation of transaldolase (Grossman et al. 2004) and was the causative variant identified in a consanguineous Turkish patient with transaldolase deficiency (Verhoeven et al. 2001). Urine polyol and sugar profiling demonstrated elevated arabitol, erythritol, ribitol, and sedoheptulose with normal xylitol and galactitol consistent with a diagnosis of transaldolase deficiency. Plasma metabolomic testing revealed elevated ribitol, ribonate, erythronate, and arabitol (*Z*-scores all greater than 4.6), numerous elevations of other compounds involved in primary and secondary bile acid metabolism consistent with liver dysfunction, and decreased levels (*Z*-scores less than −2.3) of compounds involved in sphingolipid metabolism.

AFP levels peaked to 319 μg/L 12 days after the last acetaminophen dose and then decreased over the following weeks, most recent level 12 μg/L 429 days after admission (Fig. 2, left axis). AST and ALT peaked 4 days after the last acetaminophen dose (maximum AST 70 units/L, ALT 73 units/L) followed by a downward trend. The patient started *N*-acetylcysteine (NAC) at 15 mg/kg/day 73 days after admission (Fig. 2, right axis) titrating the dose to a treatment goal of 100 mg/kg/day (Rodan and Berry 2016). AFP levels have continued to decrease (Fig. 2).

Discussion

Transaldolase deficiency is a rare inborn error that can progress unrecognized until significant liver damage has been acquired. *Taldo1*$^{-/-}$ mouse studies demonstrate increased sensitivity to acetaminophen toxicity (Hanczko et al. 2009; Perl et al. 2011) – consistent with our clinical observation of nodular hepatosplenomegaly with elevated AFP after administration of standard doses of acetaminophen in a transaldolase-deficient patient. Our report is the first description of suggested increased sensitivity to acetaminophen in humans with transaldolase deficiency.

Prior to his presentation, our patient's symptoms were mild, and his liver was not noted to be enlarged. His parents deny acetaminophen administration until the time of the illness that lead to his hospitalization at 13 months old. The stable nodular hepatomegaly in our patient 1 year after his admission, despite now near-normal AFP levels, suggests the acetaminophen and possibly the viral infection acutely exacerbated underlying liver disease. There are case reports of children with transaldolase deficiency with the presence of liver cirrhosis presenting at ages younger than our proband although the exposure of these patients to acetaminophen is unknown (Eyaid et al. 2013; Balasubramaniam et al. 2011; Verhoeven et al. 2005).

Urine organic acids on our patient were suggestive of a disturbance in mitochondrial energy metabolism, with increased fumarate, glutarate, and significantly elevated 2-ketoglutarate, similar to results in other patients with transaldolase deficiency (Engelke et al. 2010). *Taldo1*$^{-/-}$ mice hepatocytes also demonstrate mitochondrial dysfunction with decreased mitochondrial membrane potential, mitochondrial mass, nitric oxide production, glutathione, NADH, and NADPH/NADP ratio (Hanczko et al. 2009). Human transaldolase-deficient lymphoblasts demonstrate reduced NADPH (Qian et al. 2008). The impaired ability to tolerate oxidative stress likely increased the pathogenicity of acetaminophen in our patient, compounded by possible reactive hepatitis which has been observed with respiratory syncytial virus infections (Oh et al. 2016). Despite our patient's markers of mitochondrial energy disturbance, the level of 5-oxoproline (pyroglutamate) on urine organic acids was within normal limits for age. This contrasts the reports of pyroglutamic acidosis with acetaminophen toxicity or chronic exposure in patients without transaldolase deficiency (Fenves et al. 2006) – however, our patient's sample was collected 12 days after exposure.

The decrease in compounds involved in sphingolipid metabolism may represent a hitherto undiscovered connec-

Fig. 1 (continued) circumference. (**c**) At 13 months of age, he had significant radiological abnormalities including a non-contrast T1-weighted MRI (*left*) demonstrating hepatomegaly and heterogeneous liver parenchyma with multiple hyperintense hepatic nodules including two within the anterior left hepatic lobe (*thick arrows*) and one within the right hepatic lobe (*thin arrow*). Representative ultrasound image of the larger left anterior hepatic nodule (*middle, between calipers*) demonstrates well-defined contour iso- to hypoechoic to surrounding fibrotic liver parenchyma with contrast enhancement pattern suggestive of regenerative nodule. Elastography stiffness measurement of 2.7 m/s correlates with moderate to advanced hepatic fibrosis. There is an additional persistent hyperechoic right-sided lesion (*right*), with MRI and ultrasound characteristics suggestive of lipid-rich adenoma versus atypical hemangioma

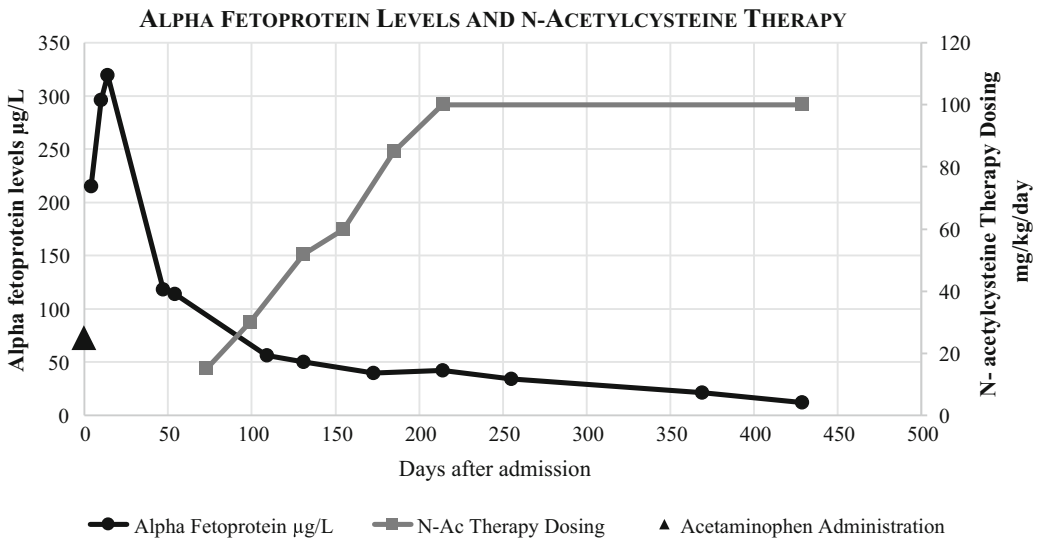

Fig. 2 Acetaminophen exposure, laboratory monitoring, and therapeutic strategy. The patient received five doses of acetaminophen over 4 days and was discontinued on the second day of admission. After acetaminophen was discontinued, the patient's alpha-fetoprotein levels continued to rise and then gradually fell over a period of 6 months (*left axis*). Therapy with *N*-acetylcysteine was titrated upward to a goal of 100 mg/kg/day (*right axis*)

tion between sphingolipid metabolism and the pentose phosphate pathway. Alternatively, this may relate to generalized liver dysfunction. For instance, in patients with chronic hepatitis B viral infection, there is a statistically significant decrease in several sphingolipid ratios independently related to the presence of cirrhosis – including ceramide d18:1/18:1 and sphingomyelin d18:1/18:1 which were also decreased in our patient (Zheng et al. 2015). The specific mechanism of these decreases has not been elucidated and may be related to the hepatitis B viral infective process rather than the cirrhosis – but this association warrants further investigation.

Acetaminophen is one of the most commonly used antipyretics and analgesics in the pediatric population with recent usage by families reported in 10–26% of children (Vernacchio et al. 2009). Children with transaldolase deficiency represent a small but increasingly recognized population in which studies suggest that acetaminophen is toxic and liver damage can be present with only mild to no elevation in transaminases, as seen in our patient and cases in literature (Verhoeven et al. 2001, 2005; Tylki-Szymańska et al. 2009). Although the development of hepatomegaly and elevated AFP correlated well with our patient's nodular hepatomegaly, AFP is not routinely sent on pediatric patients and most acetaminophen administration occurs outside the hospital setting. Therefore, significant liver damage may occur prior to diagnosis.

Taldo1$^{-/-}$ mice are highly susceptible to development of hepatocellular carcinoma and acetaminophen-induced liver failure; however, these deleterious effects were blocked by lifelong administration of NAC (Hanczko et al. 2009). NAC in humans is well tolerated and was associated with decreasing AFP values in a transaldolase-deficient patient (Rodan and Berry 2016). Our patient's elevated AFP and ALT were resolving prior to initiation of NAC treatment (Fig. 2). This is possibly the result of his liver recovery after injury or the natural decline of AFP by age seen in other patients with transaldolase deficiency (Lipiński et al. 2018), rather than the presence of therapeutic dosing levels of NAC treatment. The impact on morbidity and mortality from NAC administration in humans remains to be studied.

Heterozygosity for loss of function *TALDO* variants is more frequent than complete loss of function. Whether or not this is of consequence under conditions of increased oxidative stress remains to be determined. The liver architecture of heterozygous *Taldo1*$^{+/-}$ mice demonstrates increased anisonucleosis and nodular dysplasia with a reduced NADPH/NADP ratio; however, *Taldo1*$^{+/-}$ mice do not demonstrate increased susceptibility to acetaminophen-induced liver failure (Hanczko et al. 2009). Our patient's parents deny any recognized adverse responses to acetaminophen.

Conclusion

We report liver damage in association with acetaminophen usage in a patient with transaldolase deficiency, supporting the clinical importance of avoidance of acetaminophen in individuals with transaldolase deficiency.

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Synopsis

Our observations emphasize the individual susceptibility of patients with rare inborn errors of metabolism and specifically support the clinical importance of avoidance of acetaminophen in individuals with transaldolase deficiency.

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Compliance with Ethics Guidelines

Conflict of Interest Statements

Jasmine Lee-Barber, Taylor E. English, Jacquelyn F. Britton, Nara Sobreira, Jason Goldstein, and David Valle declare that they have no conflict of interest. Hans Tomas Bjornsson is a consultant for Millennium Pharmaceuticals, Inc.

Informed Consent

Our study was approved by the Johns Hopkins Medicine Institutional Review Board. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of the proband for being included in the study. Additional informed consent was obtained from the parents of the proband for which identifying information is included in this article.

Institutional Committee for Care and Use of Laboratory Animals

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

Details of the Contributions of Individual Authors

Drs. Lee-Barber and Bjornsson conceived the study, wrote the manuscript, and provided clinical data. Drs. Sobreira and Valle performed sequencing and analyzed sequencing data. Drs. English and Goldstein and Mrs. Britton provided clinical data. All authors were involved in revisions.

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RESEARCH REPORT

Sialuria: Ninth Patient Described Has a Novel Mutation in *GNE*

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Abstract Sialuria is a rare autosomal dominant inborn error of metabolism characterized by cytoplasmic accumulation and urinary excretion of gram quantities of free sialic acid due to failure of feedback inhibition of the rate-limiting enzyme in the sialic acid synthesis pathway, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE/MNK). To date, eight cases had been published worldwide, all with heterozygous missense variants at the allosteric site, specifically at Arginine 294 (formerly 263) and Arginine 297 (formerly 266) of GNE. The described cases so far have rather homogeneous clinical features which include developmental delay, mildly coarse features, hepatomegaly and prolonged neonatal jaundice. The apparent rarity of this disorder is hypothesized to be due to the variable and sometimes transient nature of the clinical features and to the absence of routine testing for urinary sialic acids. Here we present the ninth case of sialuria diagnosed in a child investigated because of clinical signs and symptoms and furthermore describe a novel pathogenic variant in the associated gene, *GNE*.

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Introduction

Sialuria (MIM 269921) is a rare autosomal dominant, inborn error of metabolism characterized by constitutive overproduction, cytoplasmic accumulation and urinary excretion (>1 g/day) of free sialic acid (Seppala et al. 1991; Hinderlich et al. 2015). This is the result of failure of feedback inhibition of the rate-limiting enzyme in the sialic acid synthesis pathway, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE/MNK) (Seppala et al. 1999).

The term sialuria is derived from the Greek 'sialos' owing to, at least in part, the identification of sialic acids in salivary mucins some 70 years ago (Varki and Schauer 2009). Sialic acids are a group of more than 50 different (Varki and Schauer 2009) negatively charged acetylated nine-carbon sugars derived from neuraminic acid, which decorate glycoproteins on most cell surfaces (reviewed in Varki 2008). They play a myriad of roles in glycobiology, including but not limited to, cellular recognition, binding of the influenza virus (Suzuki 2005) and metastatic cancers (Bull et al. 2014; Chen et al. 2016). The critical role of sialic acids in vivo is epitomized by the early embryonic lethality of the GNE loss of function murine model (Schwarzkopf et al. 2002). Despite this, loss of expression in vitro appears to be better tolerated (Abeln et al. 2017). Equally, the murine model overexpressing mutated GNE resulting in overexpression of sialic acids perhaps goes some way towards explaining the neurocognitive features seen in patients with sialuria (Kreuzmann et al. 2017).

GNE/MNK in humans is encoded by *GNE* gene on 9p13.3. Structurally, GNE/MNK is a bifunctional enzyme containing both an epimerase and a kinase domain. Importantly, the epimerase domain has an allosteric site known to bind its downstream product and negative

regulator, cytidine monophosphate (CMP)-sialic acid (Chen et al. 2016). Heterozygous missense variants at the allosteric site, specifically at Arginine 294 and Arginine 297 [NM_001128227.2; NP_001121699.1, hGNE2 protein isoform (formerly Arginine 263 and Arginine 266; NM_005476.5; NP_005467.1, hGNE1 protein isoform)], result in sialuria (Champaigne et al. 2016; Kurochkina et al. 2010). Conversely mutations elsewhere in GNE/MNK, which lead to decreased sialylation, have been implicated in neuronal development and hereditary inclusion body/GNE myopathy (Leroy 2004; Cho et al. 2017; Kurochkina et al. 2010).

Furthermore, of the disorders of sialic acid metabolism, the allelic sialic acid storage disorders (SASD) – infantile sialic acid storage disease (ISSD) and its milder version Salla disease – share many clinical features with sialuria (reviewed in Varki 2008). These however are attributable to mutations in *SLC17A5*, which results in defective transport of sialic acids from the lysosomes to the cytosol and thereby in lysosomal accumulation (Adams and Gahl 2003).

Since the first report of a French patient with sialuria in 1968 (Montreuil et al. 1968), there have been a total of eight cases published worldwide (Champaigne et al. 2016; Wilcken et al. 1987; Don and Wilcken 1991; Seppala et al. 1991; Krasnewich et al. 1993; Ferreira et al. 1999; Leroy et al. 2001; Enns et al. 2001). Most of these patients have been identified in childhood based on variable and often transient clinical findings and through biochemical tests, which are not routine, making it likely that this is an underrepresentation of the true prevalence. Signs and symptoms of sialuria may include mild coarse facies, prolonged neonatal jaundice, hepatomegaly, microcytic anaemia, frequent upper respiratory tract infections and gastroenteritis, failure to thrive, developmental delay, hypotonia, seizures and delayed bone age. Most recently, a relationship between sialuria and intrahepatic cholangiocarcinoma has also been proposed (Champaigne et al. 2016). The previous patient identified through our local services here, who was the second patient with sialuria in the literature (Wilcken et al. 1987), was last seen at age 20 years. She then demonstrated ongoing hepatosplenomegaly, mild distinctive features and moderate developmental delays. Interestingly, the eighth patient in the literature was diagnosed through whole exome sequencing (Champaigne et al. 2016), and we envisage that further patients will be identified in this way.

Here we describe a ninth patient with sialuria who was also identified in childhood on the basis of his clinical picture. Furthermore, we also propose a new pathogenic variant in *GNE*.

Materials and Methods

The patient was initially seen at Sydney Children's Hospital, Randwick, NSW Australia. Urine metabolic screen was conducted through The Children's Hospital at Westmead, NSW Australia. Oligosaccharide analysis by thin layer chromatography and sialic acid quantification was carried out by the SA Pathology, National Referral Laboratory Department of Biochemical Genetics North Adelaide, South Australia. Cell fractionation was undertaken by Dr. Tim Wood at Greenwood Genetic Centre, Greenwood, South Carolina, USA. *GNE* mutation analysis was through Prevention Genetics, Marshfield WI. *SLC17A5* mutation analysis was through Medical Neurogenetics, Atlanta, Georgia, USA.

The Case

The patient, a boy, was diagnosed with sialuria at 2 years and 4 months. He was born at 36 weeks and 3 days gestation by normal vaginal delivery to a then G1P1 mother. His parents are non-consanguineous, of European descent, and with pertinent family history. The pregnancy was complicated throughout by nausea. Antenatal ultrasounds were unremarkable. At birth his weight was 2.385 kg (above the 10th centile), length was 48 cm (above the 50th centile) and head circumference was 32 cm (10th centile). He did not require resuscitation. At 2 days of age, he developed unconjugated hyperbilirubinaemia and required phototherapy for 3 days. Jaundice then recurred, and he was readmitted to hospital for a further 4 days of phototherapy. Investigations for recurrent jaundice identified hepatosplenomegaly associated with elevation in plasma alanine aminotransferase (ALT) 199 U/L, aspartate aminotransferase (AST) 242 U/L and gamma-glutamyl transferase (GGT) 765 U/L. Infectious serology for hepatitis A, B and C, as well as toxoplasmosis, cytomegalovirus and herpes simplex, was negative. Jaundice gradually resolved over the first 3 months of life; however his hepatic transaminitis and hepatosplenomegaly persisted.

As an infant he had several viral illnesses, with predilection for upper respiratory tract infections. Although he usually recovered well, during some of these episodes, he experienced febrile convulsions. The frequency of illnesses and seizures generally improved with age. However, he had a further episode of tonic-clonic seizures in the context of a febrile viral illness at 6 years and 3 months of age. This occurred after having 1.5 years seizure-free. The patient was also briefly troubled by obstructive sleep apnoea as an infant and reviewed by an otolaryngologist. It was postulated that his obstructive symptoms were likely

secondary to enlarged tonsils. He did not undergo adenotonsillectomy.

A mildly decreased IgG (3.74 g/L) was noted during routine investigations at 1 year of age. IgM and IgA levels were in the normal ranges. Influenza immunization was recommended. Repeat immunoglobulin levels at 6 years and 10 months of age demonstrated complete normalization – IgG 5.13 g/L, IgA 1.34 g/L and IgM 0.89 g/L. Additionally, iron deficiency anaemia (haemoglobin 123 g/L, mean corpuscular volume 71.8 fL, ferritin 15 μg/L) and dyslipidaemia with elevated cholesterol (5.7 mmol/L) and triglycerides (4.6 mmol/L) were detected on routine bloods at 1 year of age. He was commenced on iron replacement, with variable compliance. Dyslipidaemia was not treated. Repeat blood samples at 6 years and 10 months demonstrated haemoglobin of 131 g/L, mean corpuscular volume 74.6 fL, ferritin 16 μg/L, cholesterol 5.1 mmol/L and triglycerides 1.2 mmol/L.

Developmentally he sat at 6 months, walked at 11 months and had single words as well as a pincer grip by 14 months. At 3 years of age, he underwent a developmental assessment and was diagnosed with mild developmental delay and autism spectrum disorder. His cognitive skills at 5 years and 2 months of age were in the low average range as per The Wechsler Preschool and Primary Scale of Intelligence – 4th edition (WPPSI-IV). Audiology and vision tests were normal. He is now 7 years of age and with ongoing early intervention attends a mainstream primary school. He continues to have restricted, repetitive behaviours with symptoms of anxiety.

Clinical examination at 6 years and 4 months of age revealed softening of his coarse features with persistence of epicanthic folds, flattening of the nasal bridge and posteriorly rotated ears. Tonsils were enlarged bilaterally. His abdomen remained protuberant with appreciable hepatomegaly and a 14 cm liver span. The spleen was approximately 8 cm long. The remainder of his examination was unremarkable.

Diagnosis The patient's diagnosis of sialuria was made when he was 2 years and 4 months of age during ongoing investigations for his persistent transaminitis and hepatosplenomegaly. A paediatric gastroenterologist also reviewed the patient at that time, and there was consideration of a liver biopsy if the extensive battery of testing did not yield a diagnosis. In brief, comparative genomic hybridization (CGH) array was normal as was his amino acid profile, organic acid profile, acylcarnitine profile, very long chain fatty acids profile, lysosomal enzymes, transferrin isoforms, copper, ceruloplasmin and coeliac serology. However, urinary oligosaccharide analysis by thin layer chromatography revealed large amounts of sugar at the position of free sialic acid. Further quantification reported a level of 2,980 μmol of free urinary sialic acid/mmol creatinine. Free sialic acid in cultured skin fibroblasts was also elevated at 34 nmol/mg protein. Cell fractionation was undertaken which demonstrated 76–83% of the sialic acid localizing to the soluble fraction that is the cytosolic component, compared to 32–47% in the control samples. Parental urinary sialic acid levels were normal, as was the urinary sialic acid level in the patient's only sibling.

Genetic testing was also pursued. No variants were reported in sequencing of *SLC17A5*. This was followed by bidirectional Sanger sequencing of *GNE*, whereby the patient was found to be heterozygous for a previously undocumented variant NM_001128227.2: c.250G>C; p.Asp84His in the *GNE* gene (MIM 603824, GeneID 10020). Additionally segregation testing in the patient's parents found this to be a de novo change. A second heterozygous variant c.51+34 T>C (refSNP number in dbSNP: rs7875447) in *GNE* was also identified in the patient. However, this variant is known to be in an intronic region, commonly found, not associated with disease states and therefore benign as per American College of Medical Genetics and Genomics (ACMG) criteria (Richards et al. 2015).

Since diagnosis the patient has undergone yearly review with our local metabolic service. His monitoring included a skeletal survey at 2.5 years of age, which was normal, and routine measurement of his liver function tests. Liver function tests at 6 years and 10 months of age demonstrated persistence of the mild derangement in the transaminases (ALT 194 U/L and AST 89 U/L). In the light of the report by Champaigne et al. (2016), a decision has been made to undertake yearly liver ultrasounds for monitoring. His imaging in 2018 again noted hepatosplenomegaly, not associated with any other specific anomaly.

Discussion

Given the apparent rarity of this condition, the identification of a ninth patient with sialuria was felt to be worth publishing. Importantly, although this patient possesses many of the clinical features associated with sialuria and SASDs, the diagnosis was not initially forthcoming lending support to the hypothesis that sialuria may be an under-recognized condition, at least when the diagnosis is based on subtle and sometimes transient clinical findings. However we envisage there is scope for further diagnoses being made through whole genomic sequencing. Indeed since we commenced proceedings for this publication, we have been made aware of a potential tenth case of sialuria worldwide.

Secondly, we report a novel de novo heterozygous missense variant in *GNE* c.250G>C, p.Asp84His

(NM_001128227.2; NP_001121699.1, hGNE2 protein isoform), which results in autosomal dominant sialuria, with biochemical correlate in this patient. Importantly, the wild-type aspartic acid residue is highly conserved among GNE proteins; among the 100 vertebrates in the Multiz alignment, only platypus has a different amino acid residue at this location. Protein structure analysis using HOPE (Venselaar et al. 2010) indicates that substitution of aspartic acid, which carries a negative charge, with histidine, which is neutral and larger, is expected to disrupt the wild-type conformation of GNE.

Further structural information on the effect of this variant can be gauged from the recently published GNE crystal structure by Chen et al. (2016). Aspartic acid 84 as denoted using the newer hGNE2 nomenclature proposed by Huizing et al. (2014) was formerly known as aspartic acid 53 using the older hGNE1 (NM_005476.5; NP_005467.1) nomenclature. The hGNE1 nomenclature has been used to report previous patients with sialuria, as well as in the crystal structure by Chen et al. (2016). The crystal structure provides evidence that aspartic acid 84 (formerly 53) also forms part of the allosteric site or at least is required for correct folding of the site (Fig. 4b of Chen et al. 2016). They demonstrate that the cytosine base of CMP-sialic acid, the negative downstream regulator, is sandwiched between the side chains of aspartic acid 84 (formerly 53) and Valine 293 (formerly 262). This suggests a possible mechanism by which variants affecting aspartic acid 84 may have similar effects to variants affecting Arginine 294 (formerly 263) and Arginine 297 (formerly 266) which are known to cause autosomal dominant sialuria.

Overall, based on the available evidence, the c.250G>C; p.Asp84His variant identified here meets the ACMG criteria (Richards et al. 2015) for PS2 (de novo variant), PM2 (absent from controls), PP2 (low rate of missense variants and where missense variants are a common mechanism of disease), PP3 (in silico support for pathogenicity), PP4 (patient's phenotype including biochemical evidence is highly specific for a condition with a single genetic aetiology) and PM1 (variant affects a critical functional region of the protein which lacks benign variation). We therefore propose that there is sufficient compelling evidence for this novel variant being classified as pathogenic and extending the known sialuria causing mutations in *GNE*.

Finally, with very rare (or rarely recognized) disorders, it is often difficult to be sure of the clinical significance of biochemical or other findings. In the case of sialuria, the clinical findings of described cases have a homogeneity which suggests that they are a real consequence of the disorder. As more cases are discovered primarily by next-generation sequencing rather than primarily by clinical features, there may be more information about the full picture and prevalence of this condition.

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Take-Home Message

Sialuria: Ninth patient described has a novel mutation in *GNE*.

Contributions of the Individual Authors

Noelia Nunez Martinez, part of the clinical team, wrote the paper, organized all outstanding investigations and approved the submitted version.

Michelle Lipke organized the original investigations, made the diagnosis and approved the submitted version.

Jacqueline Robinson, part of the clinical team, organized the patient follow-up and approved the submitted version.

Bridget Wilcken headed the clinical team, edited the paper and approved the submitted version.

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None of the authors has any competing interest.

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The investigations were all clinically required, and no ethical approval was necessary.

The patient's parents consented to all investigations.

No laboratory animals were involved.

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RESEARCH REPORT

Stability of the ABCD1 Protein with a Missense Mutation: A Novel Approach to Finding Therapeutic Compounds for X-Linked Adrenoleukodystrophy

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Abstract Mutations in the *ABCD1* gene that encodes peroxisomal ABCD1 protein cause X-linked adrenoleukodystrophy (X-ALD), a rare neurodegenerative disorder. More than 70% of the patient fibroblasts with this missense mutation display either a lack or reduction of the ABCD1 protein because of posttranslational degradation. In this study, we analyzed the stability of the missense mutant ABCD1 proteins (p.A616T, p.R617H, and p.R660W) in X-ALD fibroblasts and found that the mutant ABCD1 protein p.A616T has the capacity to recover its function by incubating at low temperature. In the case of such a mutation, chemical compounds that stabilize mutant ABCD1 proteins could be therapeutic candidates. Here, we prepared CHO cell lines stably expressing ABCD1 proteins with a missense mutation in fusion with green fluorescent protein (GFP) at the C-terminal. The stability of each mutant ABCD1-GFP in CHO cells was similar to the corresponding mutant ABCD1 protein in X-ALD fibroblasts. Furthermore, it is of interest that the GFP at the C-terminal was degraded together with the mutant ABCD1 protein. These findings prompted us to use CHO cells expressing mutant ABCD1-GFP for a screening of chemical compounds that can stabilize the mutant ABCD1 protein. We established a fluorescence-based assay method for the screening of chemical libraries in an effort to find compounds that stabilize mutant ABCD1 proteins. The work presented here provides a novel approach to finding therapeutic compounds for X-ALD patients with missense mutations.

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Introduction

X-linked adrenoleukodystrophy (X-ALD) (OMIM 300100) (Bezman et al. 2001) is a human genetic disorder caused by mutation of the *ABCD1* gene (Mosser et al. 1993) that encodes the peroxisomal membrane protein ABCD1, which consists of 745 amino acids. To date, more than 340 nonrecurrent missense mutations of the *ABCD1* gene have

been identified (http://www.x-ald.nl). In these missense mutations, approximately 70% of the ABCD1 proteins are either not detected or reduced in amount in X-ALD fibroblasts. We previously reported that the mutant ABCD1 protein is degraded via the proteasome pathway (Takahashi et al. 2007), at least in part, because the degradation of the missense mutant ABCD1 was partially inhibited by treatment with MG132. Zhang et al. have reported that X-ALD fibroblasts with certain temperature missense mutation recovered their function by incubating them at a low temperature (Zhang et al. 2011). These results indicate that a part of the mutant ABCD1 protein, which is incorrectly folded and becomes degraded in the cytosol, might have a capacity to recover its function by stabilization. Thus, a chemical compound with the capacity to stabilize the mutant ABCD1 protein is a potential therapeutic agent for X-ALD.

Certain strategies and candidate drugs for X-ALD treatment have been reported (Morita et al. 2011). To date, however, no effective therapeutic drugs for X-ALD have been developed. Thus, development of a high-throughput system for screening of therapeutic compounds based on direct stabilization and stimulation of functional activity of mutant ABCD1 is of paramount importance for this condition. In this study, we prepared CHO cells expressing missense mutant ABCD1 fused with GFP and found that GFP at the C-terminal was posttranslationally degraded together with the mutant ABCD1 protein. Using these model cells, we demonstrate a novel approach to finding therapeutic compounds for X-ALD by stabilizing the ABCD1 protein having a missense mutation.

Materials and Methods

Materials

pEGFPN-1 was purchased from Clontech Laboratories (Mountain View, CA). The KOD-plus mutagenesis kit was from Toyobo (Osaka, Japan). The MTT assay kit was from Roche Applied Science. Bortezomib was purchased from Cell Signaling Technology (Danvers, MA). [1-^{14}C] lignoceric acid (53 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). ECL Plus, a Western blotting detection system, and Fluorolink Cy3-labeled goat anti-rabbit IgG were purchased from GE Healthcare (Buckinghamshire, England). The mouse anti-human ALDP/ABCD1 monoclonal antibody (MAB2162) and the rabbit anti-catalase antibody were purchased from Millipore (Billerica, MA) and Immunochemicals (Gilbertsville, PA), respectively. The rabbit anti-PMP70/ABCD3 antibody was raised against the C-terminal 15 amino acids of rat PMP70/ABCD3 (Imanaka et al. 2000). The chemical library of existing drugs was a gift provided by the Drug Discovery Initiative at the University of Tokyo.

Plasmid Construction

pEGFP vectors, each harboring a missense ABCD1 mutant, were constructed with a KOD-plus mutagenesis kit using pEGFP/ABCD1 as the template (Takahashi et al. 2007). The oligonucleotide primer sets were designed on the basis of their sequences (Suppl. Table 1). The mutation in the constructs was confirmed by the dye-terminator cycle sequencing method using an ABI PRISM310 DNA sequencer (Life Technologies Corporation, Carlsbad, CA).

Cell Culture and Stable Transfection

CHO-K1 cells were cultured in F12 medium (Nissui, Tokyo, Japan) with 10% FCS containing streptomycin and penicillin at 37°C and 5% CO_2. CHO cells expressing wild or mutant ABCD1-GFP were prepared by the transfection of pEGFP/wild ABCD1 or pEGFP/mutant ABCD1 into CHO cells. The transfection procedures were performed using Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. At 48-h post-transfection, the medium was replaced with the same medium containing G418 (500 μg/mL). In the G418-resistant cells, we selected several clones expressing wild ABCD1-GFP or each mutant ABCD1-GFP as determined by means of fluorescence imaging. The cell lines expressing mutant ABCD1-GFP were identified by the observation of GFP fluorescence under a condition in which the CHO cells were incubated in the presence of MG132 (20 μM) for 20 h.

Human skin fibroblasts from a healthy individual and X-ALD patients with a missense mutation (R617H, A616T, and R660W) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) containing 10% FCS.

Fluorescence-Based Assay Method for the Screening of Chemical Libraries

The CHO cells stably expressing mutant ABCD1-GFP (referred to as CHO/mutABCD1-GFP) were seeded in a 96 well plate (Greiner, blk/clr bottom) with 100 μL of DMEM/F12 (phenol red-free) containing 10% FCS (4×10^4 cells/well). Chemical compounds were added to each well on the next day. One μL of stock solution (2 mM in DMSO) was mixed with the culture medium to give a final yield of 20 μM. CHO/mutABCD1-GFP was cultured in the wells from columns 1 to 11, and CHO/wildABCD1-GFP was in cultured column 12 as a positive control. DMSO was added

to each well in columns 1 and 12, and each compound was added to 80 wells from columns 2 to 11. After 48-h incubation, culture medium was discarded, and cells were washed with 170 μL of 1 × Hank's balanced salt solution (HBSS). After the addition of 100 μL of 1 × HBSS, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm with Filter Max F5 (Molecular Devices, Sunnyvale, CA).

Immunofluorescence Analysis

Immunofluorescence analysis was performed as described previously (Morita et al. 2013). The primary antibodies were rabbit antibodies against catalase (1:200) and mouse antibodies against ABCD1 (1:200). Alexa 488-conjugated goat anti-rabbit (1:500) was utilized to label the anti-catalase antibodies. Cy3-conjugated goat anti-mouse was used to label the anti-ABCD1 antibodies. The cells were mounted in Vectarshield with DAPI (Vector Laboratories, Burlingame, CA) to allow examination under confocal microscopy (confocal microscope LSM780, Carl Zeiss Microscopy, Tokyo, Japan).

Other Methods

Fatty acid β-oxidation was measured essentially as described by Watkins et al. (Morita et al. 2013; Watkins et al. 1991). Immunoblotting was performed as described previously using ECL Plus Western blotting detection reagent (Kurisu et al. 2003). The protein concentration was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as the standard.

Results

Stability of Missense Mutant ABCD1 Proteins in X-ALD Fibroblasts

We analyzed the stability of mutant ABCD1 proteins in X-ALD fibroblasts with a missense mutation of A616T, R617H, and R660W (Suppl. Fig. 1). We selected these mutant ABCD1 proteins because they had been demonstrated to be temperature-sensitive mutations and culturing X-ALD cells harboring these mutations at lower temperature partially restored their function (Zhang et al. 2011). Indeed, when these X-ALD fibroblasts were treated with MG132, a proteasome inhibitor, the mutant ABCD1 proteins A616T and R617H partially recovered (Fig. 1a), indicating that these mutant ABCD1 proteins are unstable due to incorrect folding and are partly degraded by proteasomes. In contrast, the mutant ABCD1 protein R660W did not recovered, indicating that p.R660W is degraded by other proteases. When these X-ALD fibroblasts were incubated at 30°C for 7 days, the mutant ABCD1 proteins A616T and R660W recovered (Fig. 1a). The recovered mutant ABCD1 protein A616T was partially localized to peroxisomes, but p.R660W was not (Fig. 1b). The VLCFA β-oxidation activity of X-ALD fibroblasts (A616T) cultured at 30°C was approximately 75% of that in the control fibroblasts (Fig. 1c), even though the expression level of the mutant ABCD1 protein was approximately 30% compared with normal fibroblasts (Fig. 1a). We previously reported that the C26:0/C22:0 ratio became near normal level by culturing at 30°C for 3 weeks (Zhang et al. 2011). These results suggest that the mutant ABCD1 protein A616T has the capacity to recover its function and can be rescued by chemical compounds that stabilize it. In contrast, the mutant ABCD1 protein R660W did not show any recovery of VLCFA β-oxidation, probably because of mislocalization (Fig. 1b). In the case of the X-ALD fibroblasts (R617H), the mutant ABCD1 protein was not recovered by incubation at a low temperature.

Stability of the Mutant ABCD1 Proteins Expressed in CHO Cells

To evaluate the stabilization of each mutant ABCD1 protein more easily, we expressed missense mutant ABCD1 proteins fused with GFP at the C-terminal (p.A616T, p.R617H, and p.R660W) in CHO cells. When CHO cells stably expressing each mutant ABCD1-GFP (CHO/mutABCD1-GFP) were cultured at 37°C, the expression of the mutant ABCD1-GFP was beneath the limit of detection (Fig. 2a and Suppl. Fig. 2). However, the mutant ABCD1-GFPs A616T, R617H, and R660W were largely recovered by treatment with MG132, suggesting that these mutant ABCD1-GFPs were posttranslationally degraded by proteasomes. However, the recovery of p.R660W was less than that of p.A616T and p.R617H, which is consistent with the findings on mutant ABCD1 proteins in X-ALD fibroblasts (Fig. 1a). In this experiment, it should be noted that the GFP at the C-terminal was degraded together with the mutant ABCD1 protein. When these cells were cultured at 30°C for 3 days, the mutant ABCD1-GFPs became detectable and co-localized with the ABCD3 protein, a peroxisomal membrane protein (Fig. 2a, b). However, peroxisomes expressing mutant ABCD1-GFPs R617H and R660W became large as if a subset of peroxisomes aggregate. The recovery of the mutant ABCD1-GFP A616T was time-dependent and reached a maximum level at 3 days (Suppl. Fig. 3). When CHO/mutABCD1-GFP (A616T) were incubated for 5 days at 30°C followed by incubation at 37°C, the mutant ABCD1-GFP was still

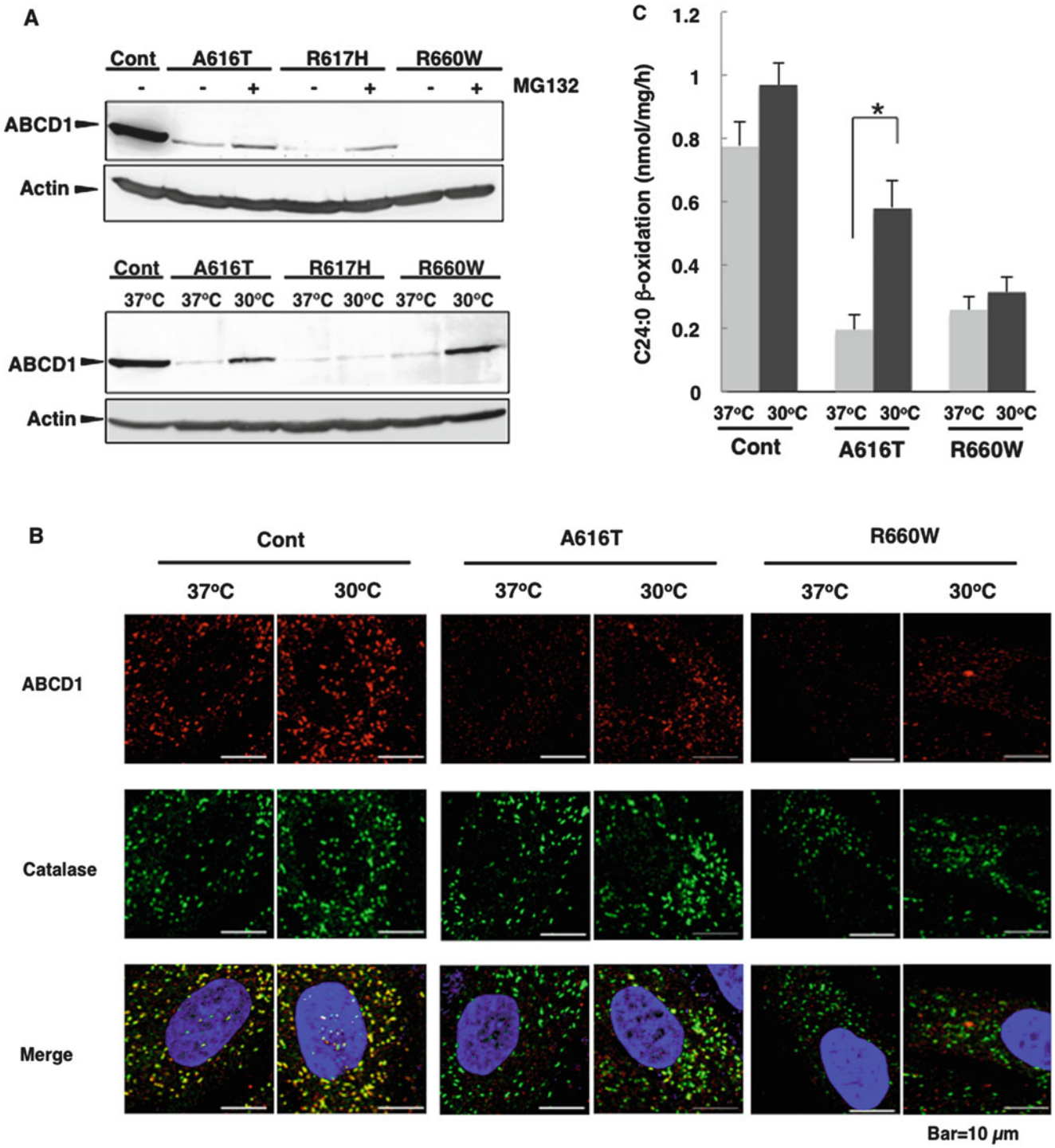

Fig. 1 Recovery of mutant ABCD1 proteins in X-ALD fibroblasts. (**a**) X-ALD fibroblasts (A616T, R617H, and R660W) were incubated at 37°C in the absence or presence of MG132 (20 μM) for 20 h or incubated at 30°C for 7 days. After the incubation, the expression of ABCD1 was analyzed by immunoblotting. Total cellular protein (100 μg protein/sample) were separated by SDS-PAGE and transferred to nitrocellulose membranes. ABCD1 and actin were stained with an anti-ABCD1 antibody and anti-β-actin antibody, respectively. (**b**) Control (Cont) and X-ALD fibroblasts (A616T and R660W) were incubated at 37°C or 30°C for 7 days and subjected to immunofluorescence analysis. ABCD1 was stained with an anti-ABCD1 antibody followed by a Cy3-labeled secondary antibody. Catalase was stained with an anti-catalase antibody followed by an Alexa 488-labeled secondary antibody. Catalase appears as green dots and ABCD1 as red dots. The figure displays a merged image. (**c**) VLCFA β-oxidation activities in control (Cont) and X-ALD fibroblasts (A616T and R660W) were measured using [1-^{14}C]C24:0 as the substrate. Control and X-ALD fibroblasts (A616T and R660W) were incubated at 37°C or 30°C. After 7-day incubation, cells were harvested and incubated with a reaction buffer containing dissolved α-cyclodextrin [1-^{14}C] C24:0. The reaction was stopped by the addition of 1M KOH for alkaline hydrolysis. After neutralization and Folch extraction, the radioactivity in the water-soluble fraction was measured by scintillation counting. Results are the means ± S.D.; $n = 3$. Statistical analysis of the data was performed with Student's t-test (*, $p < 0.02$)

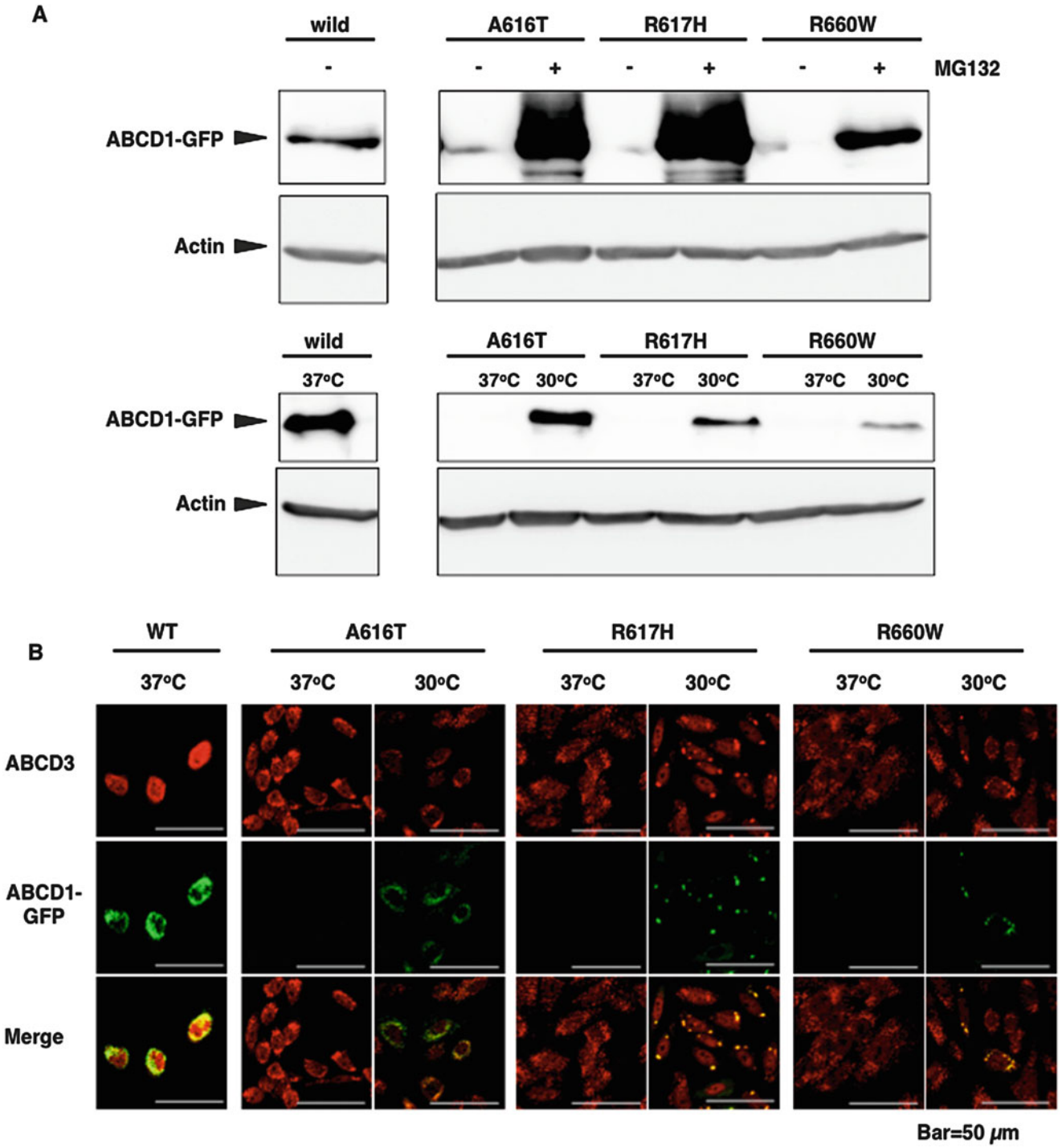

Fig. 2 Missense mutant ABCD1-GFPs recovered by treatment with MG132 or incubation at a low temperature. CHO cells expressing wild or mutant ABCD1-GFPs (A616T, R617H, and R660W) were cultured in the presence of MG132 (20 μM) for 20 h or at 30°C for 5 days. After the incubation, the expression of ABCD1-GFP was analyzed by immunoblotting (**a**) or immunofluorescence (**b**). ABCD1-GFP and actin were detected by using an anti-GFP antibody or anti-β-actin antibody, respectively. Peroxisomes were stained with an anti-ABCD3 antibody followed by a Cy3-labeled secondary antibody. ABCD1-GFP appears as green dots and ABCD3 as red dots. The antibody used in this experiment did not react with endogenous ABCD1 in CHO cells

detectable for at least up to 24 h (Suppl. Fig. 4), indicating that the mutant ABCD1 protein is relatively stable once it localizes to peroxisomes.

The stability of each mutant ABCD1 protein in X-ALD fibroblasts was similar to that of mutant ABCD1-GFP in CHO cells, suggesting that CHO/mutABCD1-GFP can be

used as a model cells. Additionally, the fluorescent intensity in CHO/mutABCD1-GFP is a good indicator of the recovery of the mutant ABCD1 protein. Therefore, these cells are very useful for the screening of chemical compounds that are able to stabilize temperature-sensitive mutant ABCD1 protein.

Screening Method Using CHO/mutABCD1-GFP

We used the CHO/mutABCD1-GFP (A616T) cells to develop a screening method to identify chemical compounds that can restore temperature-sensitive ABCD1 mutants (Fig. 3). The GFP fluorescent was detected by culturing at a low temperature or with MG132 (Suppl. Fig. 5). The average Z-factor for the fluorescence intensity was calculated to be more than 0.5 in the CHO/wildABCD1-GFP and CHO/mutABCD1-GFP (A616T). The compounds were first screened by measuring the fluorescence intensity with a spectrofluorometer. In this screening, we selected the chemical compounds that exhibited a greater than 30% of the fluorescence intensity of CHO/wildABCD1-GFP. Next, the positive compounds were analyzed for their capacity to recover the mutant ABCD1-GFP by immunofluorescence or by immunoblot analysis to exclude the false-positive compounds. Finally, the ability of the positive compounds to recover mutant ABCD1 protein was analyzed in X-ALD fibroblasts (A616T).

Screening of Existing Drugs

In the present study, we screened 1,948 compounds from the Drug Discovery Initiative library of the University of Tokyo and found 19 compounds that exhibited the increase in the fluorescent intensity. These 19 drugs were used to study their effect on the peroxisomal localization of mutant ABCD1 proteins in X-ALD patient fibroblasts. In this experiment, the concentration of drugs was decreased to 5 μM because higher doses altered the cell morphology. Four drugs, including the anthracycline anticancer agents (doxorubicin, idarubicin, and aclarubicin) and bortezomib, induced the recovery of mutant ABCD1-GFP in perox-

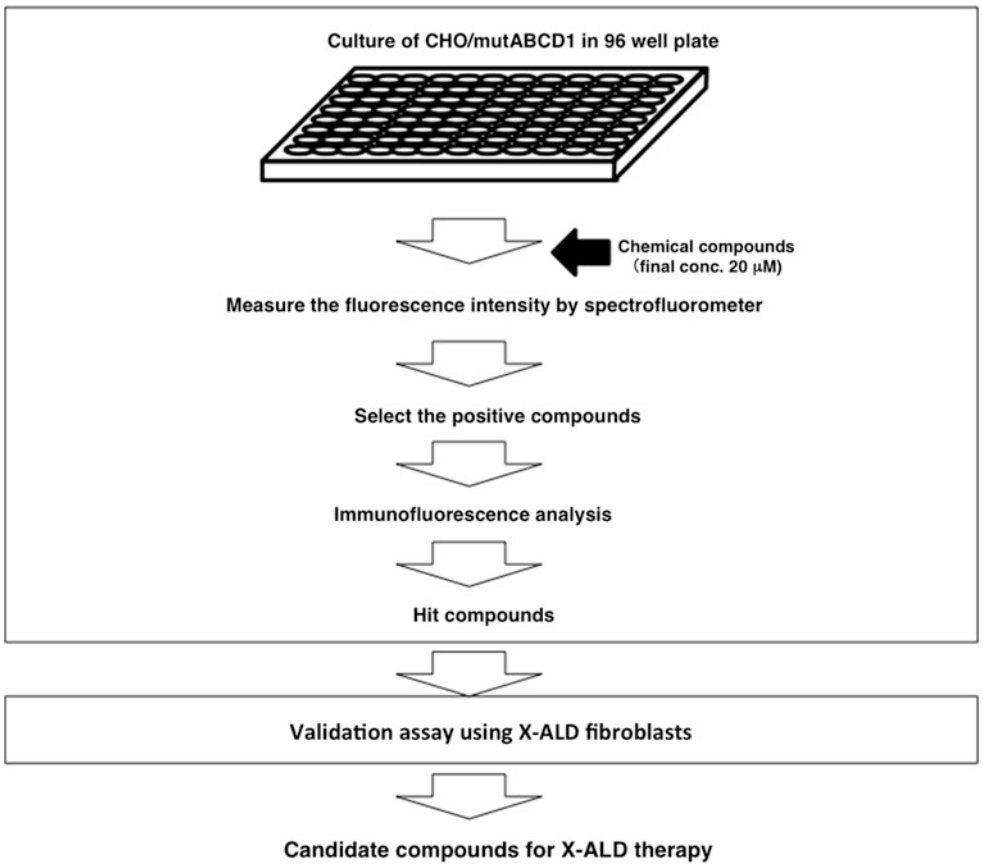

Fig. 3 Flowchart for the screening of chemical compounds. CHO/wildABCD1-GFP and CHO/mutABCD1-GFP (A616T) cells cultured in 96 well plates (4×10^4 cells/well) were treated with chemical compounds (final conc. 20 μM) and incubated for 2 days. After washing with 1 × HBSS, their fluorescent intensity was directly measured with a spectrofluorometer. Next, the effect of positive compounds on the localization of mutant ABCD1-GFP was determined by immunofluorescence analysis. Hit compounds from the fluorescence-based assay were finally validated on ALD fibroblasts

isomes (Suppl. Fig. 6). The other 15 drugs did not elicit any fluorescence in peroxisomes (data not shown). When X-ALD fibroblasts (A616T) were incubated with bortezomib and doxorubicin, only bortezomib induced the recovery of mutant ABCD1 proteins (Fig. 4a). Other anthracycline antibiotics such as idarubicin and aclarubicin did not bring

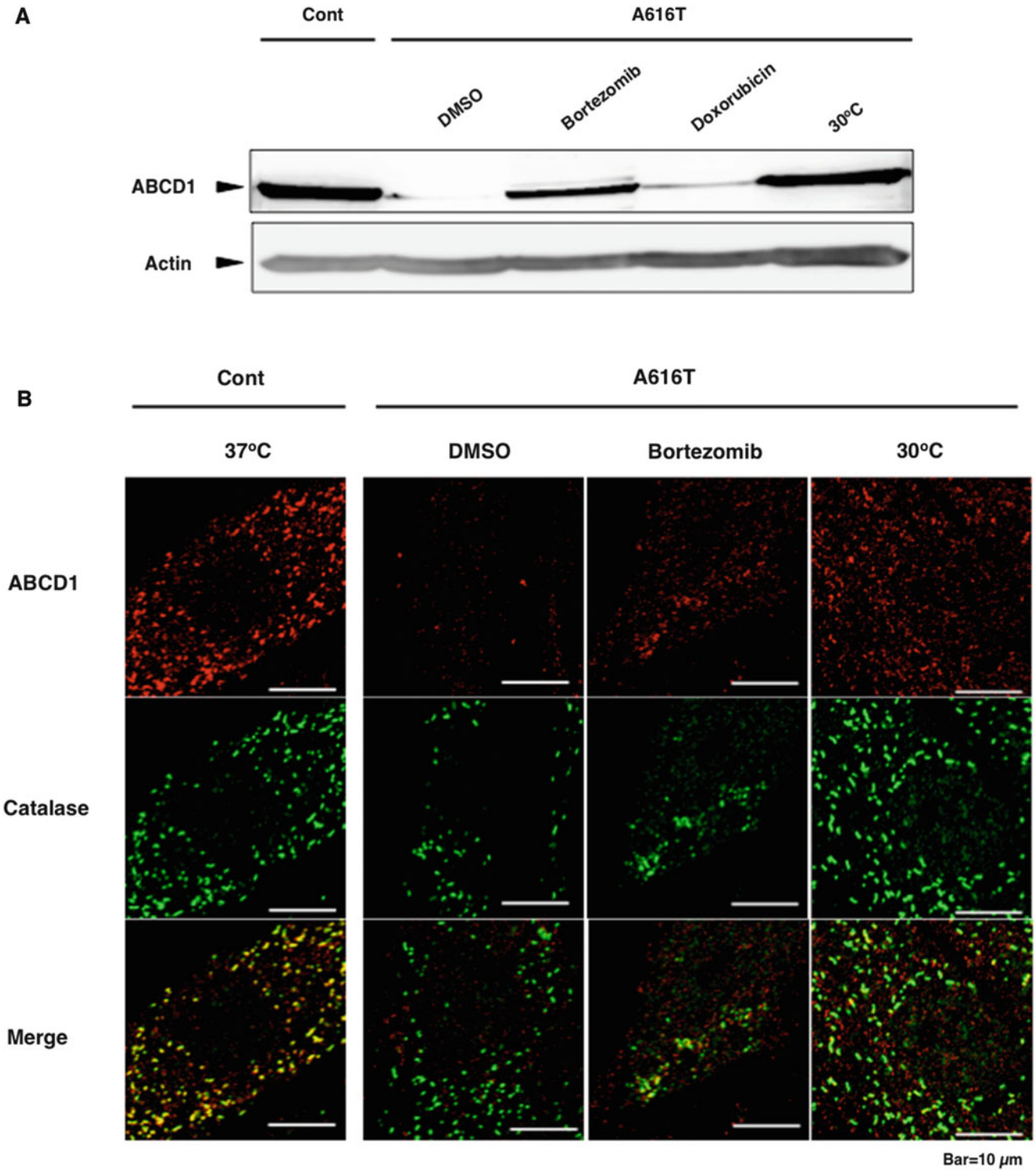

Fig. 4 Recovery of mutant ABCD1 (A616T) by the treatment of bortezomib in X-ALD fibroblasts. (**a**) X-ALD fibroblasts (A616T) were incubated in the presence or absence of bortezomib (50 nM) or doxorubicin (5 μM) for 2 days or incubated at 30°C for 7 days. After the incubation, the cells were analyzed as indicated in Fig. 1a. (**b**) X-ALD fibroblasts (A616T) were incubated in the presence or absence of bortezomib at 50 nM for 2 days or incubated at 30°C for 7 days. After the incubation, they were subjected to immunofluorescence analysis as indicated in Fig. 1b

about any recovery (data not shown). This discrepancy between X-ALD fibroblasts and CHO/mutABCD1-GFP is due to the promoter induction by anthracycline antibiotics (Kurisu et al. 2003). We found that the transcription of the *mutant ABCD1-GFP* gene as well as *GFP-SKL* gene was stimulated by the treatment of doxorubicin (Suppl. Fig. 7). This result suggests that anthracycline antibiotics such as doxorubicin, idarubicin, and aclarubicin activate the CMV promoter derived from the pEGFP vector, resulting in the induction of a large amount of mutant ABCD1-GFP. Therefore, we believe anthracycline antibiotics do not have the capacity to stabilize the mutant ABCD1 protein. In contrast, bortezomib exhibited a dose- and time-dependent recovery of mutant ABCD1 proteins in X-ALD fibroblasts (Suppl. Fig. 8a, b) without significant effects on cell viability up to 500 nM for 2 days (Suppl. Fig. 9). When ALD fibroblasts (A616T) were incubated with bortezomib at 50 nM for 2 days, the mutant ABCD1 protein A616T partially localized to peroxisomes, although the expression of catalase was relatively decreased (Fig. 4b).

Discussion

Recently, small molecules with chaperone activity have garnered attention for clinical importance in protein folding diseases with missense mutations (Loo and Clarke 2007). In lysosomal storage diseases, pharmacological chaperone therapy is currently being investigated as a potential therapeutic approach (Sawkar et al. 2006; Fan 2008). Furthermore, several missense mutant ABC proteins, including ABCG2 (BCRP), ABCB4 (MDR3), ABCC7 (CFTR), ABCC1 (MRP1), and ABCC8 (SUR1), can be recovered by chemical chaperones (Loo et al. 2005, 2011; Sampson et al. 2011; Yu et al. 2011; Zhang et al. 2012).

In the missense mutations in X-ALD, approximately 70% of the mutant ABCD1 proteins were posttranslationally degraded. However, some mutant proteins still have the capacity for functional recovery by stabilization. Indeed, in cases in which mutant ABCD1 proteins could be rescued by culturing X-ALD fibroblasts at lower temperature, they showed residual biological activity (Zhang et al. 2011). This indicates that stabilization of the mutant protein is an attractive therapeutic approach. In the present study, we characterized three missense mutant ABCD1 proteins: R617H is located in the Walker B motif, A616T in the ABC signature motif, and R660W in the C-terminal downstream from the Walker B motif (Suppl. Fig. 1). Among them, the mutant ABCD1 protein A616T was functionally recovered by incubation at a low temperature. These data are in line with an earlier study that reported that the function of p.A616T could be restored when X-ALD fibroblasts were cultured at 30°C (Zhang et al. 2011). These data suggest that pharmacological chaperone therapy is adaptable to X-ALD patients with a temperature-sensitive missense mutation.

The expression of GFP fused with a desired protein in living cells is useful for monitoring the fate of the protein. Although it should be noted that the CMV promoter that is genetically transfected in CHO/mutABCD1-GFP can be stimulated by chemical compounds such as anthracycline antibiotics (Kinoshita et al. 2008), CHO/mutABCD1-GFP allows monitoring of the stabilization of mutant ABCD1 proteins. In these cells, GFP at the C-terminal is degraded along with the mutant ABCD1 protein, indicating that GFP fluorescence is detected only when they are stabilized. Based on these properties, we established a screening method and found bortezomib, a potent proteasome inhibitor, which is commonly used as a drug for multiple myeloma patients (Dou and Goldfarb 2002). Recently, bortezomib has been reported to improve the function of mutant lysosomal α-glucosidase in fibroblasts from Pompe disease patients (Shimada et al. 2011) and also to stabilize the deltaF508-cystic fibrosis transmembrane conductance regulator (CFTR) (Wilke et al. 2012). In the present study, the mutant ABCD1 proteins were recovered and partially localized in peroxisomes by treatment with bortezomib (Fig. 4b). Unfortunately, bortezomib did not show significant recovery of peroxisomal fatty acid β-oxidation in X-ALD fibroblasts (A616T) (data not shown). The failure to increase the peroxisomal fatty acid β-oxidation might be due to cellular damage caused by the treatment of bortezomib. In the CFTR, pharmacological chaperones, together with proteostasis regulators, that bind directly to the mutant CFTR and indirectly affect the cellular pathways, are being investigated as a novel pharmacological strategy (Hanrahan et al. 2013). At present, bortezomib seems to be not applicable for therapeutic drugs for X-ALD patients due to the significant side effects. Instead, bortezomib provides a piece of supporting evidence that small molecule interventions that stabilize a subset of ABCD1 mutant proteins could have therapeutic value for X-ALD in the future. Testing in animal model is required for verifying the possibility of therapeutic use.

The discovery of bortezomib confirms that the fluorescence-based screening protocol presented here is useful for the screening of a large number of chemical compounds for drug candidates that can stabilize the missense mutant protein. It should be considered that our assay method is only effective to a part of mutant ABCD1 proteins with missense mutation. Nevertheless, the present study provides a novel approach to finding therapeutic compounds for X-ALD.

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Take-Home Message

A novel approach to finding therapeutic compounds for X-ALD patients.

Conflict of Interest

Masashi Morita, Shun Matsumoto, Airi Sato, Kengo Inoue, Dzmitry G. Kostsin, Kozue Yamazaki, Kosuke Kawaguchi, Nobuyuki Shimozawa, Stephan Kemp, Ronald J. Wanders, Hirotatsu Kojima, Takayoshi Okabe, and Tsuneo Imanaka declare that they have no conflict of interest.

Informed Consent

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

Animal Rights

The article does not contain animal subjects.

Author Contributions

TI conceived and supervised the study; TI, MM, AH, HK, TO, NS, SK, and RJW designed the experiments; MM, SM, AS, KI, DGK, and KY performed the experiments; and MM, SM, and DGK wrote the manuscript, which was discussed by all authors.

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RESEARCH REPORT

Psychosocial Functioning in Parents of MPS III Patients

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Abstract *Background*: Mucopolysaccharidosis type III (MPS III or Sanfilippo syndrome) is a lysosomal storage disease resulting in progressive neurocognitive decline during childhood and early demise. Its diagnosis may have a great impact on parents, potentially leading to psychosocial problems such as anxiety, depression, parental distress, and posttraumatic stress.

Methods: Twenty-six mothers and 19 fathers of 34 Dutch MPS III patients completed the "Hospital Anxiety and Depression Scale" (HADS), the "Distress Thermometer for Parents" (DT-P), and the "Self-Rating Scale for Posttraumatic Stress Disorders" (SRS-PTSD). Independent-sample T-tests and chi-square tests were used to assess differences between parents of MPS III patients and reference groups regarding anxiety and depression (HADS), distress (DT-P), and posttraumatic stress (SRS-PTSD).

Results: Mothers met the criteria for clinically relevant anxiety (50%) and depression (34.6%) more frequently compared to reference mothers ($p = 0.001$). Fathers more often met the criteria for clinically relevant depression (36.8%) compared to reference fathers ($p = 0.022$). Clinically relevant distress was highly prevalent in mothers (84.6%) and fathers (68.4%) of MPS III patients compared to reference parents ($p < 0.01$). Finally, the prevalence of PTSD was strikingly higher in both mothers (26.9%) and fathers (15%) than reported in the general Dutch population (respectively, $p < 0.001$ and $p < 0.05$).

Conclusions: We report a clinically relevant impact of parenting an MPS III patient on psychosocial functioning, which is demonstrated by high levels of anxiety, depression, distress, and a remarkably high prevalence of PTSD. Structural monitoring of the psychosocial functioning of MPS III parents is therefore essential and may be beneficial for the whole family.

Introduction

Mucopolysaccharidosis type III (MPS III or Sanfilippo syndrome) is a lysosomal storage disease primarily characterized by progressive neurocognitive decline during childhood (Shapiro et al. 2016). The first phase of the disease manifests after a seemingly normal development during the first 2 years of life, followed by a slowing of cognitive development. The second phase is characterized by severe sleeping problems, behavioral problems, and progressive cognitive decline. In the final phase of the disease, motor skills are lost and patients become fully care dependent and bedridden (Cleary and Wraith 1993). No disease-modifying treatment is yet available and patients usually die in the second or third decade of life (Shapiro et al. 2016). The diagnosis of this devastating disease may have great impact on the parents and the family. Raising a chronically ill child requires parents to act in multiple roles involving complex responsibilities, such as management of the disease and caring for healthy siblings (Hatzmann et al. 2008). Parents of chronically ill children are at a greater risk

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for psychosocial problems such as depression, anxiety, cognitive problems, and parental distress (Cousino and Hazen 2013; Murphy et al. 2007; van Oers et al. 2014). Finally, parents frequently face potentially traumatic events (e.g., receiving the initial diagnosis), followed by short- or long-term stress responses (Kazak et al. 2006). Studies evaluating the psychosocial functioning of parents of MPS III patients reported elevated levels of parental distress, depression, and anxiety (Grant et al. 2013; Kalkan Ucar et al. 2010; Malcolm et al. 2012; Somanadhan and Larkin 2016). However, these studies comprised small sample sizes and results were not compared with data on parents of healthy children. In addition, previous studies made no distinction between mothers and fathers, which may be of interest as studies focusing on other disorders identified significant gender differences in psychosocial experiences of parents (Clarke et al. 2009; Marchal et al. 2017). As detailed knowledge about the psychosocial functioning of parents of MPS III patients will help to organize appropriate (psychosocial) support and interventions, we aimed to assess anxiety, depression, and parental distress, as well as posttraumatic stress symptoms, in mothers and fathers of MPS III patients compared to Dutch reference groups.

Methods

Participants and Procedures

Parents of all living MPS III patients under care at the Academic Medical Center (AMC), Amsterdam, were invited by letter to participate in this cross-sectional study. Parents who gave permission to participate received an e-mail with a personal link to online questionnaires. Before starting the questionnaires, online informed consent was obtained. The data collection was performed in accordance with the regulations of the Medical Ethics Committee of the AMC, the Netherlands.

Measures

Sociodemographic Characteristics

Since this study involves a relatively small sample size and the members of our research group know all parents, we did not collect sociodemographic data of nonparticipating parents to guarantee the anonymous nature of the study. Age, gender, educational level, and marital status from participating parents were collected with a sociodemographic questionnaire.

Anxiety and Depression

Anxiety and depression were measured with the "Hospital Anxiety and Depression Scale" (HADS) (Bjelland et al. 2002). This questionnaire consists of 14 items with a four-point Likert scale (0–3) divided into two subscales measuring symptoms of anxiety and depression experienced during the previous week, resulting in scores from 0 to 21 for each subscale. Mean scores on the subscales were calculated, and the proportion of parents with clinically relevant anxiety and/or depression (score of >8) was reported. The Dutch version of the HADS has shown to be valid and reliable (Spinhoven et al. 1997). The Cronbach's alpha values in the present study were good (0.81–0.91). Results were compared to Dutch reference parents (Vingerhoets 2012).

Parental Distress

Parental distress was measured with the "Distress Thermometer for Parents" (DT-P) (Haverman et al. 2013). The DT-P consists of a thermometer score where parents were asked to rate their overall distress (0 = *no distress* to 10 = *extreme distress*). Distress was indicated as clinically relevant from a score ≥ 4. The thermometer was accompanied by a problem list (parents indicated whether they had experienced any of the listed problems during the previous week) divided over six problem domains: practical, family/social, emotional, physical, cognitive, and parenting. The problem domain scores were the sum of the dichotomous items (0 = *no* and 1 = *yes*) in each problem domain. Three additional questions about perceived support and wish for referral were asked.

The DT-P is a well-validated short screening instrument to identify the level of distress in parents of children with a chronic health condition (Haverman et al. 2013). The Cronbach's alpha values in the present study were moderate to good (0.65–0.88). Results of parental distress were compared to Dutch reference parents of healthy children (van Oers et al. 2017).

Posttraumatic Stress

Posttraumatic stress symptoms were measured with the "Self-Rating Scale for Posttraumatic Stress Disorders" (SRS-PTSD) questionnaire (Carlier et al. 1998). Parents were asked to think of an event related to their child's illness that has had the most impact on them. The SRS-PTSD is a self-reported questionnaire for adults and contains 17 items corresponding to the diagnostic DSM-IV

symptoms of PTSD: reexperiencing, avoidance, and hyperarousal. Symptoms experienced during the last 4 weeks were registered on a three-point Likert scale (0–2). Higher scores represent more posttraumatic stress symptoms. Parents met the criteria for PTSD if at least one reexperiencing, three avoidance, and two hyperarousal symptoms were present during the previous 4 weeks. The SRS-PTSD has shown to have adequate psychometric properties (Carlier et al. 1998). In the present study, Cronbach's alpha values were good (0.76–0.91). The prevalence of PTSD among the general Dutch population, measured with the same questionnaire, is known from the literature (Bronner et al. 2009).

Statistical Analyses

Statistical Package for Social Sciences (SPSS) (version 23.0, SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. First, descriptive statistics were used to describe the sociodemographic characteristics of parents and the reference groups. Baseline differences between parents and the reference groups were analyzed with independent-sample T-tests for continuous data and chi-square tests/Fisher's exact tests for categorical data. Second, independent-sample T-tests were performed for continuous data and chi-square tests/Fisher's exact tests for categorical data to assess differences between parents of MPS III patients and reference parents on the outcomes of the questionnaires. Effect sizes (*d*) were calculated by dividing the difference in mean scores between the groups by the pooled standard deviation of both groups, to report the strengths of the differences. *P*-values <0.05 were considered statistically significant in all statistical analyses.

Results

Sociodemographic Characteristics

Fifty-seven parents of 37 patients with MPS III were invited for this study. Four parents declined participation as they indicated they were afraid that participation would be too stressful. Eight parents did not complete the online questionnaires (response rate 78.9%). Sixteen parent couples participated in this study (Table 1). Mothers and fathers were significantly older than those in the reference groups ($p \leq 0.001$). However, as the age of MPS III parents was not correlated with the scores on the questionnaires, no correction for age was used.

Table 1 Sociodemographic characteristics of mothers and fathers of MPS III patients and reference groups

	Mothers			Fathers		
	MPS III, $N = 26$	Reference HADS, $N = 368$	Reference DT-P, $N = 671$	MPS III, $N = 19$	Reference HADS, $N = 368$	Reference DT-P, $N = 463$
Parents						
Age in years, M (SD)	48.7 (10.2)	40.0* (8.2)	38.7* (6.4)	50.1 (9.6)	43.1* (8.5)	41.7* (7.4)
Educational level[a], N (%)						
Low	7 (26.9)	121 (32.9)	88 (13.1)	2 (10.5)	116 (31.5)	72 (15.6)
Intermediate	9 (34.6)	147 (39.9)	300 (44.7)	7 (36.8)	129 (35.1)	193 (41.7)
High	10 (38.5)	100 (27.2)	281 (41.9)	10 (52.6)	123 (33.4)	190 (41.0)
Marital status, N (%)						
Married/living together	23 (88.5)	337 (91.6)	604 (90.0)	18 (94.7)	353 (95.9)	449 (97.0)
Single/separated	3 (11.5)	29 (7.9)	66 (9.8)	1 (5.3)	10 (2.7)	14 (3.0)
Other	0	2 (0.50)	1 (0.20)	0	5 (1.4)	0
Child ($N = 34$)						
Age in years, M (SD) age range	19.76 (9.5) 5–38					
Gender, male, N (%)	18 (52.9)					
MPS phenotype, N (%)						
Rapidly progressing (RP)	9 (26.5)					
Slowly progressing (SP)	25 (73.5)					

*$p \leq 0.001$, according to independent-sample T-tests

[a] Highest level completed. *Low* primary education, lower and middle general secondary education, *intermediate* middle vocational education, higher secondary education, preuniversity education, *high* higher vocational education, university (CBS 2012)

Anxiety and Depression

Mothers of MPS III patients reported significantly higher mean levels of anxiety ($p < 0.001$) and depression ($p < 0.001$) than mothers in the reference group. In addition, they reported more frequently clinically relevant anxiety ($p = 0.001$) and clinically relevant depression ($p = 0.001$) than the reference group. Fathers reported more frequently clinically relevant depression compared to fathers in the reference group ($p = 0.022$) (Tables 2 and 3).

Parental Distress

Mothers and fathers of MPS III patients reported higher mean DT-P thermometer scores and more frequently clinically relevant distress compared to reference parents ($p < 0.01$). Moreover, mothers and fathers reported higher scores on all domains compared to reference parents ($p < 0.001$–$p < 0.05$), except for the social, physical, and cognitive domain for fathers. Analyses of the individual problem domain items revealed that mothers of MPS III patients reported significantly more often problems on 24 out of 34 items ($p < 0.001$–$p < 0.05$) and fathers on 15 out of 34 items ($p < 0.001$–$p < 0.05$). The results of the additional questions showed that both mothers and fathers reported more often than reference parents to have problems with receiving sufficient support from people around them ($p < 0.001$) and indicated more often a (probable) wish to talk to a professional about their situation ($p < 0.001$–$p < 0.01$). Fathers more often reported that people in their surrounding react with a lack of understanding to their situation compared to reference fathers ($p < 0.05$) (Table 4).

Posttraumatic Stress

Ten parents (22%, 7 mothers and 3 fathers) met the criteria for the diagnosis of PTSD (Table 5). When compared to the prevalence of PTSD among the general Dutch population (3.8%) (Bronner et al. 2009), the prevalence in this study is significantly higher among mothers of MPS III patients compared to Dutch women (26.9% vs. 5.3%, chi-square $p < 0.001$) and among fathers of MPS III patients compared to Dutch men (15.8% vs. 2.2%, Fisher's exact $p < 0.05$).

Discussion

Our study investigated the psychosocial functioning of mothers and fathers of Dutch MPS III patients by measuring levels of anxiety, depression, parental distress, and posttraumatic stress. Firstly, we demonstrate significantly higher mean levels of anxiety and depression among mothers compared to reference mothers. Although not significant, the mean level of anxiety and depression among fathers are also notably higher compared to the reference

Table 2 Anxiety and depression (mean scores) in mothers and fathers of MPS III patients in comparison to reference parents[a]

	Mothers				Fathers			
	MPS III, $N = 26$ M (SD)	Reference HADS, $N = 368$			MPS III, $N = 19$ M (SD)	Reference HADS, $N = 368$		
		M (SD)	p	d		M (SD)	p	d
Anxiety	8.0 (4.3)	4.8 (3.5)	**<0.001**	0.90	5.7 (3.9)	4.1 (3.7)	0.067	0.43
Depression	6.0 (3.6)	3.1 (3.3)	**<0.001**	0.87	5.8 (4.9)	3.6 (3.6)	0.069	0.60

Effect size: d
Significant differences at $p < 0.05$ are presented in bold, according to independent-sample T-tests
[a] Higher scores represent higher levels of anxiety and depression.

Table 3 Clinical scores of anxiety and depression in mothers and fathers of MPS III patients compared to reference parents[a]

	Mothers							Fathers						
	MPS III, $N = 26$		Reference HADS, $N = 368$					MPS III, $N = 19$		Reference HADS, $N = 368$				
	N	%	N	%	p	OR	95% CI	N	%	N	%	p	OR	95% CI
Anxiety	13	50	76	20.7	**0.001**	2.42	1.57–3.74	6	31.6	64	17.4	0.128	1.82	0.90–3.65
Depression	9	34.6	44	12.0	**0.001**	2.90	1.59–5.26	7	36.8	56	15.2	**0.022**	2.42[a]	1.28–4.57

Significant differences at $p < 0.05$ are presented in bold, according to chi square tests
[a] Cutoff point for clinically relevant anxiety and depression: score of ≥8

Table 4 Parenting distress in mothers and fathers of MPS III patients compared to reference parents

	Mothers					Fathers				
	MPS III, $N = 26$	Reference, $N = 671$	p	OR/ES	95% CI	MPS III, $N = 19$	Reference, $N = 463$	p	OR/ES	95% CI
Thermometer score, M (SD)	5.96 (2.74)	3.51 (2.70)	<**0.001**	0.91		5.00 (2.75)	2.83 (2.53)	<**0.001**	0.86	
Clinical, %	84.6	42.3	<**0.001**	2.0	1.66–2.41	68.4	32.2	**0.001**	2.13	1.52–2.97
Total problem score, M (SD)	11.38 (7.43)	5.42 (5.07)	<**0.001**	1.15		7.32 (6.52)	3.73 (4.17)	**0.029**	0.85	
Practical problems, M (SD)	2.31 (2.13)	1.06 (1.31)	**0.007**	0.93		2.00 (2.36)	0.80 (1.18)	**0.041**	0.98	
Housing, %	15.4	5.5	0.060	2.79	1.07–7.25	15.8	3.7	**0.039**	4.3	1.38–13.43
Work/study, %	26.9	25.3	0.855	1.06	0.56–2.03	42.1	25.9	0.117	1.63	0.94–2.81
Finances/insurance, %	11.5	16.7	0.601	0.69	0.24–2.03	15.8	14.5	0.747	1.10	0.38–3.16
Housekeeping, %	46.2	21.6	**0.003**	2.14	1.38–3.32	26.3	12.1	0.078	2.18	0.99–4.80
Transport, %	19.2	4.6	**0.008**	4.16	1.76–9.83	15.8	3.9	**0.044**	4.06	1.31–12.61
Child care/child supervision, %	50.0	10.1	<**0.001**	4.93	3.16–7.70	31.6	5.4	**0.001**	5.85	2.73–12.55
Leisure activities/relaxing, %	61.5	22.4	<**0.001**	2.75	1.97–3.85	52.6	14.9	<**0.001**	3.53	2.19–5.70
Social problems, M (SD)	1.04 (1.48)	0.39 (0.74)	**0.035**	0.83		0.68 (1.16)	0.28 (0.63)	0.144	0.62	
Dealing with (ex)partner, %	23.1	12.4	0.128	1.87	0.90–3.87	10.5	11.7	1.00	0.90	0.24–3.43
Dealing with family, %	26.9	10.9	**0.022**	2.48	1.27–4.83	21.1	6.7	**0.041**	3.14	1.24–8.01
Dealing with friends, %	23.1	3.7	**0.001**	6.19	2.78–13.79	15.8	1.5	**0.005**	10.44	2.93–37.27
Interacting with your child(ren), %	30.8	11.8	**0.010**	2.61	1.42–4.82	21.1	7.8	0.063	2.71	1.07–6.83
Emotional problems, M (SD)	4.19 (2.77)	1.77 (2.12)	<**0.001**	1.13		2.42 (1.98)	1.08 (1.64)	**0.001**	0.81	
Controlling emotions, %	53.8	27.4	**0.003**	1.96	1.35–2.86	52.6	11.9	<**0.001**	4.43	2.71–7.26
Self-confidence, %	46.2	22.7	**0.006**	2.04	1.31–3.16	15.8	12.7	0.724	1.24	0.43–3.60
Fears, %	53.8	10.7	<**0.001**	5.02	3.31–7.62	47.4	6.5	<**0.001**	7.31	4.07–13.15
Depression, %	65.4	31.9	<**0.001**	2.05	1.52–2.77	36.8	22.2	0.162	1.66	0.90–3.06
Feeling tense or nervous, %	69.2	36.1	**0.001**	1.92	1.46–2.53	36.8	26.3	0.311	1.40	0.76–2.57
Loneliness, %	30.8	7.7	**0.001**	3.97	2.11–7.48	15.8	3.7	0.**039**	4.30	1.38–13.43
Feelings of guilt, %	26.9	17.4	0.200	1.54	0.80–2.97	0.0	7.3	0.385	–	–
Use of substances (e.g., alcohol, drugs, and/or medication), %	3.8	2.7	0.519	1.43	0.20–10.34	10.5	3.0	0.127	3.48	0.85–14.24
Intrusive/recurrent thoughts about a specific event, %	69.2	20.4	<**0.001**	3.39	2.52–4.56	26.3	13.8	0.170	1.90	0.87–4.18
Physical problems, M (SD)	2.81 (2.00)	1.80 (1.71)	**0.003**	0.59		1.68 (2.19)	1.33 (1.46)	0.491	0.24	
Eating, %	23.1	12.4	0.128	1.87	0.90–3.87	5.3	4.8	0.612	1.11	0.16–7.79
Weight, %	19.2	26.2	0.425	0.733	0.33–1.63	10.5	16.6	0.752	0.63	0.17–2.39
Sleep, %	57.7	29.7	**0.002**	1.95	1.37–2.76	42.1	21.4	**0.046**	1.97	1.13–3.43

(continued)

Table 4 (continued)

	Mothers					Fathers				
	MPS III, $N = 26$	Reference, $N = 671$	p	OR/ES	95% CI	MPS III, $N = 19$	Reference, $N = 463$	p	OR/ES	95% CI
Fatigue, %	76.9	55.7	**0.033**	1.38	1.11–1.72	42.1	44.1	0.866	0.96	0.56–1.64
Out of shape/condition, %	53.8	20.9	**<0.001**	2.58	1.76–3.79	26.3	19.0	0.386	1.39	0.64–3.01
Pain, %	26.9	24.3	0.759	1.11	0.58–2.12	21.1	18.1	0.762	1.16	0.48–2.83
Sexuality, %	23.1	10.6	0.057	2.18	1.05–4.55	21.1	8.9	0.091	2.38	0.95–5.96
Cognitive problems, M (SD)	1.04 (0.87)	0.40 (0.70)	**0.001**	0.91		0.53 (0.84)	0.25 (0.55)	0.170	0.50	
Concentration, %	57.7	17.9	**<0.001**	3.23	2.24–4.66	26.3	11.2	0.061	2.34	1.06–5.19
Memory, %	46.2	22.4	**0.005**	2.07	1.33–3.20	26.3	13.6	0.167	1.93	0.88–4.25
Parenting problems[a]*, M (SD)*	2.27 (1.80)	0.34 (0.78)	**<0.001**	2.30		2.42 (1.54)	0.32 (0.82)	**<0.001**	2.48	
Dealing with your child, %	38.5	9.1	**<0.001**	3.53	2.06–6.06	36.8	7.8	**0.002**	3.79	1.95–7.37
Dealing with the feelings of your child, %	57.7	7.7	**<0.001**	6.21	4.09–9.44	63.2	6.9	**<0.001**	7.30	4.53–11.77
Talking about the disease/consequences with your child, %	30.8	2.5	**<0.001**	10.14	4.82–21.30	31.6	2.2	**<0.001**	11.68	4.75–28.77
Independence of your child, %	69.2	6.3	**<0.001**	9.23	6.26–13.60	78.9	6.0	**<0.001**	10.43	6.82–15.96
Following advice about treatment/giving medication, %	30.8	2.8	**<0.001**	9.07	4.39–18.75	31.6	2.4	**<0.001**	10.62	4.40–25.65
Additional questions										
Enough support from surroundings, %	65.4	92.1	**<0.001**	0.71	0.54–0.94	63.2	93.3	**<0.001**	0.67	0.48–0.96
People react with a lack of understanding, %	23.1	11.3	0.109	2.04	0.98–4.24	26.3	10.2	**0.043**	2.59	1.17–5.77
Would like to talk to a professional about situation, yes/maybe %	69.2	17.1	**<0.001**	4.04	2.98–5.48	36.8	12.5	**0.008**	2.94	1.56–5.56

Total problem score and problem domain scores were analyzed with independent-sample T-tests. The presence of a clinical thermometer score (≥4) and of reported problems (individual items) was analyzed with chi-square tests. Significant differences at $p < 0.05$ are presented in bold

[a] Control parents in the domain "parenting problems" consisted of 560 mothers and 370 fathers

Table 5 Posttraumatic stress (symptoms) in mothers and fathers of MPS III patients

	Mothers ($N = 26$)		Fathers ($N = 19$)	
	N	%	*N*	%
Intrusions (≥1 symptom)	23	88.5	17	89.5
Avoidance (≥3 symptoms)	7	26.9	5	26.3
Hyperarousal (≥2 symptoms)	12	46.2	7	36.8
PTSD[a]	7	26.9	3	15.8

[a] Criteria PTSD are met if at least one intrusion, three avoidance, and two hyperarousal symptoms have been present in the previous 4 weeks

fathers. Half of the mothers meet the criteria for clinically relevant anxiety and approximately one third of both mothers and fathers meet the criteria for clinically relevant depression. Twice as many fathers meet the criteria for clinically relevant anxiety compared to reference fathers, although this is not significant. These high levels of anxiety and depression are in line with previous research among parents of MPS III patients (Grant et al. 2013; Kalkan Ucar et al. 2010).

Secondly, more clinically relevant parental distress and problems on all life domains are found among both mothers and fathers compared to mothers and fathers of healthy children. The reported levels of parental distress are strikingly higher in comparison to those found in other studies on parents with chronically ill children (Basart et al. 2017; Haverman et al. 2013; Limperg et al. 2016). For instance, clinically relevant distress is reported in 63% of mothers and 59% of fathers of patients with pediatric cancer (Schepers et al. 2018) compared to, respectively, 85% and 68% of the mothers and fathers in our study. This is probably due to the fact that, in contrast to most of the other investigated disorders, MPS III is an invariably progressive, neurodegenerative, and ultimately fatal disorder with no disease-modifying treatment available (Shapiro et al. 2016). Thus, after receiving the diagnosis, parents face a very grim and uncertain future, without any hope for improvement or cure. In addition, severe behavioral difficulties and sleeping problems, which are common in MPS III patients (Valstar et al. 2008), are also reported to be associated with increased parental distress (Malcolm et al. 2012; Neece 2014; Somanadhan and Larkin 2016). Another striking conclusion is that approximately one third of both mothers and fathers indicate that they do not receive enough support from their surroundings.

Thirdly, the majority of the parents in this study report posttraumatic stress symptoms related to their child's illness and an astonishing high percentage meet the criteria for PTSD.

We believe that our study adds important and new information to the existing scarce literature. First, we used a relatively large sample size compared to earlier quantitative studies. In addition, almost as many fathers as mothers participated in this study, whereas earlier studies included mostly mothers. The distinction between mothers and fathers is important, as the differences in experiences in psychosocial functioning should be addressed in the care for parents of chronically ill children (Marchal et al. 2017). Finally, we are the first to report on posttraumatic stress symptoms as a potential major factor in the psychosocial impact of being a parent of an MPS III patient, which may warrant a specific therapeutic approach.

Some limitations of the present study need to be discussed. Firstly, four parents declined participation as they felt too burdened, which could have led to selection bias. However, these parents may be even more affected by psychosocial distress than the participants, leading to an underestimation of the problem. Secondly, due to the fact that all data were coded, we are unaware of the disease phases the patients are currently in. Therefore, we cannot correlate the patients' disease phase with the psychosocial functioning of the parents. However, we believe that not the disease phase in particular is correlated to PTSD but the fact that these parents are exposed to prolonged stress due to multiple potential traumatic events (e.g., diagnosis, disease progression, disappointment about treatment possibilities) over the trajectory of the illness (Malcolm et al. 2012) which may impede with the normal diminishing stress response over time. Since this study only included parents of patients who are alive, a greater proportion of the patients with a rapidly progressing phenotype will have passed away, leading to an overrepresentation of patients with a slowly progressive phenotype. Finally, we do not know which event parents had in mind while completing the SRS-PTSD questionnaire. We did not want to introduce bias by providing potential events, such as the moment of diagnosis, since this varies per person.

It is noteworthy that, in our clinical experience, most parents do not have professional psychosocial support, even though 69% of the mothers and 37% of the fathers indicate that they (probably) would like to talk to a professional about their situation. Most parents indicate that the well-being of their child is the most important, which was also demonstrated in a previous study which reported that parents of MPS III patients often force themselves to retain a positive outlook in order to keep the family together (Somanadhan and Larkin 2016). This indicates that most parents are strong and resilient and have efficient coping strategies.

Despite this admirable coping, our data stress the importance of structural monitoring of the psychosocial functioning of these parents in daily clinical practice, as this may help to improve the well-being of parents and also of healthy siblings. Since parents often experience anxiety and stress following their child's diagnosis (Somanadhan and Larkin 2016), we propose to incorporate a medical psychologist consultation as standard of care immediately after the diagnosis. In addition, by using a short screening instrument such as the DT-P (Haverman et al. 2013), parents could be structurally monitored and those parents who need support may thus be identified (van Oers et al. 2014), followed by psychosocial support (for instance, referral to a clinical psychologist or social worker). Although most psychologists are not familiar with MPS III, local services should be able to provide treatment for anxiety, depression, distress, and/or PTSD.

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Synopsis

Mothers and fathers of MPS III patients have an impaired psychosocial functioning, demonstrated by increased levels of anxiety, depression, distress, and a remarkably high prevalence of PTSD.

Details of Author Contributions

Thirsa Conijn and Stephanie Nijmeijer were involved in conception and design of this study, in analyses and interpretation of the data, and drafting the article. Lotte Haverman, Hedy van Oers, and Frits Wijburg were involved in the conception and design of this study, in analyses and interpretation of the data, and critically revising the article. All authors are in agreement with submission of this draft to JIMD reports. Frits Wijburg is the guarantor for this article.

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Competing Interest Statement

The authors have no competing interests to declare.

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Ethics Approval

The study was conducted in compliance with ethical standards.

Patient Consent

Informed consent was obtained from the participating parents.

Institutional Committee for Care and Use of Laboratory Animals

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

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RESEARCH REPORT

The Second Case of Saposin A Deficiency and Altered Autophagy

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Abstract Krabbe disease is a lysosomal storage disease caused by galactosylceramidase deficiency, resulting in neurodegeneration with a rapid clinical downhill course within the first months of life in the classic infantile form. This process may be triggered by the accumulation of galactosylceramide (GalCer) in nervous tissues. Both the enzyme galactosylceramidase and its in vivo activator molecule, saposin A, are essential during GalCer degradation. A clinical manifestation almost identical to Krabbe disease is observed when, instead of the galactosylceramidase protein, the saposin A molecule is defective. Saposin A results from posttranslational processing of the precursor molecule, prosaposin, encoded by the PSAP gene. Clinical and neuroimaging findings in a 7-month-old child strongly suggested Krabbe disease, but this condition was excluded by enzymatic and genetic testing. However, at whole exome sequencing, the previously undescribed homozygous, obviously pathogenic PSAP gene NM_002778.3: c.209T>G(p.Val70Gly) variant was determined in the saposin A domain of the PSAP gene. Fibroblast studies showed GalCer accumulation and the activation of autophagy for the first time in a case of human saposin A deficiency. Our patient represents the second known case in the literature and provides new information concerning the pathophysiology of saposin A deficiency and its intralysosomal effects.

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Introduction

Lysosomal degradation of glycosphingolipids begins with the cleavage of monosaccharide units from the nonreducing ends of the oligosaccharide chain. This reaction is catalyzed by exohydrolases exhibiting activity in acidic pH optima. Several enzymes require glycoprotein molecules known as low molecular weight activator proteins for these reactions (Kolter and Sandhoff 2005; Sandhoff 2016)

Activator proteins are glycoproteins that provide the structural modifications necessary for binding the enzyme and substrate during degradation of lysosomal glycosphingolipids, which combine with the enzyme or the substrate or sometimes with both. These permit selective degradation of membrane lipids in the intraendosomal and intralysosomal membrane pool without compromise of lysosomal integrity (Spiegel et al. 2005).

These proteins are divided into two main groups, sphingolipid activator proteins (SAPs) and GM2 activator protein (Kolter and Sandhoff 2005). With posttranslational

modification, the molecule prosaposin encoded by the PSAP gene is separated into four homologous mature proteins – saposins A–D. These are acidic, enzymatically inactive, thermostable, protease-resistant glycoproteins, 8–11 kDa in size. Despite all their structural similarities, they possess distinct, specific activator functions (Kishimoto et al. 1992).

Inherited activator protein function deficiency leads to the accumulation of undegraded membrane glycosphingolipids in the lysosomal compartments and to lysosomal storage disease (Kolter and Sandhoff 2005). Saposin A is responsible for the activation and stabilization of galactosylceramide beta-galactosidase (GalCer degradation) (Harzer et al. 1997; Morimoto et al. 1989; Pankiv et al. 2007) Deficiency of saposin A results in a Krabbe-like manifestation (Kolter and Sandhoff 2005; Matsuda et al. 2001; Spiegel et al. 2005).

Rather than being the last stage in degradation, the lysosome has been shown to have cellular functions. These include coordinating several intracellular signal pathways, including autophagy, a mechanism necessary for cellular survival (Matsuda et al. 2001; Seranova et al. 2017; Settembre et al. 2008). The impaired degradation mechanism in lysosomal storage diseases compromises autophagic flux, and this has been shown to be one of the mechanisms in the etiopathogenesis of lysosomal storage diseases (Lieberman et al. 2012). Mouse studies have been performed on this subject, but there has been very little examination of saposin A deficiency and its effect on autophagy (Sun and Grabowski 2013).

We report a case of a newly described homozygous mutation in the saposin A domain in the PSAP gene in an infant exhibiting clinical findings of Krabbe disease. To the best of our knowledge, this is the second case following Spiegel et al.'s case report published in 2005, and the first case to be reported from Turkey. This study also presents, for the first time, autophagic findings in human saposin A deficiency.

Material and Methods

Case Report

A 7-month-old girl, with first-degree consanguinity between her parents, presented to our clinic due to refractory convulsions. Head control was present at the age of 1 month, but this was lost at the age of 4 months, while feeding difficulty occurred at 5 months and generalized tonic convulsions started at 6 months. At examination on arrival, her head circumference was in the 3rd percentile, weight in the 3rd to 10th percentile, and height in the 25th percentile. No marked dysmorphic feature was observed, and no organomegaly was present. Deep tendon reflexes were increased, and hypertonicity was present. Ammonia, lactate, plasma amino acids, blood spot carnitine and acylcarnitine, and urine organic acid analyses were normal. Cerebrospinal fluid (CSF) proteins were 135 mg/dL (normal for age, 20–50 mg/dL). Bilateral ventricular enlargement, hyperintense lesions in cerebral white matter, and thickening in the optic nerves were determined at cerebral imaging. No increase in cerebral metabolites was observed at cerebral magnetic resonance spectroscopy (Fig. 1). Severe axonal polyneuropathy was determined by electromyography. Galactosylceramidase enzyme analysis was performed due to compatibility with Krabbe disease. Galactosylceramidase activity in dried blood was low (Table 1). No mutation was determined at GALC gene analysis performed due to clinical and cerebral

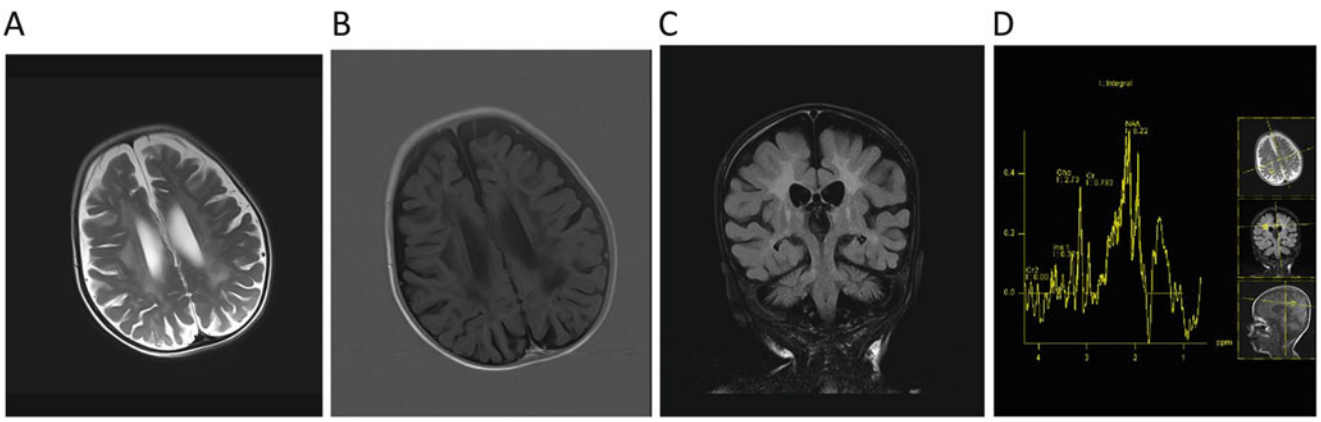

Fig. 1 Magnetic resonance imaging of the patient. (**a**) Axial T2 image; hyperintensity at the periventricular white matter and centrum semiovale. (**b**) Axial T1 IR (inversion recovery) image; hypointensity at the periventricular white matter and centrum semiovale. (**c**) Coronal FLAIR image; hyperintensity at deep white matter, subcortical u-fiber is spared. (**d**) Multivoxel proton MR spectroscopy; choline peak at the level of right centrum semiovale

Table 1 Lysosomal enzymes (dry blood spot)

Enzyme (nmol/mL/h)	Proband	Mother	Father	Brother	Normal values
Galactosylceramidase	0.2	1.1	0.88	0.97	0.55–3.55
Beta-hexosaminidase A	18	28.2	17	13.5	7–70
Beta-galactosidase	9.1	12.3	9.7	9.3	6–24

Table 2 Lysosomal enzymes in white blood cells

Enzyme	Proband	Father	Mother	Brother	Normal values
Galactosylceramidase (nmol/17 h/mg protein)	3.74	21	23.6	32	Infantile Krabbe: 0–0.8 Adult Krabbe: 2.2–3.5
Arylsulphatase A (nmol/h/mg protein)	38.3	36.1	42	27.4	22–103
Beta-galactosidase (nmol/h/mg protein)	234	354	278.4	310	163–378
Tripeptidyl peptidase I (nmol/h/mg protein)	309	101	219	81.3	42–339
Palmitoyl protein thioesterase (nmol/h/mg protein)	57	26.2	102	94.3	17–139

imaging findings compatible with Krabbe disease. Intraleukocytic galactosylceramidase levels investigated for confirmation of enzyme levels were low (Table 2). However, the patient's intraleukocytic galactosylceramidase enzyme activity was higher than the enzyme levels of patients with Krabbe disease. When comparison with other lysosomal enzymes was performed, glucocerebrosidase, beta-galactosidase, hexosaminidase A, hexosaminidase B, alpha-glucosidase, and sphingomyelinase enzyme levels were normal. Assessed in the light of the MR and clinical findings, the suspicion of Krabbe disease was very powerful, but since no mutation was determined at genetic analysis, whole exome sequencing was investigated with a preliminary diagnosis of saposin A deficiency, known to follow a similar clinical course to that of Krabbe disease. At whole exome sequencing, the pathogenic NM_002778.3: c.209T>G (p.Val70Gly) variant not previously described in the PSAP gene was identified and determined as homozygous. This pathogenic variant was confirmed at PSAP gene investigation using Sanger sequencing. Eight pathogenic predictions were demonstrated from DANN, GERP, dbNSFP.FATHMM, MetaLR, MetaSVM, MutationAssessor, MutationTaster, and PROVEAN (vs one benign prediction from LRT) by in silico assessment tools. Allele was not found in Broad gnomAD exomes. Screening of the patient's mother, father, and sibling identified the same pathogenic variant as heterozygous. The patient's and family members' PSAP mutation Sanger sequencing patterns and the patient's Integrative Genomics Viewer presentation are shown in Fig. 2.

Biochemical Studies

Enzyme Assay

Galactosylceramidase activity was measured by UHPLC MS/MS (Waters Acquity™ UPLC I-Class system) method (Orsini et al. 2012). Substrate was kindly provided by CDC.

Cell Culture

Fibroblasts were obtained from forearm skin with punch biopsy. Fibroblasts were cultured and maintained in DMEM high glucose (Gibco) supplemented with 20% FBS (Gibco) and 1% (vol/vol) penicillin/streptomycin (Gibco). Passage-matched fibroblasts (passages 3–5) were used in all experiments.

Glycosphingolipid (GSL) Extraction and Thin Layer Chromatography

GSLs were extracted from proband and healthy control primary skin fibroblasts, separated and chemically stained as described previously (Sandhoff et al. 2002) with minor modifications. The cells were washed with 1 × PBS and resuspended in 1 mL distilled water. They were then subjected to three successive rounds of freeze-thawing by incubation in a −80°C freezer for 5 min and thawing in cold water. The sample was sonicated for 3 min, followed by lyophilization and extraction two times with 2 mL of acetone.

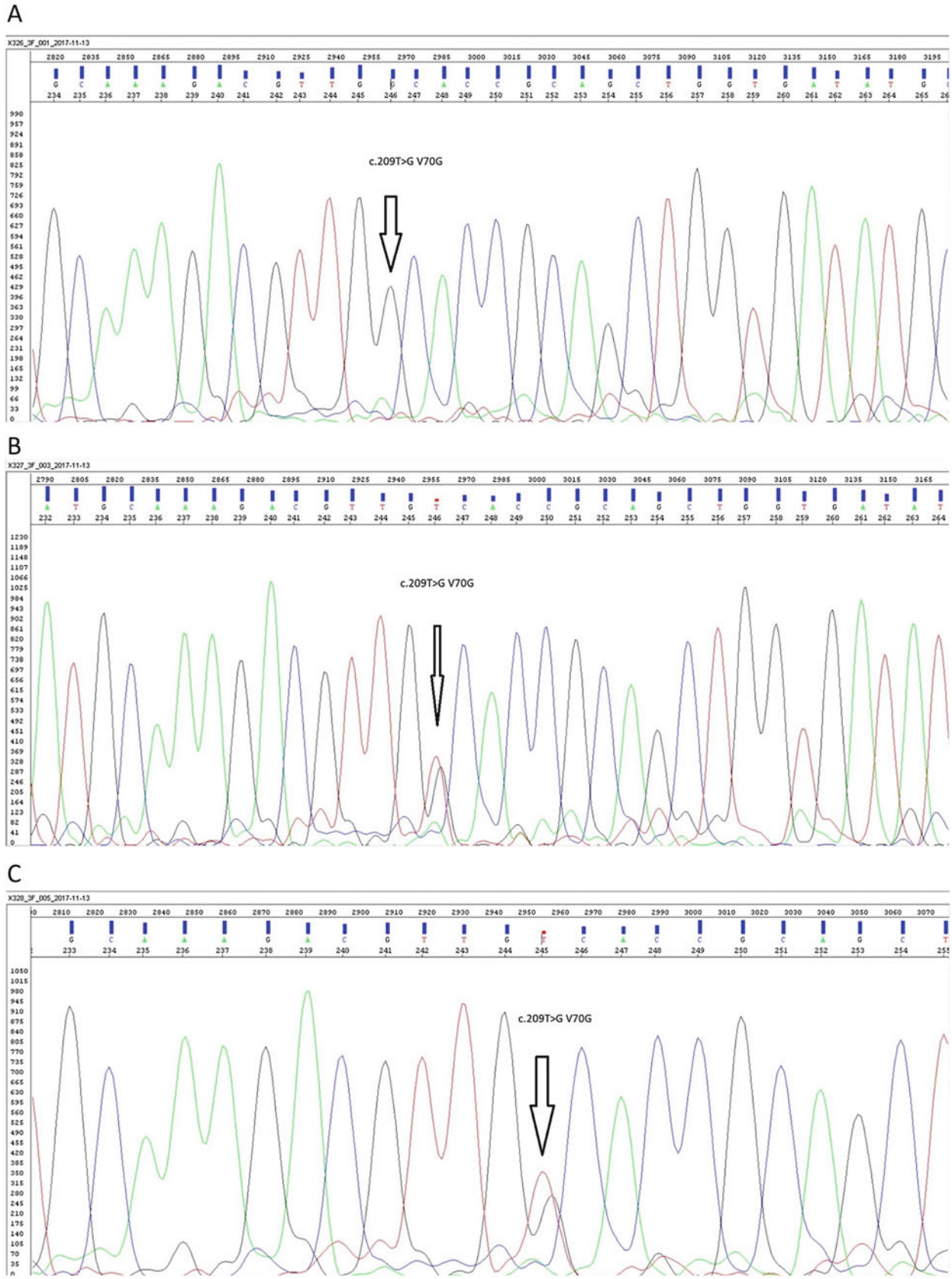

Fig. 2 Sanger sequencing images of proband (**a**), father (**b**), mother (**c**), brother (**d**), and Integrative Genomics Viewer presentation of PSAP gene of proband (**e**)

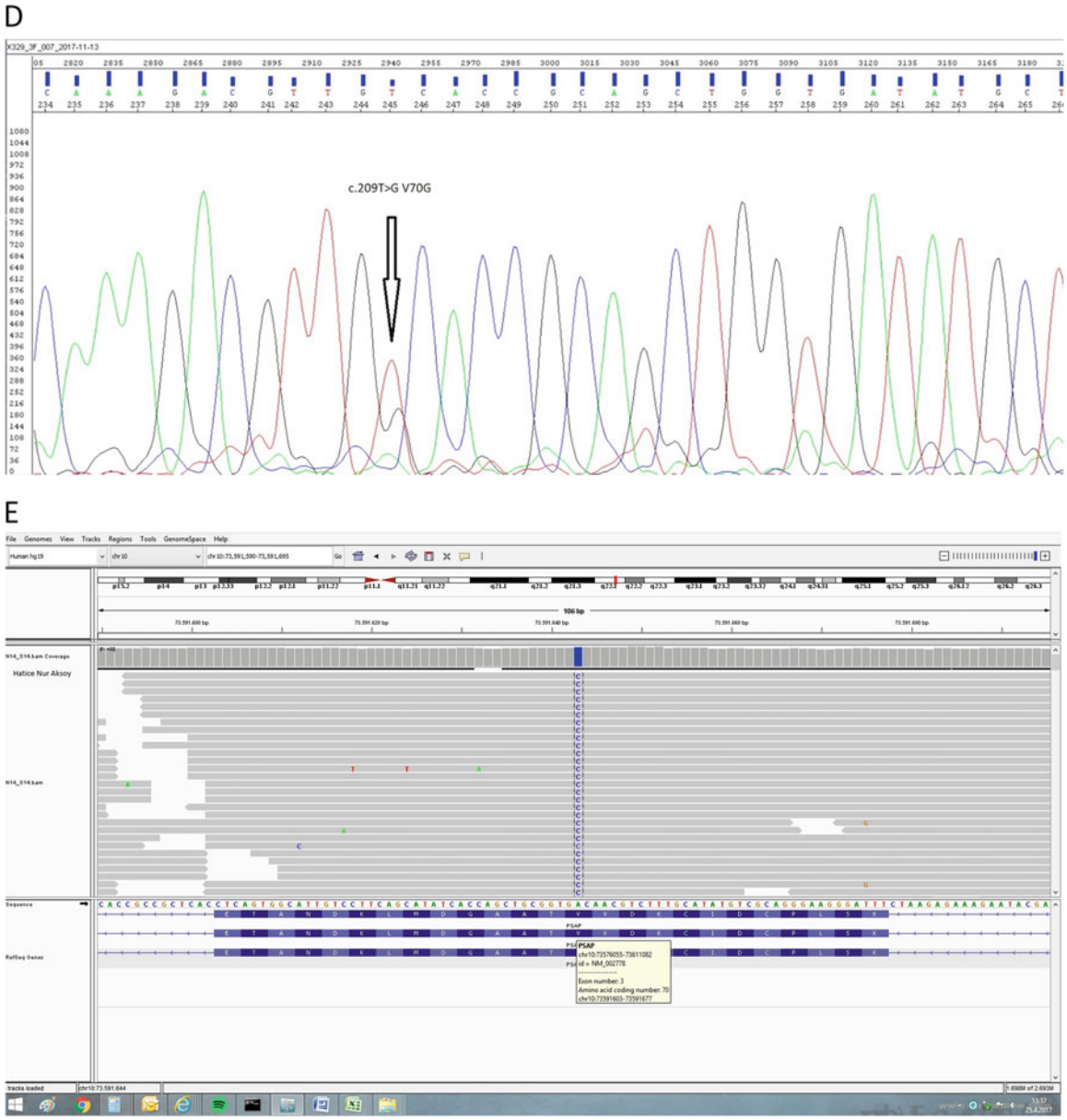

Fig. 2 (continued)

The pellets were re-extracted with solvent mixtures of chloroform/methanol/water, twice in a ratio of 10:10:1 and twice in a ratio of 30:60:8, and all supernatants were pooled. Neutral GSLs were separated on DEAE A-25 as the flow-through and methanol wash. The solvent was evaporated, and the dried lipids were dissolved in the solvent mixture of chloroform/methanol/water in a ratio of 10:10:1. TLC plates, loaded with lipid extracts, were developed with chloroform/methanol/0.2% aqueous CaCl2 (60/35/8) and then dried and stained with orcinol to reveal sugar containing compounds.

Immunofluorescence

Proband and healthy control primary skin fibroblasts were grown on microscope slides. The cells were washed with 1 × PBS and fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. They were then washed three times with PBS and permeabilized using PBS containing 0.3% TritonX100 at room temperature, followed by blocking for 1 h in PBS containing 10% goat serum and 0.3% TritonX100. Anti-LAMP1 (1:500, abcam – ab24170),

Anti-SQSTM1/p62 (1:500, Thermo – PA5–20839), and Anti-hLC3/MAP 1LCA (1:20, R&D Systems – MAB8558) primary antibody incubation was performed overnight at 4°C. The cells were extensively washed with PBS containing 0.05% Tween 20. Alexa Fluor 488-conjugated goat anti-rabbit (1:500, Abcam – ab150077) and Alexa Fluor 568-conjugated goat anti-rat (1:250-Abcam- ab175476) secondary antibody incubation was performed for 1 h at room temperature. The cells were mounted with Fluoroshield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI, Abcam – ab104139). Images were acquired by fluorescence microscopy (OLYMPUS). Colocalization analysis between p62 and LC3A and between LC3A and LAMP1 was performed using Coloc 2 based on Pearson's coefficients on ImageJ.

Statistical Analysis

GraphPad statistical software was used for statistical analysis. All values are expressed as mean ± S.E.M. Differences were tested using one-way ANOVA. A p-value of less than 0.05 was considered statistically significant.

Results

Altered Ganglioside Pattern in Saposin A-Deficient Fibroblasts

We showed the levels of neutral glycosphingolipids in fibroblasts from saposin A patient (proband) and healthy family members using thin layer chromatography (Fig. 3a). We detected significant increases in the levels of GalCer (Fig. 3b), LacCer (Fig. 3c), Cer (Fig. 3d), and GlcCer (Fig. 3e) in the proband fibroblast cell compared to the control group, 3.5-fold, 1.5-fold, 2-fold, and 1.4-fold, respectively.

Increased Numbers of Lysosomes in Saposin A-Deficient Fibroblasts

Fluorescence microscopic imaging of lysosomal-associated membrane protein (LAMP1) revealed increased numbers of lysosomal vesicles in proband fibroblast (Fig. 4c), compared to healthy family members (Fig. 4a, b). The lysosomal vesicles in the fibroblast were measured using ImageJ. The lysosomal intensity measured per cell was 105 for the father, 96 for the son, and 207.5 for the proband. There are at least twofold increases in the proband fibroblast.

Increased Autophagosome Numbers in Saposin A-Deficient Fibroblast

We determined twofold enhanced LC3 and p62 in the proband fibroblast (Fig. 5b, c, respectively) compared to healthy family members (father and son) (Fig. 5a). Autophagosome maturation was confirmed by colocalization of p62 and LC3 (Filimonenko et al. 2007; Pankiv et al. 2007; Aflaki et al. 2016) (Fig. 5a, d). Autophagosome-lysosome fusion is impaired in saposin A-deficient fibroblasts. We demonstrated that the extent of LAMP1/LC3A colocalization was reduced (ranging from 50 to 60%) in the proband fibroblast compared to those of the father and brother (Fig. 6a, b).

Discussion

This report describes a case of clinical Krabbe disease, uncommon for the molecular defect sparing the GALC gene but affecting the PSAP gene by a homozygous pathogenic variant in the gene's saposin A domain (saposin A deficiency, OMIM #611722) highly compatible with infantile Krabbe disease. Krabbe disease (globoid cell leukodystrophy, OMIM #245200) was first described by Krabbe in 1916 and is an autosomal recessive lysosomal storage disease leading to globoid cell accumulation in brain tissue. Deficiency of the saposin A molecule leads to a Krabbe-like manifestation (Spiegel et al. 2005). Following the first detection of saposin B by Mehl and Jatzkewitz in 1964, diseases caused by deficiency of these small molecules with a very important role in glycosphingolipid degeneration also began being described (Kishimoto et al. 1992). Since saposin A activates galactosylceramidase together with saposin C in vitro, a deficiency of saposin A alone was not thought to lead to a manifestation of disease (Harzer et al. 1997). However, in their 2001 mouse study, Matsuda et al. identified saposin A as a major activator of galactosylceramidase in the brain and showed that saposin A deficiency results in a Krabbe disease-like clinical manifestation (Matsuda et al. 2001). Spiegel et al. reported the first human case of saposin A deficiency in 2005 (Spiegel et al. 2005). Interestingly, Matsuda et al. observed a late-onset clinical phenotype in the sap A−/− mice, while the Spiegel et al. case had an early infantile phenotype. Matsuda et al. attributed this difference to intact saposin C, which could, in addition to its other functions, have compensated for saposin A to some extent but was unable to perform its entire function (Matsuda et al. 2001).

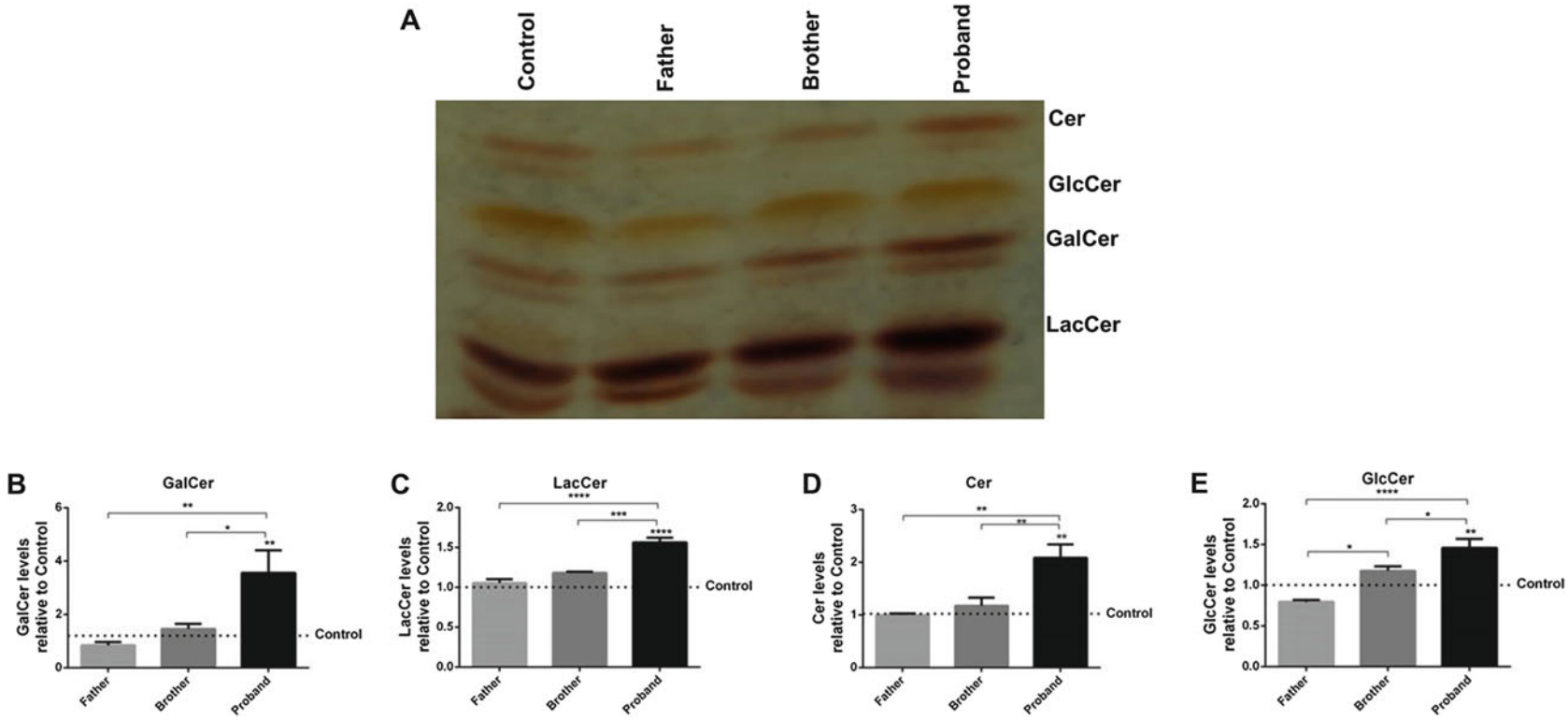

Fig. 3 Thin layer chromatographic analysis of neutral glycosphingolipid from fibroblasts of saposin A patient (proband) compared to healthy family members (father and brother) and control group (**a**). GalCer (**b**), LacCer (**c**), Cer (**d**), and GlcCer (**e**) ratio from proband and her healthy family controls with thin layer chromatography. The relative intensities of the bands on the TLC were analyzed using ImageJ. The data are represented as the mean ± S.E.M. One-way ANOVA was used for statistical analysis (*$p < 0.05$, **$p < 0.025$, ***$p < 0.0025$, and ****$p < 0.001$)

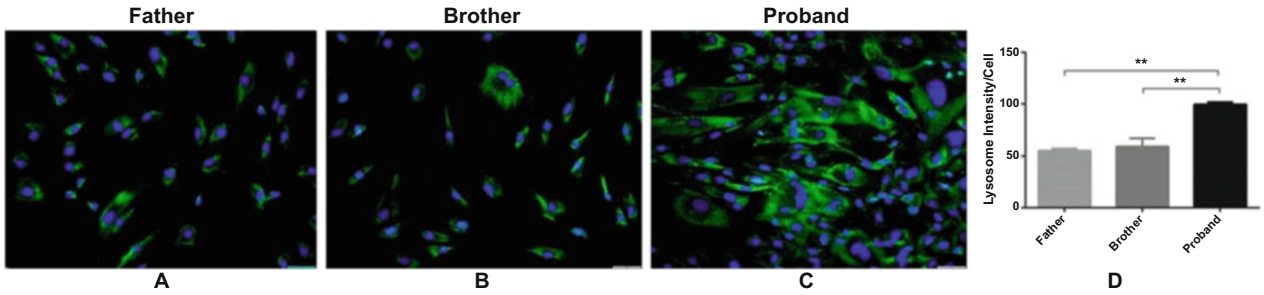

Fig. 4 Increased number of lysosomes in saposin A patient fibroblasts (proband) compared to healthy controls (father and brother). Immunofluorescence signals of LAMP1 were enhanced in fibroblast of proband (**c**) compared with her healthy family members (**a**, **b**, respectively). Lysosomes (green) were stained with Anti-Lamp1 antibody. Nuclei (blue) were stained with DAPI. 20× microscopic images. An increased number of LAMP1 vesicles in proband fibroblasts were analyzed using ImageJ (**d**). The data are represented as the mean ± S.E.M. One-way ANOVA was used for statistical analysis (**$p < 0.025$)

In contrast, classic infantile Krabbe findings were observed in Spiegel et al.'s case (2005). Classic infantile Krabbe findings were also present in our patient. Neuromotor development was normal until the 4th month, after which a severe neurodegenerative process commenced. The reason for this pronounced difference between human and mouse models is still unclear, although in a study from 2013, Sun et al. reported a shorter life span of sap A−/− mice and rapid neurodegeneration based on their own experiences (Sun and Grabowski 2013; Sun et al. 2003).

Since our patient's disease was highly compatible with a clinical manifestation of Krabbe disease but incompatible with the results of enzymatic and GALC gene analysis, all enzymatic and genetic investigations were repeated twice due to a strong suspicion of that disease. Saposin A defect was primarily considered, and the whole exome sequencing method was employed to exclude any other Krabbe-like leukodystrophy. No other relevant change associated with the clinical manifestation was determined in addition to the pathogenic variant identified in the PSAP gene. The considerable similarities between the clinical manifestation in our case and that published by Spiegel et al. initially directed us to the hypothesis of saposin A deficiency (Spiegel et al. 2005). Another problematic point is that our patient's enzyme level at dried blood and intraleukocytic investigation was higher than that expected in classic disease, but lower than that in normal controls. This is an unexpected finding considering the nature of the activator protein. For example, normal arylsulfatase B levels have been shown in cases of saposin B deficiency (Deconinck et al. 2008). However, in Spiegel et al.'s study, galactosylceramidase activity at intraleukocytic investigation was lower than that normally expected and was normal in fibroblast measurements. It has therefore been postulated that saposin A is required not only for activation but also for stabilization and that the enzyme requires an activator during intraleukocytic measurements. However, due to the use of in vitro detergents during fibroblast measurement, the activator does not affect the level of lack of protein (Spiegel et al. 2005). Additionally, Bradova et al. described low GALC activity in combined saposin A, B, C, and D deficiency (Bradova et al. 1993). Saposin A is very likely to have also in in vitro assays (despite the presence of detergents or liposomes with the GalCer substrate), not only in vivo, some activating influence on GALC activity. Thus, in saposin A deficiency, the missing influence (missing saposin A interaction with GALC and/or GalCer substrate) explains lowered GalCer degradation also in vitro, despite genetically intact GALC enzyme.

The identification of more new cases of saposin A and other activator protein deficiency will permit a better understanding of these proteins' tissue levels and lysosomal functions and will help clarify the existing uncertainties.

Galactosylsphingosine (psychosine) accumulation is particularly responsible for neurotoxicity and central nervous system involvement in Krabbe disease. Psychosine accumulates, not alone, but together with GalCer. However, accumulation of psychosine has particularly been observed in brain tissue (Harzer et al. 2002). Psychosine accumulation could not be investigated in our study since the specimen taken from our patient was fibroblast tissue, and brain tissue specimens would be needed. Galactosylceramide levels are a significant and specific marker of insufficient galactosylceramidase activity. In vivo studies have shown that saposin A also plays a role in the degradation of GalCer to Cer and also of LacCer to GlcCer in saposin A-deficient mice (Sun et al. 2013). We detected significant increases in the levels of GalCer (Fig. 3b), LacCer (Fig. 3c), Cer (Fig. 3d), and GlcCer (Fig. 3e) in the proband fibroblast cell compared to the control group, 3.5-fold, 1.5-fold, 2-fold, and 1.4-fold, respectively. The absence of any mutation in the GALC gene that codes the protein galactosylceramidase is further evidence that GalCer accumulation is due to saposin A deficiency. Since

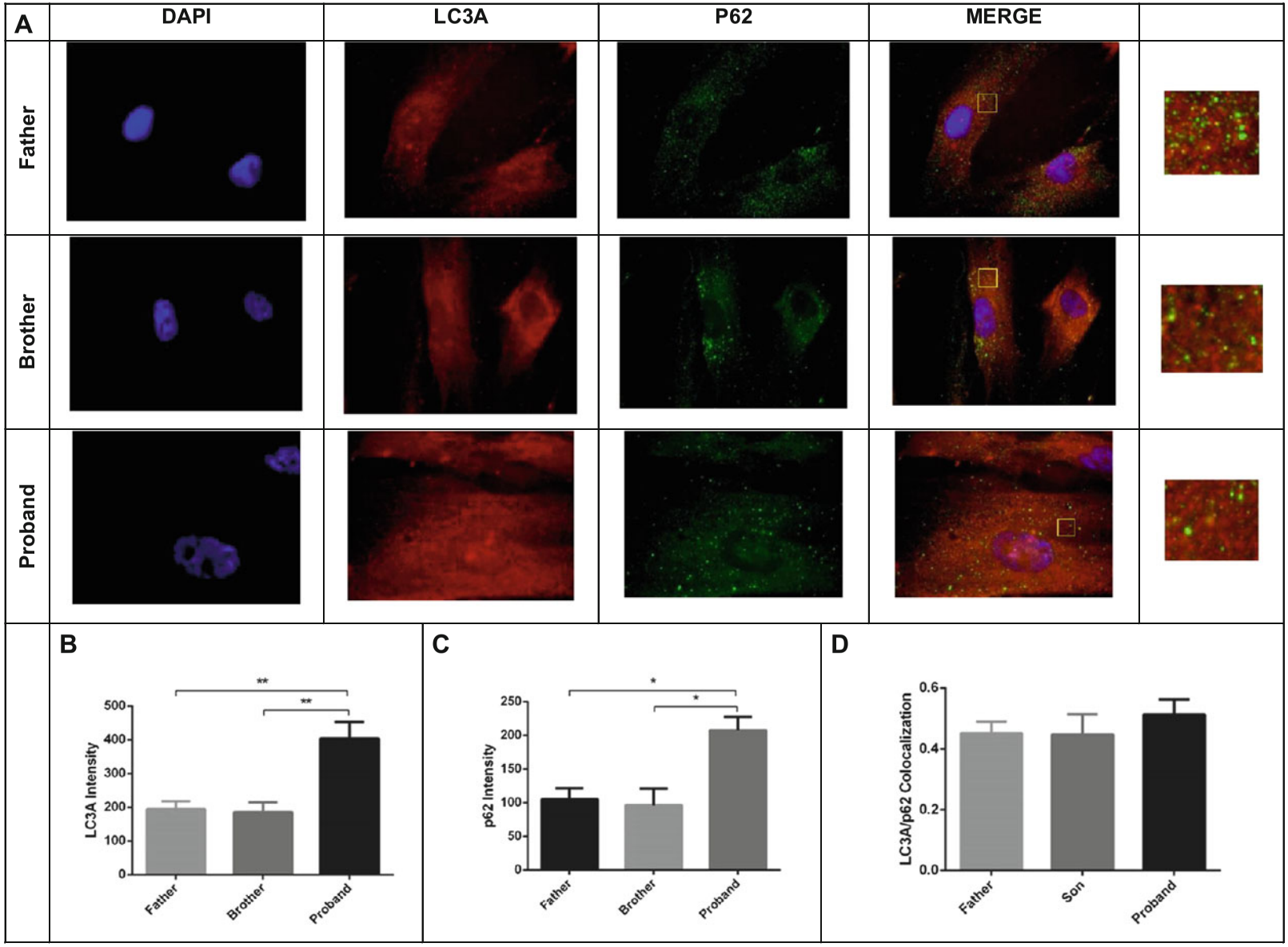

Fig. 5 Autophagosomes produced both in proband fibroblasts and in healthy control group (father and brother) (**a**). Double immunocytochemical staining of fibroblast cells showed colocalization of LC3 with p62. LC3 protein was visualized in red, p62 in green, nuclei in blue, and autophagosomes in *yellow*. A yellow signal signifies colocalization of LC3A and p62. In order to better visualize the fluorescent signals present within the large merged images, zoom images were shown. In proband fibroblast increased the number of autophagy markers; LC3A (**b**) and p62 (**c**) were analyzed using ImageJ. Production of autophagosomes was shown healthy fibroblast cells and proband fibroblast cells. Colocalization analysis of LC3 with p62-labeled lysosomes was performed on ImageJ based on Coloc 2 analysis and Pearson value (**d**). The data are represented as the mean ± S.E.M. One-way ANOVA was used for statistical analysis (*$p < 0.05$, **$p < 0.025$)

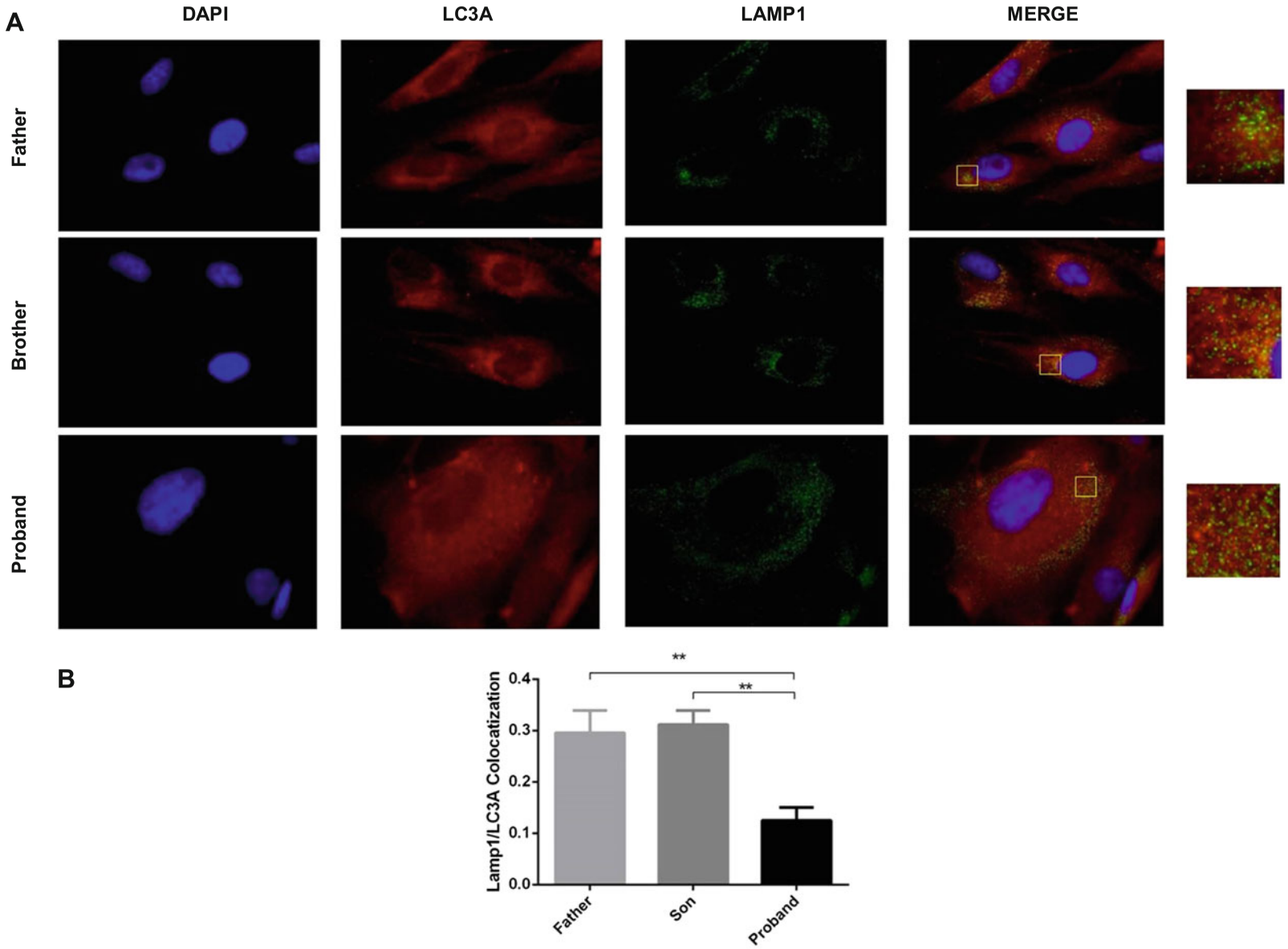

Fig. 6 Autophagosomes and lysosomes failed to fuse in saposin A patient fibroblasts compared to healthy controls (father and brother). (**a**) Double immunocytochemical staining of fibroblast cells showed the colocalization of LC3A with LAMP-1 antibodies by fluorescence microscopy. LC3A protein was visualized in red, LAMP1 in green, and nucleus in blue. Yellow signal signifies colocalization of LC3A and LAMP-1. In order to better visualize the fluorescent signals present within the large merged images, zoom images were shown. Fusion between autophagosomes and lysosomes (*yellow*) was evident in healthy fibroblast cells, but very little in proband fibroblast cells. Colocalization analysis of LC3 with LAMP-1-labeled lysosomes was performed on ImageJ based on Coloc 2 analysis and Pearson value (**b**). The data are represented as the mean ± S.E.M. One-way ANOVA was used for statistical analysis (**$p < 0.025$)

the specimen taken from our patient was obtained from fibroblast tissue, we think that the accumulation might have been greater had brain tissue or peripheral nerve tissue specimens been investigated.

Galactosylceramidase-deficient (Krabbe) skin fibroblasts have been reported to contain increased LAMP1- and LAMP2-positive vesicles in their fibroblasts (Lim et al. 2016). We observed increased numbers of lysosomal vesicles in proband fibroblast (Fig. 4c), compared to healthy family members (Fig. 4a, b). Increases of at least twofold occur in the proband fibroblast for lysosomal intensity. These data clearly indicate increased expression of LAMP1 and lysosomal vesicles in our saposin A-deficient fibroblast patient, similar to other lysosomal storage diseases.

LC3 and p62 are widely used autophagy markers. LC3 binds specifically to autophagic membranes and remains bound throughout the pathway. p62 functions to deliver ubiquitinated substrates to the autophagosome. p62 interacts with LC3 and keeps it in the inner and outer autophagosome membranes. Autophagosomes then fuse with lysosomes to form autophagolysosomes, which act to degrade their contents. p62 is also degraded in the autophagolysosomes (Aflaki et al. 2016). Several studies have shown accumulation of autophagosome via increased levels of LC3 and p62 in various lysosomal storage disorders such as Fabry disease, Gaucher's disease, and Pompe disease (Ward et al. 2016). GSL accumulations have also been shown to affect autophagy in lysosomal storage disorder, and saposin A deficiency is also the primary factor for autophagy. Enhancement of two autophagy markers, p62 and LC3, has been observed in the brains of saposin A-deficient mice (Sun and Grabowski 2013; Sun et al. 2013). Ours is the first report of impaired autophagy in the saposin A-deficient patient fibroblast. We determined twofold enhanced LC3 and p62 in the proband fibroblast (Fig. 5b, c, respectively) compared to healthy family members (father and son) (Fig. 5a). These features were attributed to a block in autophagic flux, since elevated levels of the autophagosomal marker LC3A and autophagic substrate p62 were detected in the proband fibroblast. Autophagosome maturation was confirmed by colocalization of p62 and LC3 (Aflaki et al. 2016; Filimonenko et al. 2007; Pankiv et al. 2007; Spiegel et al. 2005) (Fig. 5a, d). These results show that no problem occurred in the formation of autophagosomes in both healthy and patient fibroblasts.

In most lysosomal storage disorders, the autophagic pathway is impaired by lysosomal dysfunction. Blocking of the autophagic pathway occurs as a consequence of a decreased ability of lysosomes to fuse with autophagosomes (Filimonenko et al. 2007; Settembre et al. 2008; Ward et al. 2016). Clearance of autophagosomes occurs via fusion with lysosomes. Here, we investigated the subcellular localization of the lysosomal marker (LAMP1) and the autophagosomal marker LC3A using fluorescence microscopy and demonstrated that the extent of LAMP1/LC3A colocalization was significantly reduced (ranging from 50 to 60%) in the proband fibroblast compared to those of the father and brother (Fig. 6a, b). Our data suggest that accumulation of autophagosomes (Fig. 5b, c) in saposin A-deficient fibroblasts is due to defective clearance caused by impaired autophagosome-lysosome fusion (Fig. 6a, b).

Autophagy has been identified as an important cause of neurodegeneration in neurodegenerative lysosomal storage diseases, including Krabbe disease (Ribbens et al. 2014). Activated autophagy is a significant indicator that the intracellular coordinated lysosomal expression and regulation (CLEAR) system is also compromised. The neuropathology is thought to occur, not solely in association with GalCer or glycosphingolipid accumulation but also with impairment of the CLEAR system (Palmieri et al. 2011). Ours is also the first study to show the presence of autophagy in a case of human saposin A deficiency.

De Duve, who first described the lysosome, said that very little mystery remained regarding its place in genetic diseases. However, many uncertainties in fact remain. The role of all activator proteins, including saposin A, inside the cell, in all likelihood exceeds what we currently know. Much still remains to be explained concerning the roles of activator proteins in the lysosomal and degradation pathways, their interactions with other proteins, and whether or not these will have a role to play in the treatment of lysosomal storage diseases in the future. Further studies and new cases are now required for these questions to be answered. Saposin A deficiency may be accurately described as one of the rarest forms of inborn errors of metabolism. Our case is the second known report in the literature. However, there are thought to be a large number of leukodystrophies still awaiting identification. The most important step in the diagnosis of saposin A and other activator protein deficiencies is clinical suspicion, and it is not possible to identify these diseases in the absence of such suspicion. Investigation directed toward saposin A deficiency must be considered when clinical and radiological diagnosis of Krabbe disease is not supported by enzymatic and genetic methods.

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Conflict of Interest

No conflict of interest was declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics

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

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RESEARCH REPORT

An Electronic Questionnaire for Liver Assessment in Congenital Disorders of Glycosylation (LeQCDG): A Patient-Centered Study

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Abstract Congenital disorders of glycosylation (CDG) are ultra-rare diseases showing a great phenotypic diversity ranging from mono- to multi-organ/multisystem involvement. Liver involvement, mostly nonprogressive, is often reported in CDG patients. The main objectives of this work were (1) to better understand liver involvement in CDG patients through a liver electronic questionnaire targeting CDG families (LeQCDG) and (2) to compare responses from LeQCDG participants with literature review regarding the prevalence of liver disease and the occurrence of liver symptoms in CDG patients. The network of patient advocacy groups, families and professionals (CDG & Allies – PPAIN) developed the LeQCDG by adapting validated published questionnaires. The LeQCDG was approved by an ethics committee, and the recruitment of patients and caregivers proceeded through social media platforms. Participants were asked to report past or present liver-related symptoms (e.g. hepatomegaly, liver fibrosis and cirrhosis) and laboratory results (e.g. biochemical and/or radiological). From 11 December 2016 to 22 January 2017, 155 questionnaires were completed. Liver disease was present in 29.9% of CDG patients. Main symptoms reported included hepatomegaly, increased levels of serum transaminases, fibrosis, steatosis and cirrhosis. The data obtained in this online survey confirm findings from a recent literature review of 25 years of published evidence ($r = 0.927$, $P = 0.02$). Our questionnaire collected large amounts of meaningful, clinical and patient-oriented data in a short period of time without geographic limitations. Internet-based approaches are especially relevant in the context of ultra-rare diseases such as CDG.

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Abbreviations

CDG & Allies	PPAIN – CDG Professionals and Patient Associations International Network
CDG	Congenital disorder(s) of glycosylation
CLDQ	Chronic liver disease questionnaire
ePROs	Electronic patient-reported outcomes
HQLQ	Hepatitis quality-of-life questionnaire
LDQoL	Liver disease quality-of-life questionnaire
LDSI 2.0	Liver disease symptom index 2.0
LeQCDG	Liver electronic questionnaire for CDG
PLD-Q	Polycystic liver disease-specific symptom questionnaire
pLTQ	Post-liver transplant quality of life

PROs	Patient-reported outcomes
QoL	Quality of life

Introduction

Congenital Disorders of Glycosylation

Congenital disorders of glycosylation (CDG) are a group of mostly autosomal recessive disorders first described in 1980 by Jaak Jaeken (Jaeken et al. 1980). It is a rapidly growing family of very rare genetic diseases comprising more than 100 different subtypes (Jaeken and Péanne 2017). In glycosylation, glycans ('sugar trees') are assembled, processed and attached into proteins or lipids. It is the most important post-translational modification of proteins and a fundamental cellular process. Glycosylation defects are divided in several groups, namely, defects in *N*- and/or *O*-linked glycosylation, in lipid glycosylation and in GPI-anchor biosynthesis. *N*-glycosylation defects are the most common type, with almost 1,000 reported patients with the most frequent PMM2-CDG.

The majority of CDG patients present multisystem organ impairment with a vast clinical diversity ranging from mild to severe dysfunction (Monticelli et al. 2016; Marques-da-Silva et al. 2017a, b; Francisco et al. 2018). Liver involvement in CDG also ranges from mild to severe. Indeed, 22% of patients with CDG subtypes can present with liver involvement such as elevated serum transaminases, hepatomegaly, steatosis, fibrosis and cirrhosis (Marques-da-Silva et al. 2017a).

Data on the prevalence, severity and long-term evolution of liver disease in CDG is sparse. This hampers clinicians and families in recognizing symptoms and signs and in the prevention of liver disease-related complications, management and treatment. A recent literature review identified only 99 publications with information relevant to the subject (Marques-da-Silva et al. 2017a). Consequently, additional strategies/sources of medical information on this group of diseases are required.

Nowadays, there is a shift, especially in rare diseases, in the patient-physician relationship, and patients are often experts in their own rights obliged to become experts of their own medical conditions (Budych et al. 2012). Therefore, gathering data directly from patients and their caregivers is a unique approach with tremendous potential.

Quality of Life in Congenital Disorders of Glycosylation

Patient-reported outcomes (PROs) are assessed through validated questionnaires, which can evaluate quality of life (QoL) (Erhart et al. 2009; D'Ambrosi et al. 2017; Zeltner et al. 2016), symptoms (Malcolm et al. 2012; Neijenhuis et al. 2016), treatment and care satisfaction/effectiveness (D'Ambrosi et al. 2017) and even compliance to medication (Geissler et al. 2017). PROs are used clinically, as secondary endpoints in clinical trials and as tools employed in natural history studies and patient registries (Coons et al. 2009; Paulsen et al. 2010). Moreover, when patients are unable to reliably report for themselves, namely, in the case of children and/or due to the severity of their illness, impaired language ability speech or cognitive functioning, PROs may be completed by a proxy such as a parent (Matza et al. 2013).

Specifically for liver disease, there are already several validated tools, such as the Hepatitis Quality of Life Questionnaire (HQLQ), the Chronic Liver Disease Questionnaire (CLDQ), the Liver Disease Quality of Life questionnaire (LDQoL), the Liver Disease Symptom Index 2.0 (LDSI 2.0), the post-liver transplant quality of life (pLTQ) and the polycystic liver disease-specific symptom questionnaire (PLD-Q), which have been validated in target populations and have been translated to various languages (Younossi et al. 2001; Gutteling et al. 2007; Van Der Plas et al. 2007; Mucci et al. 2010; Saab et al. 2011; Neijenhuis et al. 2016). Indeed, in a recent study in which both CLDQ and a general HQoL questionnaire were taken from a heterogeneous population of patients with chronic liver disease, it was shown that combining PROs analysis with clinical objective scores might improve disease diagnosis, management as well as therapeutic effects (Obradovic et al. 2017).

Performing clinical research in rare diseases, like CDG, poses many challenges: there is complexity in collecting robust data in small-sized populations, that are globally dispersed, which is compounded by lack of funding (Augustine et al. 2013). Nevertheless, technology offers innovative approaches that help to overcome these hurdles. Electronic PROs (ePROs) have been gaining popularity and, a sudy comparing paper-based and ePROs revealed that both had the same reliability. The ePROs also presented advantages over the paper-based PROs, including the ease of data collection and processing while being more user-friendly (Coons et al. 2009). Additionally, social media has been emerging as a medical research recruitment platform, which is particularly helpful for geographically dispersed populations, like rare disease communities. Indeed, social media has repeatedly been used to conduct research surveys and questionnaires with high adherence and good results (Schumacher et al. 2014; Davies 2016; Topolovec-Vranic and Natarajan 2016; Burton-Chase et al. 2017). With 40% of the world population having access to the Internet, social media-based medical research is gaining support due to its accessibility, anonymity, simplicity, affordability, outreach

and engagement capabilities (Fenner et al. 2012; Davies 2016; Burton-Chase et al. 2017).

Employing social media in clinical research, we implemented an electronic questionnaire aiming to:

1. Better understand liver involvement in CDG patients through a liver electronic questionnaire targeting CDG families (LeQCDG) where participants were asked to report past or present liver-related symptoms (e.g. hepatomegaly, liver fibrosis and cirrhosis) and the laboratory findings (e.g. biochemical and/or radiological).
2. Compare data reported by LeQCDG participants with previously published literature review regarding the reported prevalence of liver disease in CDG and the occurrence of liver symptoms in CDG patients.

The questions implemented in the LeQCDG were based on a recent literature review focusing on liver involvement in CDG and inspired us to adapt the existing PROs instruments to assess liver disease (Marques-da-Silva et al. 2017a).

Methods

Literature Search

To construct the liver electronic questionnaire for CDG (LeQCDG), we performed a literature search using Google Scholar and PubMed platforms. The keywords presented in Table S1 (Supplementary Material) were used to identify validated tools to evaluate liver-related symptoms and signs. This search was also performed to find validated tools to measure quality of life in liver disease patients. To maximize and refine the number and quality of the retrieved tools, an additional search was performed as explained in Table S2 (Supplementary Material). The identified instruments were characterized according to the following parameters: generic/specific disease questionnaire, main domains, specific domains, mode of administration, number of items, score system, completion time, available translations (cross-cultural studies) and validated tools.

Building and Testing the LeQCDG

Based on the information collected from the above-mentioned literature search, a liver questionnaire was constructed. Since we specifically wanted to collect data from CDG patients, the tool was then tailored to this patient group. For this, we used information available from our recent published work (Marques-da-Silva et al. 2017a) that describes the main liver symptoms reported in CDG patients. The questionnaire integrated the following sections: 'Participants Data', 'Liver Signs and Symptoms', 'Liver Symptoms', 'Impact of Liver Involvement', 'Liver Transplantation' and 'Awareness and Information About Liver Involvement in CDG'.

The online platform used to implement the questionnaire was SurveyMonkey (http://www.surveymonkey.net – Copyright©1999–2018 SurveyMonkey) which allowed construction of the questionnaire with different question types such as multiple choice, text insertion or classification scale (Fig. S1 in Supplementary Material). The SurveyMonkey platform also allows automatic capture of responses. The questionnaire was an open survey and directed to CDG patients and caregivers, henceforth referred to as 'participants'.

Using the SurveyMonkey platform, the LeQCDG was pilot tested by relevant partners: two researchers, two physicians and six CDG caregivers. The pilot testing included face validity concerning the relevance of the items as they appear to participants and possible effects of literacy on reading comprehension. Suggestions derived from the pilot allowed us to refine the questionnaire to record not only the views of patients with liver disease but also patients that had clinical liver-related symptoms, thus expanding the scope of participants. The sample was derived from participants reporting on CDG patients with liver disease, who answered to specific questions regarding liver symptoms (e.g. incidence of fibrosis, cirrhosis and portal hypertension).

Questions related to the impact of liver disease on the wellbeing of the CDG patients were also suggested by our CDG partners during the piloting phase. A glossary, explaining different liver disease symptoms, was made available to participants (Fig. S2 in Supplementary Material).

LeQCDG Recruitment, Dissemination and Analysis

The LeQCDG was launched online on 11 December 2016 and was available for participation for 42 days, until 22 January 2017. The survey was available in different languages (English, French, Spanish and Portuguese) and took between 15 and 20 min to complete. To assure the participants' anonymity, the IP identification number of respondents was not recorded. Multiple entries from the same individual were avoided choosing the SurveyMonkey option 'the questionnaire cannot be answered several times from the same device'. The respondents could review and change their answers (in this case, through a 'Prev.' button).

The LeQCDG participants were recruited from clinics and from the CDG community. Due to geographic limitations, social networks like Facebook, LinkedIn, Twitter and the Rareconnect platform (https://www.rare-connect.org/en/community/cdg) were used to disseminate the LeQCDG to the CDG community. In December 2016 at

the LeQCDG launching time, the CDG community included 720 participants of the 'CDG Global Alliance', a closed group on Facebook, and 1,100 followers of the 'Sindrome CDG' Facebook page. The Facebook page was started in 2000, and the members have been responsive to activity calls. An example of patient engagement in research activities is the constant participation of the CDG community in conferences and workshops.

An example of the survey announcement is shown in Fig. S3 (Supplementary Material). The participants identified themselves as CDG caregivers or as CDG patients and as having liver disease or being on a transplant list. Medical records and disease confirmation were not required for participation, as we considered 'random' participation of non-CDG patients/caregivers very unlikely.

Epidemiological data suggest that at least 20–30% of CDG patients may present liver disease symptoms (Marques-da-Silva et al. 2017a). Therefore, the sample size should contain, with high probability (e.g. 95%), sufficient numbers of positive and negative cases of liver disease. To achieve these conditions and the desired significance level of $P < 0.05$, a sample size of minimum 138 participants was calculated to be necessary, in order to achieve enough power to allow differentiation of the major liver symptoms in CDG patients (Lachin 1981).

Data Analysis

LeQCDG responses were exported from SurveyMonkey to excel. All questionnaires were analysed, except for participants reporting unknown CDG types or NGLY1 deficiency since NGLY-1 is a congenital disorders of deglycosylation (CDDG) which is different from CDG. These exclusion criteria were based on the fact that these patients do not qualify as 'diagnosed CDG patient'. Descriptive statistics were used to analyse and report the data. In addition, correlation between the survey and bibliographic data was evaluated with Pearson's linear correlation coefficient and correspondent P value (Prob>F) using OriginPro 8.5 software (OriginLab Corp., Northampton, USA).

Ethical approval for this study was obtained from the Ethics Committee at the Faculty of Psychology, Lisbon University (20/07/2016), and an informed consent was obtained from all participants. The online survey was conducted in accordance with the CHERRIES checklist (Eysenbach 2004).

Results

During 42 days, 203 participants accessed the LeQCDG. Four participants did not give informed consent and were excluded from further participation. According to the CHERRIES checklist (Eysenbach 2004), the completion rate was 77.9% (a total of 155 out of 199 participants who agreed to participate).

Participants

All but 1 participant were caregivers (1 was a CDG patient); 90% (162 out of 180) of participants knew that CDG patients can be affected by liver disease. Patients of both sexes were represented in a very similar percentage, 52.8% (95 out of 180) female patients and 47.2% (85 out of 180) male patients (Table S3 in Supplementary Material). The majority of CDG patients they represented were 10 years or younger (61.2% – 109 out of 178) (Table S4 in Supplementary Material). The major CDG types represented were PMM2-CDG (76.1% – 137 out of 180) and ALG6-CDG (4.4% – 8 out of 180) (Fig. S4 in Supplementary Material). These results were expected since PMM2-CDG is the most prevalent CDG followed by ALG6-CDG as the second most prevalent one.

Liver Disease in CDG

Among the CDG patients represented by the participants, 29.9% (53 out of 177) had liver disease, 41.8% (74 out of 177) did not have liver disease, and in 28.3% (50 out of 177) of patients, the participant did not know whether the CDG patient suffered from liver disease or not. However, in only 63.6% (105 out of 165) of all CDG patients reported, biochemical and/or radiological examinations were used to diagnose liver disease. For 96.1% (49 out of 51) of CDG patients with liver disease, the diagnosis of liver disease was made before or at the age of 10 years, and in 49% (25 out of 51) of the cases, liver problems were diagnosed before 12 months of age.

Monitoring rate of liver disease is detailed in Table 1. Hepatomegaly was described in 29% (47 out of 162) of CDG patients; additional reported symptoms and pathology

Table 1 Monitoring rate of liver function in all CDG patients

Characteristic	Value
Monitoring rate of liver function	
Twice a year	19.9% (32)
Once a year	37.3% (60)
Once in 2 years	9.9% (16)
Other	30.4% (49)
Don't know/specify	2.5% (4)

'Other' includes every 2 weeks, 6–12 times per year, 3–4 times per year, once in every 3 years, once and never/no needed. The results are expressed as percentage and absolute number; the total number of participants answering this question was 161

findings were steatosis, ascites, splenomegaly and oesophageal varices (Fig. 1).

Liver Symptoms in CDG

Within the population of CDG patients with known liver disease ($n = 53$), 17.6% (9 out of 51) answered that there was an alternative cause for liver disease put forward besides CDG; 27.5% (14 out of 51) noticed hepatic disturbances concomitant with an infectious syndrome. 10.2% (5 out of 49) were taking medication specifically for their liver condition at the time of the questionnaire. Liver symptoms were reported as shown in Fig. 2, with a high incidence of hepatomegaly (61.2% – 30 out of 49) and increased levels of serum transaminases (51% – 26 out of 51). Within this group of patients, liver transplantation was proposed to 5 patients (10.4% – 5 out of 48), 2 of whom had MPI-CDG and 3 had PMM2-CDG.

In the LeQCDG, 30.2% (39 out of 129) PMM2-CDG patients reported liver disease, and a specific subgroup analysis on these patients was performed. The main symptoms were also hepatomegaly (56.4% – 22 out of 39) and increased levels of serum transaminases (51.3% – 20 out of 39) (Fig. 2). In fact, for PMM2-CDG, we observed a significant correlation between the results obtained through the LeQCDG and the descriptions retrieved from the medical literature (reviewed in Marques-da-Silva et al. 2017a) (Fig. 3).

Within this subgroup of 39 patients with PMM2-CDG, liver transplantation was suggested to 3 patients; however only 1 patient reported severe liver disease in the LeQCDG supporting the suggestion of liver transplantation with

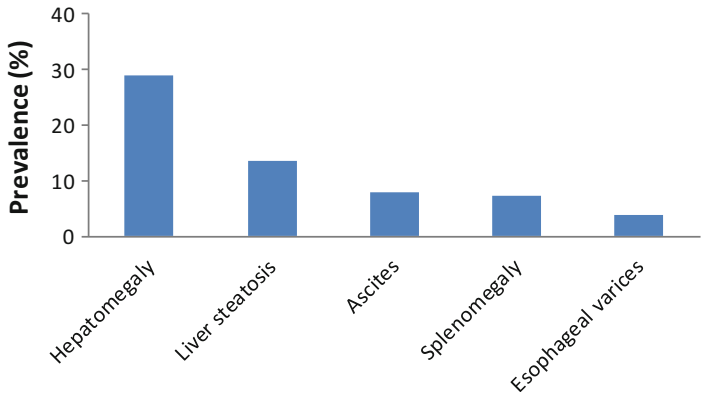

Fig. 1 Prevalence of liver symptoms in all CDG patients represented in the LeQCDG

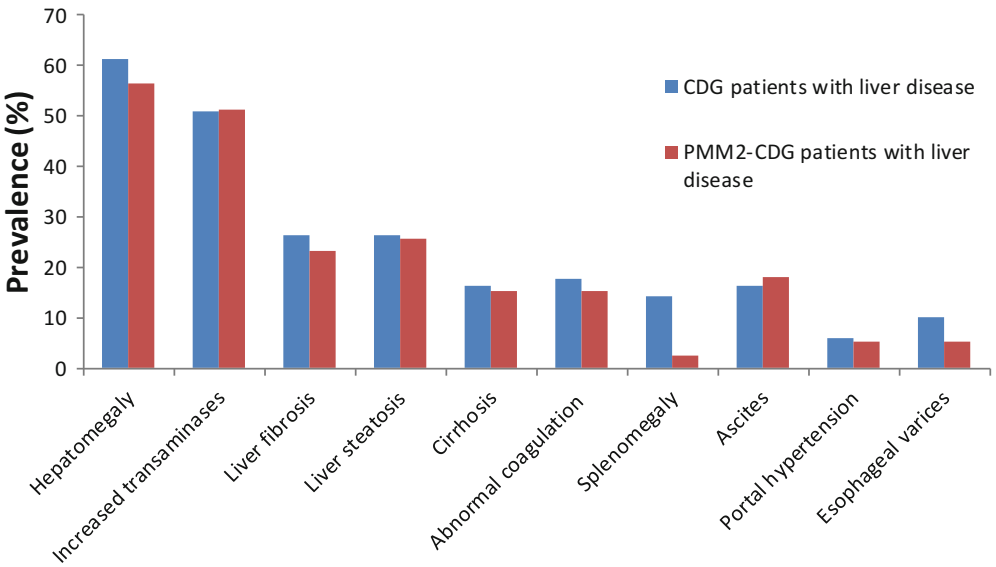

Fig. 2 Prevalence of liver symptoms obtained in the LeQCDG for CDG patients with PMM2-CDG (red) and with other CDG (blue)

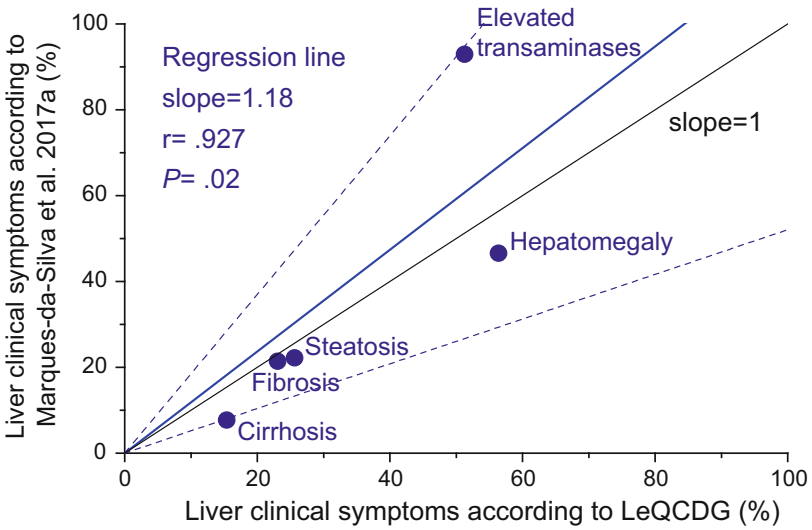

Fig. 3 Scatterplot and regression line (in blue) for the relation between the prevalence of liver symptoms in PMM2-CDG patients obtained from LeQCDG results and from literature data (Marques-da-Silva et al. 2017a). The slope of the regression line was found 1.18 ± 0.24. The 95% confidence interval is plotted as confidence bands (dashed lines) around the regression line. Pearson's correlation coefficient (r) and observed significance of the test (P) are indicated. The line with slope = 1 corresponds to perfect agreement between both methods

symptoms that include hepatomegaly, increased levels of transaminases, liver fibrosis, steatosis and cirrhosis. The other two participants reported only increased levels of transaminases and in one case also the occurrence of ascites.

Impact of Liver Involvement

Liver disease-associated symptoms in CDG patients can be found in Table 2; most symptoms were not present. In line with this, the participants reported that the liver disease has no or only minor impact on the patient's wellbeing and functioning (Table S5 in Supplementary Material) or emotional impact (Table S6 in Supplementary Material). Besides the low impact of liver disease on the physical and emotional health of CDG patients, most respondents (all but one being caregivers) reported their constant concerns regarding physical health and emotional wellbeing of the patient (Table S7 in Supplementary Material).

Awareness and Information About Liver Involvement in CDG: All Participants

We found that 50% of the participants that did not know if the CDG patient they reported on had liver disease also reported that the possibility of having liver disease has never been mentioned to the CDG patient. This is at least in part due to a lack of awareness in the medical community regarding CDG and related signs and symptoms. The participants classify the CDG patient's doctor's knowledge about CDG as represented in Table 3. Most participants think that the medical community should be trained better about CDG signs and symptoms (42.6% – 66 out of 155) and/or refer a patient to a more experienced colleague, in order to help in diagnosis and management (40% – 62 out of 155).

The treating physicians were reported to have recommended the patient to visit a hepatologist in 23.2% (36 out of 155) of cases. Approximately 62.6% (97 out of 155) of the participants feel they should know more about liver involvement in CDG because this allows them to communicate and share information with the doctor responsible for the CDG patient (43.8% – 67 out of 153). The majority of LeQCDG participants (i.e. 60.6%) gathered information about liver involvement in CDG themselves. The main information sources reported were 'to talk with CDG treating physician', 'information from a patient group website' and 'information from a practical CDG guide'. However, only 7.7% (12 out of 155) of participants have an informative document regarding liver disease in CDG. From all participants, 57.4% (89 out of 155) of the participants plan to try to learn more about liver involvement in CDG on the next scheduled medical check-up.

Discussion

Principal Results

The results show that 29.9% of the reported CDG patients have liver disease; in 49% of these patients, the liver problems were diagnosed before 12 months of age. We analysed the entire cohort but also analysed the subgroup of

Table 2 Associated liver symptoms in CDG patients with liver disease

Classification rate	0	1	2	3
Abdominal distension	53% (27)	31% (16)	8% (4)	8% (4)
Feeling sleepy or drowsy during the day (particularly after eating)	55% (28)	24% (12)	14% (7)	8% (4)
Having difficulties sleeping during the night	55% (28)	20% (10)	16% (8)	10% (5)
Nausea	53% (27)	24% (12)	8% (4)	16% (8)
Body temperature fluctuations (too low temperature or fever)	61% (31)	12% (6)	20% (10)	8% (4)
Jaundice (yellow discoloration of the whites of your eyes)	88% (45)	4% (2)	2% (1)	6% (3)
Itching	80% (41)	12% (6)	6% (3)	2% (1)
Bad breath or body odour	69% (35)	20% (10)	4% (2)	8% (4)
Loss of appetite	55% (28)	18% (9)	14% (7)	14% (7)
Pain or discomfort	59% (30)	20% (10)	10% (5)	12% (6)
Fatigue or low levels of energy	31% (16)	33% (17)	16% (8)	20% (10)

The results are expressed as percentage and absolute number; the total number of participants answering this question was 51. Scoring system used: 0, symptom not present; 1, mild/rare; 2, moderate/sometimes; 3, severe/often

Table 3 Opinion of participants about CDG patient's doctor's knowledge about CDG

Question	Value
How would you rate the CDG patient's doctor's knowledge about CDG?	
Excellent	14.2% (22)
Good	26.5% (41)
Neutral	20.6% (32)
Fair	16.1% (25)
Poor	22.6% (35)

The results are expressed as percentage and absolute number; the total number of participants answering this question was 155

PMM2-CDG patients, as this subtype made up 76.1% of our cohort of participants.

Limitations of This Work

The number of known CDG patients worldwide is unknown but estimated to be between 1,500 and 2,000. The number of CDG subtypes that are actually known has reached a count of 125 and is steadily increasing (Jaeken and Péanne 2017). These facts make it difficult to estimate the representative participation of CDG patients overall and of all CDG subtypes to this questionnaire. One hundred and fifty-five participants completed the questionnaire, representing 21 CDG types, and each CDG (apart from PMM2-CDG) was represented by only a few participants. This urged us to focus on a sub-analysis of PMM2-CDG, the CDG with the highest number of participants (76.1%). To increase the total number of participants and of CDG subtypes in future work, the recruitment strategy could be refined, and additional languages could be used.

This is a patient-oriented questionnaire focused on the patient/caregiver perspective. Due to the lack of a structured platform to keep files confidentially and in a secure way, medical records and pathology confirmation were not reviewed for confirmation of the reported data.

Comparison Between LeQCDG and Literature Data

The major liver disease symptoms identified, within all CDG or within the subgroup of PMM2-CDG patients, were hepatomegaly, increased transaminases, liver fibrosis, steatosis and cirrhosis and changes in coagulation factors. These are the same symptoms as we found in our recent literature review (Marques-da-Silva et al. 2017a). The good correlation between both methods (Fig. 3) even for symptoms with lower prevalence (up to 30% in cirrhosis, fibrosis and steatosis) indicates that the population sample in the query was at least as adequate as that in the medical literature, to allow differentiation of patients with less common symptoms.

The data from the LeQCDG show prevalence to be slightly higher to those from the literature review, except for increased transaminases. This is probably because not all CDG patients with liver disease are reported, leading to

fewer reports for hepatomegaly, liver fibrosis, steatosis and cirrhosis in the literature than actually found in the population. Regarding serum transaminases, the higher incidence of increased levels obtained in literature may be due, at least in some cases, to non-hepatic conditions. Liver transplantation was suggested for PMM2-CDG and MPI-CDG patients, indicating that liver disease in CDG can become life-threatening. Inversely, liver disease seems to have no or minimal impact on the patient's wellbeing and functioning in the large majority of patients which can be related to the low severity of liver disease-associated symptoms for CDG patients with liver disease described in this questionnaire. This is in accordance with previous studies that correlated quality of life and liver disease severity (Younossi et al. 2001; Saffari et al. 2016; Neijenhuis et al. 2016). This validates the experience of LeQCDG participants to evaluate CDG patients' quality of life.

The fact that 90% of the LeQCDG participants knew that CDG may affect the liver and more than half of the participants will try to learn more about liver disease in CDG in the next medical check-up of the CDG patient is important in the context of patient empowerment. In fact, in an open question requesting additional data from blood analysis, some participants added quantitative values for specific parameters, e.g. transaminases. This detailed information means we can have confidence in the respondents' knowledge about the CDG patient they are reporting on.

CDG Electronic Questionnaires as an Additional Source of Patient Information

The Internet, as a fast source of information and with the possibility to share health information, represents a potent tool for collecting data from families of children affected by rare diseases (Tozzi et al. 2013), such as CDG. Internet-based electronic questionnaires are increasingly being used among the rare diseases community to better understand, for example, the disease impact in terms of health (Molster et al. 2016; Price et al. 2016) and economy (Chevreul et al. 2016). In this electronic questionnaire, besides the information about clinical symptoms related to liver disease and its impact on the wellbeing of patients, relevant data regarding CDG awareness and information related to the disease was also gathered. In doing so, we learned that CDG awareness and information are lacking within this community meaning that there is a need to educate physicians and create public awareness on CDG. However, we observed that regarding clinical information in the LeQCDG, the families presented additional information that is not described in the literature. This adds value to available medical CDG information.

Conclusions

The results obtained with this online questionnaire confirmed the data obtained in the previous literature review (Marques-da-Silva et al. 2017a) in terms of prevalence, severity and phenotype of liver disease, with a high incidence of hepatomegaly and increased levels of serum transaminases. In addition, the consequences of liver disease on the patient's wellbeing and the emotional impact are considered minor, although physical health and emotional wellbeing of the CDG patient are both important concerns.

The information was collected in 42 days and confirmed data representing 25 years of scientific publications dedicated to CDG patients. It should be noted that all the information was given by the participants that were mostly family caregivers. This work demonstrated that the participants are empowered and capable of completing validated tools about CDG, clinical symptoms and their impact on the patient's quality of life.

We believe that the recent vision on patient-centered health will move patient associations to embrace patient/caregiver-driven research within their community. Our study shows that geographic limitations can be overcome and that large amounts of meaningful, representative clinical and patient-oriented data can be collected, in a short period of time, when patient advocacy groups, families and professionals (CDG & Allies – PPAIN) work closely together. These findings and the model of this questionnaire can be especially relevant for other ultra-rare diseases.

Note

This study is a result of a collaborative study between patient advocacy groups, families and professionals (CDG Professionals and Patient Associations International Network; CDG & Allies – PPAIN).

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Synopsis

Liver involvement in CDG was accessed through a CDG family-targeted online questionnaire based on a recent literature review. Main symptoms reported included hepatomegaly, increased levels of serum transaminases, fibrosis,

steatosis and cirrhosis. Electronic questionnaires can boost knowledge on rare diseases, improving possible treatments and management. This is also useful with regard to future clinical trials.

Compliance with Ethics Guidelines

Conflict of Interests

Vanessa dos Reis Ferreira is the president and founder of the Portuguese Association for CDG and other Rare Metabolic Diseases (APCDG-DMR). All other authors declare no competing financial interests.

Details of the Contributions of Individual Authors

Dorinda Marques-da-Silva participated in planning, conducted the work, analysed data, conceived the article outline, participated in the conception and design, drafted all the manuscript including figures and tables, obtained final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Rita Francisco participated in conducting the work; conceiving the article outline, conception and design; and drafting the manuscript.

Vanessa dos Reis Ferreira participated in planning and conducting the work; conceiving the article outline, conception and design; and critically revising it for important intellectual content.

Liz Forbat participated in conception and design and in critically revising it for important intellectual content.

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Ethical Guidelines, Human and Animal Rights and Consents

This work has been carried out in accordance with The Code of Ethics of the World Medical Association. Ethical approval for this study was obtained from the Ethics Committee at the Faculty of Psychology, Lisbon University (20/07/2016). Besides, the informed consent was obtained from participants, and the privacy rights of human subjects were ensured.

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RESEARCH REPORT

Acute Hepatic Porphyrias in Colombia: An Analysis of 101 Patients

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Abstract *Background*: There is minimal information available about acute hepatic porphyrias (AHPs) in developing countries. The aim of this study was to describe the demographics, clinical features, and mortality of AHPs in Colombia.

Patients and methods: 121 patients with presumed diagnosis of AHPs were reported in Colombia between 1944 and 2018. A pooled analysis of 53 patients with confirmed diagnosis was performed to evaluate the demographics, clinical features, and mortality of AHPs in the country. Selected variables were compared by periods (1952–2000 and 2001–2018).

Results: Most attacks occurred in women (66%), with a women-to-man ratio of 39/14. 96% of the patients were diagnosed with AHPs between 15 and 40 years of age. Precipitants were identified in 71% of attacks and more than one precipitant in 41% of them. Drugs (85%) and infections (44%) were the most common precipitants. 11% of women had premenstrual attacks. Abdominal pain was the most common symptom (96%). Cortical blindness, posterior reversible encephalopathy syndrome, and rhabdomyolysis were described. 70% of attacks were confirmed by qualitative test only. 67% of attacks were treated with intravenous heme. The use of heme increased from 4 to 85% in the last two decades. Mortality decreased about twofold in relation to the increase in the use of heme. Severe motor neuropathy was associated with increased mortality. Gonadorelin analogues, heme prophylaxis, and orthotopic liver transplantation have been used to prevent recurrent attacks.

Conclusions: Diagnosis and treatment of AHPs in Colombia have improved in recent decades. However, there are still important shortcomings to address.

Abbreviations

AHPs	Acute hepatic porphyrias
AIP	Acute intermittent porphyria
ALA	Aminolevulinic acid
ALAS1	Aminolevulinic acid synthase 1
DNA	Deoxyribonucleic acid
GnA	Gonadorelin analogs
HCP	Hereditary coproporphyria
HMBS	Hydroxymethylbilane synthase
NAPOS	Norwegian Porphyria Centre
OLT	Orthotopic liver transplantation
PBG	Porphobilinogen
PRES	Posterior reversible encephalopathy syndrome
RNA	Ribonucleic acid
VP	Variegate porphyria

Introduction

Acute hepatic porphyrias (AHPs) are rare diseases caused by genetic mutations that lead to enzymatic deficiencies in the heme pathway. The most common AHPs are acute intermittent porphyria (AIP, OMIM 176000), hereditary coproporphyria (HCP, OMIM 121300), and variegate porphyria (VP, OMIM 176200).

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Clinical penetrance of mutations is low, so less than 10% of carriers develop acute attacks of neurovisceral manifestations. Attacks occur mainly in women of childbearing age and rarely before puberty. Symptoms and signs (e.g., abdominal pain, vomiting, hypertension, paresis, dysesthesia, and behavioral changes) are nonspecific and indistinguishable among AHPs and other more common diseases. These are precipitated by factors that induce aminolevulinic acid synthase 1 (ALAS1, EC.2.3.1.37) (e.g., hormones, drugs, infections, starvation, stress) (Bissell et al. 2017). Patients with HCP and VP may also develop blistering skin lesions either concurrently or in the absence of attacks.

Diagnosis of attacks is only possible by demonstrating elevated urinary porphobilinogen (PBG). A normal PBG excludes that concurrent symptoms are caused by AHPs (Woolf et al. 2017). Identification of individual porphyrias requires additional tests [analyses of porphyrins, hydroxymethylbilane synthase (HMBS, EC.2.5.1.61) enzymatic activity, or DNA] (Whatley et al. 2009).

Intravenous heme (Panhematin®, Recordati Rare Diseases; Normosang®, Orphan Europe) is the only specific treatment available for attacks. It must be administered to all patients with AHPs and severe attacks. Carbohydrate loading (oral or intravenous) is also a suitable therapy for mild symptoms (Stein et al. 2013).

A minority of patients develop severely debilitating recurrent attacks (≥4 attacks requiring hospitalization per year). Gonadorelin analogs (GnA) are administered to prevent premenstrual cyclic attacks. Heme is given prophylactically to prevent recurrent attacks unrelated to menstruation or refractory to GnA. Orthotopic liver transplantation (OLT) is an effective, but still experimental, treatment for patients who are disabled by frequent hospitalizations for attacks that are resistant to GnA or prophylactic heme (Balwani et al. 2017). There is minimal information available about AHPs in developing countries, such as Colombia. Clinical features and mortality of Colombian patients with symptomatic AHPs have been described only in case reports and small series from single centers. However, many patients in these studies were erroneously diagnosed, so results are inaccurate (Jaramillo-Calle 2017).

In this study, we reviewed 121 patients with presumed diagnosis of AHP who were reported in Colombia between 1944 and 2018. The demographics, clinical characteristics, diagnosis, treatment, and mortality of AHPs in the country were described.

Methods

Sources of information: PubMed, Scopus, Embase, Google Scholar, SciELO, and LILACS were searched using the terms "porphyria" and "Colombia" without restrictions (From inception to January 2018). Case reports and series describing patients with AHPs were evaluated if the primary author affiliation was in Colombia. References of selected articles were checked manually to identify unrecovered studies.

Patients: 121 patients with presumed AHPs were reported in Colombia between 1944 and 2018 (full list of references in Supplementary Material). Five reports were excluded for duplication and one for being about a cutaneous porphyria. 115 reports were reviewed, of which 14 (12%) were excluded due to unconfirmed diagnosis of AHPs. At the end, we included 101 patients with confirmed AHPs. For each patient, there was information available about a single attack.

Definitions: Confirmed diagnosis of AHPs was defined as (1) compatible symptoms of attacks and elevated PBG (qualitatively or quantitatively) or (2) confirmatory tests to identify the type of porphyria (analyses of porphyrins, HMBS enzymatic activity, or DNA) (Whatley et al. 2009). Porphyrinogenic risk of medications was evaluated in the NAPOS drug database (www.drugs-porphyria.org) (complete list in Supplementary Material). Attacks were defined as severe if any of the following manifestations occurred: paresis, bulbar palsy, respiratory failure, hyponatremia, seizures, psychosis, or lethargy (Stein et al. 2013; Pischik et al. 2004).

Statistical analysis: A pooled analysis of 53 patients with individual information available was performed to evaluate demographics, clinical features, diagnosis, treatments, and outcomes of attacks. Descriptive statistics were generated. In the analysis of each variable, only patients with information available were considered. Mortality of attacks was calculated as the number of patients who died during the reported attack divided by the total number of patients. Analyses of attacks severity, diagnosis, treatment, and mortality were stratified by periods (1952–2000 and 2001–2018) to assess changes. Comparisons were performed by Wilcoxon rank sum test for continuous variables and Fischer's exact test for categorical variables. Two-sided p values <0.05 indicated statistically significant differences. Analyses were performed using Stata Software v.14.2.

Results

Geographical distribution: Fig. 1 presents the geographic distribution of 101 Colombian patients with AHPs by place of birth and diagnosis. As expected, most patients were identified in densely populated capital cities with the greatest access to tertiary-level medical centers (Medellín, Bogotá, Cali).

Sex and age: Most attacks occurred in women (66%), with a women-to-man ratio of 39/14. Median age at diagnosis was 26 years [women, 27 years (15–50) vs.

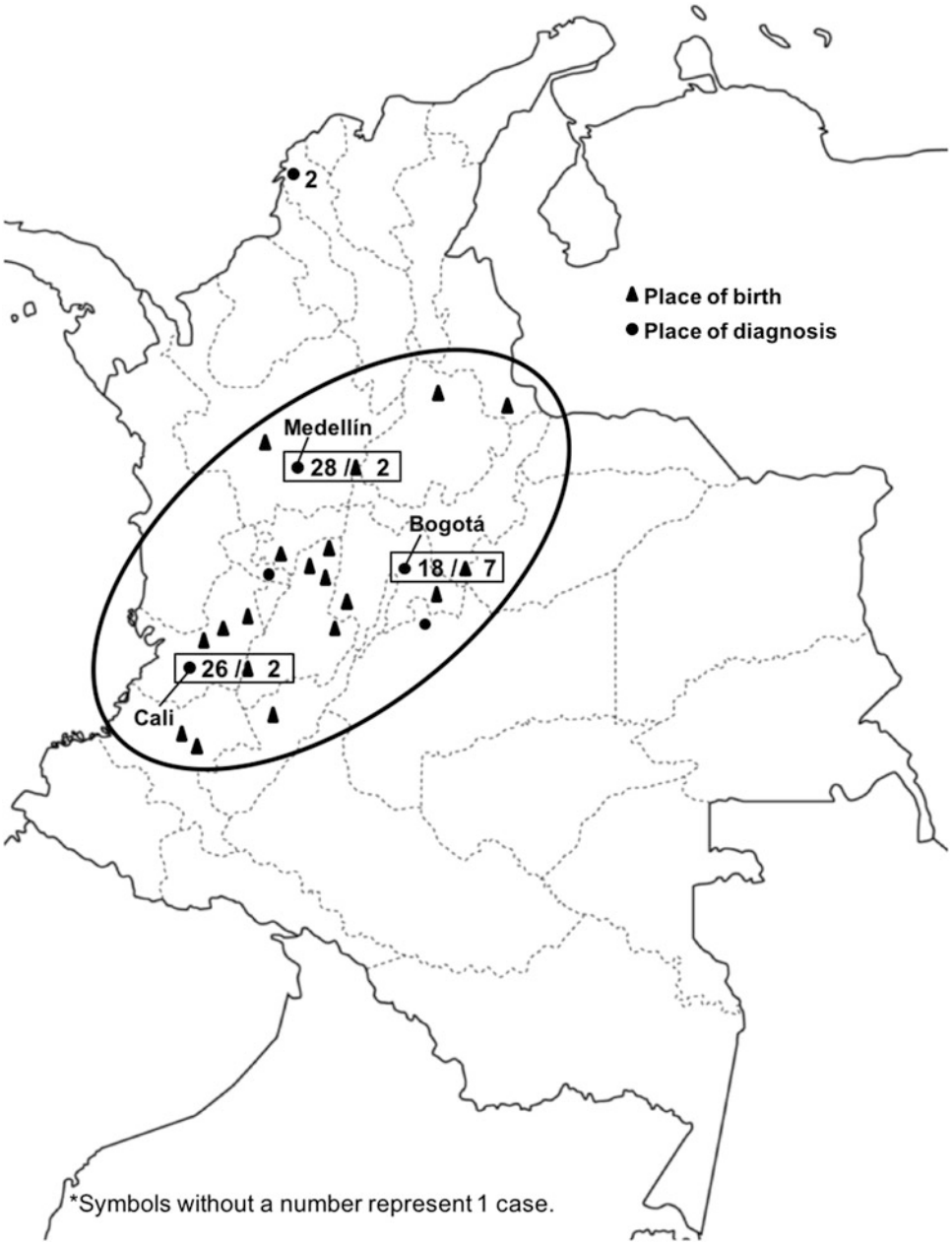

Fig. 1 Places of birth and diagnosis of patients with acute hepatic porphyrias in Colombia. This figure shows that most patients were born ($n = 27$, triangles) or diagnosed ($n = 74$, circles) in the central region of the country (black oval), with predominance in the three largest and densely populated capitals of Colombia (Medellín, Bogotá y Cali)

men, 25 years (15–42), $p = 0.24$]. 51 (96%) patients were diagnosed with AHPs between 15 and 40 years of age.

Precipitants: Precipitants were identified in 71% of attacks (>1 precipitant in 41%) (Fig. 2). The most common precipitants were drugs (85%) and infections (44%). 11% of women had premenstrual attacks. Eight pregnancies occurred and were complicated by attacks during or soon after pregnancy in three instances. One woman with four pregnancies had a mild attack probably due to the use of a porphyrinogenic compound (*laudanum*: Alcohol and opium tincture) (Ramírez 1959); another woman with two pregnancies had two attacks in which precipitants were not identified (Contreras-Zúñiga and Zuluaga-Martínez 2006); another woman with one pregnancy had one attack due to a postpartum endometritis (Mendoza et al. 1995); and another woman with one pregnancy had no attacks (Argüello et al. 1978).

Clinical manifestations: 85% of attacks were severe. Severity was not significantly different between periods (1952–2000, 81% vs. 2001–2018, 93%; $p = 0.4$) or sexes

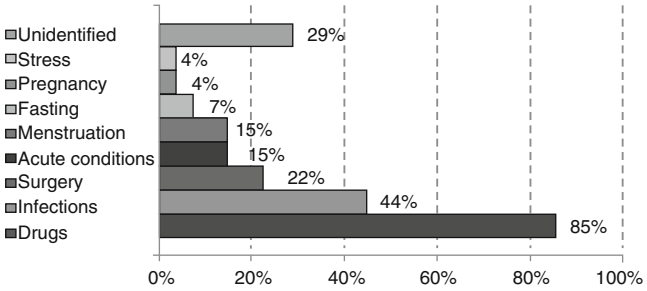

Fig. 2 Frequency of precipitant factors of 53 attacks in 53 patients with acute hepatic porphyrias in Colombia

(women, 88% vs. men, 80%; $p = 0.6$). 96% of patients had abdominal pain alone or in combination with other manifestations (Table 1). Three women presented posterior reversible encephalopathy syndrome (PRES) (Adams and Amaya 2014; Uribe et al. 2007; Enríquez-marulanda et al. 2016), and one of them had also acute rhabdomyolysis (Adams and Amaya 2014).

Diagnosis: In 96% of patients, diagnosis of AHPs was confirmed by elevated PBG. Quantitative PBG tests were used in 30% of attacks, increasing from 24% before 2001 to 42% afterward. In one patient, diagnosis of AHPs was confirmed by DNA analysis, in which a HMBS gene mutation (c.1084delT) compatible with AIP was identified (Angel et al. 2010). Of the patients confirmed to have AHPs, 98% were diagnosed with AIP based exclusively on clinical manifestations and an elevated PBG, without further testing to differentiate whether they might have HCP or VP instead of AIP.

Treatment: The use of heme in Colombia was reported for the first time in 1980 (Pradilla et al. 1980). Since then, 67% of attacks were treated with heme. The use of heme increased from 4% of attacks before 2000 to 85% afterward ($p < 0.001$). 18% of attacks were treated with carbohydrates loading (oral or intravenously) and heme, 21% with carbohydrates loading only, and 47% with symptomatic measures only (e.g., analgesics, antispasmodics, anticonvulsants, antiemetics, among others). Median hospital stay length was 21 days (7–70 days).

Prevention: 55% of the patients reported a history of multiple episodes of compatible symptoms of attacks before the diagnosis of AHPs was confirmed, being this more frequent among women (75% vs. 69%). One woman suffered severely debilitating premenstrual cyclic attacks, with more than 14 attacks per year on average. Despite receiving GnA and heme prophylaxis, she continued to present recurrent attacks and additionally developed serious side effects. OLT was performed with the subsequent disappearance of symptoms and normalization of urinary ALA and PBG (Jaramillo-Calle et al. 2018).

Mortality: Mortality of attacks during the period covered by the study was 32%. It was 38% between 1952 and 2000, with minimal changes within the period (1952–1979, 38%; 1980–2000, 40%), and decreased to 14% between 2001 and 2018. Patients who did not receive heme died approximately twofold more frequently than patients treated with heme (31% vs. 17%). Two patients died among those who received heme (80% success rate). The most common cause of death was respiratory paralysis with severe pulmonary sepsis (85%). Median age at diagnosis did not differ significantly between patients who survived and those who died [survivors, 27 years (16–50) vs. dead, 24 years (17–43) $p = 0.8$]. Patients who died developed severe motor neuropathy more frequently than patients who survived [paresis (36% vs. 0%; $p = 0.04$), quadriparesis (42% vs. 0%; $p = 0.007$), bulbar palsy (48% vs. 0%; $p = 0.001$), respiratory failure (53% vs. 4%; $p = 0.0004$)]. There were no significant differences in other severe manifestations [hyponatremia (20% vs. 27%; $p = 0.7$), seizures (9% vs. 31%; $p = 0.2$), and consciousness impairment (50% vs. 21%; $p = 0.12$)].

Discussion

Sex and age: Attacks occurred more often in women (66%) and exclusively at age 15 or later, which makes it unlikely prepubertal. This predominance of attacks in women and fertile ages is attributed in part to the effect of sex hormones, which are important precipitants of attacks and increase substantially during puberty (Andersson et al. 2003).

Menstruation and pregnancy: 11% of women in this study had premenstrual attacks, which are attributed in part to an increase in progesterone during the luteal phase of the menstrual cycle. Pregnancy theoretically raises the risk of attacks, since estrogen and progesterone levels increase prominently. Four of the women in this study had a total of eight pregnancies; three of these women had attacks during a pregnancy each. Nevertheless, only the attacks in one woman were precipitated by pregnancy while the attacks in the other two women were precipitated by a porphyrinogenic compound and an infection. This supports that, despite the higher concentrations of hormones, most pregnancies in women with AHPs are not complicated by attacks. When attacks occur during pregnancy, they are mainly due to exposure to typical precipitants (Marsden and Rees 2010; Kauppinen and Mustajoki 1992).

Clinical manifestations: Almost all patients had abdominal pain, as in most studies. Paralysis and respiratory failure were more common in this study than other studies (Table 1). Hypertension was the most frequent clinical sign instead of

Table 1 Clinical manifestations of patients with symptomatic acute hepatic porphyrias in different studies

Signs and symptoms (%)	Current	A	B	C	D	E	F	G	Median	(min–max)
Abdominal pain	96	95	95	100	97[a]	86	74	93	95	(74–100)
Paralysis/quadriparesis	60	31	–	59	–	–	–	26	45	(26–60)
Psychiatric[b]	49	40	25	18	1	29	55	40	34.5	(1–55)
Dark urine	47	74	90	–	–	–	–	64	69	(47–90)
Respiratory failure	45	7	20	–	–	–	–	–	20	(7–45)
Constipation	40	48	80	37	27	41	60	46	43.5	(27–80)
Hypertension	38	36[c]	55[d]	–	74[e]	–	40	–	40	(36–74)
Palpitations/tachycardia	32	80	85	30	37	10	50	53	43.5	(10–85)
Nauseas/vomiting	30	43	80	11	79	36	73	47	45	(11–80)
Sensory impairment	28	26	25	–	–	7	–	39	26	(7–39)
Seizures	26	20	20	30	–	1	9	–	20	(1–30)
Myalgia	17	50[f]	70	–	–	30	–	63	50	(17–70)
Muscle paresis/weakness	10	29	50	48	–	20	63	59	48	(10–63)
Diarrhea	8	5	5	–	–	–	29	–	6.5	(5–29)
Fatigue	–	–	–	–	–	42	–	72	57	(42–72)
Headache	–	5	–	–	–	13	–	52	13	(5–52)
Fever	–	9	–	–	–	–	18	–	13.5	(9–18)
Anorexia	–	–	–	37	–	–	–	–	37	–

A Stein and Tschudy (1970), *B* Mustajoki and Koskelo (1976), *C* De Siervi et al. (1999), *D* Hift and Meissner (2005), *E* Bylesjö et al. (2009), *F* Bonkovsky et al. (2014), *G* Mykletun et al. (2014)

[a] Any pain

[b] Including anxiety, depression, behavioral changes, hallucinations, confusion

[c] Diastolic >90

[d] Diastolic ≥100

[e] Systolic >130

[f] Pain or paresthesia

tachycardia. PRES occurred in three women (Adams and Amaya 2014; Uribe et al. 2007; Enríquez-marulanda et al. 2016). Numerous reports of PRES during an attack have emerged in recent years with the increased availability of neuroimaging in the emergency department. One patient had cortical blindness, which has been previously reported (Kupferschmidt et al. 1995; Garg et al. 1999; Bhat et al. 2010). Severe acute rhabdomyolysis occurred in an Afro-American woman with hyponatremia and hypokalemia (Adams and Amaya 2014). Other five patients with rhabdomyolysis during an attack have been reported in the literature (Marsden and Peters 2004; García-Martul et al. 2008; Yrjönen et al. 2008; Chen et al. 2015; Devars du Mayne et al. 1987). Potential pathophysiological mechanisms for these manifestations during an attack are discussed elsewhere (García-Martul et al. 2008; Olivier et al. 2017).

Diagnosis: Of the 115 symptomatic patients with presumptive diagnosis of AHPs, 14 (12%) were excluded because there was no evidence to support the diagnosis. Of these 14 patients, one had liver disease, and the diagnosis of AHP was based only on elevated porphyrins in urine without having measured the PBG (Ordoñez 1944), which could have been explained by a secondary porphyrinuria due to hepatic dysfunction (Doss 1987). Another patient had elevated urinary aminolevulinic acid (ALA) and normal PBG (Mendoza et al. 1995), which can occur in ALA dehydratase porphyria, lead poisoning, and type 1 hereditary tyrosinemia. The remaining 12 patients had normal or unmeasured PBG despite being symptomatic, and no other evidence (i.e., personal or family history or other porphyria tests) was informed to support the diagnosis of AHPs (Latorre and Muñoz 1988). These findings suggest that many subjects diagnosed with AHPs in Colombia might not really have these diseases. The diagnosis of AIP in 98% of the patients confirmed to have AHPs was based exclusively on clinical manifestations and elevated PBG, without further testing to differentiate whether they might have HCP or VP instead of AIP. These patients should have been diagnosed with unspecified AHP. In only one patient, AIP was diagnosed by analysis of the HMBS gene in which a small deletion mutation was found (c.1084delT). This patient had previously presented symptoms compatible

with an attack, but measurements of urinary PBG were not available (Angel et al. 2010). The same mutation was identified in a 16-year-old female with a confirmed attack in the USA (Leung-Pineda and Wilson 2017). The mutation changes a stop codon and produced a mutant HMBS protein longer than the normal enzyme, which is predicted to result in no-go decay (Chen et al. 2018). Finally, 70% of attacks were evaluated using qualitative test only (i.e., Watson-Schwartz or Hoesch tests), which experts no longer recommend due to the high risk of false results (Woolf et al. 2017; Balwani et al. 2017; Deacon 2011). This is probably explained by the higher cost and lower availability of quantitative test. The previously mentioned findings suggest important limitations in availability, use, and interpretation of diagnostic tests to confirm the attacks and identify the types of AHPs in Colombia.

Treatment and prognosis: Mortality of attacks decreased from 38 to 14% in relation to increase in the use of intravenous heme, supporting that the prognosis of attacks has improved since the introduction of heme therapy (Jeans et al. 1996; Kauppinen and Mustajoki 1992). Two patients treated with heme died in this study, probably because the delayed administration of the drug (20 and 24 days after admission) (Pradilla et al. 1980; Buitrago and Santa 2009). Heme is more effective when administered early, since it cannot reverse established nerve damage (Mustajoki and Nordmann 1993). In addition to heme therapy, overall improvement in the quality of life and healthcare in Colombia during the last decades is likely to have contributed to the decrease in mortality of attacks, especially the greater availability of intensive care units and mechanical ventilatory support. Mortality was significantly higher among patients who developed severe motor neuropathy (i.e., paresis, quadriparesis, or respiratory failure), which occurs in very advanced and untreated attacks. This supports that these clinical manifestations predict a poor prognosis in attacks (Pischik et al. 2004).

Prevention of recurrence: A woman with AIP and severe premenstrual cyclic attacks received GnA to suppress menstruation, but the attacks continued to recur (Jaramillo-Calle et al. 2018). Studies have shown that the effectiveness of GnA in ameliorating attacks related to menstruation varies among women; although most women can perceive a complete or partial improvement, some few do not experience any change (Schulenburg-Brand et al. 2017; Innala et al. 2010). It is possible that, in addition to the hormonal changes, other unrecognized precipitants contribute to the attacks of these women. Also, some unknown genetic factor related to the severity and frequency of attacks might modulate the response to GnA. Monthly heme infusions were also given prophylactically to this patient, but they were ineffective to stop the recurrent attacks. This is consistent with studies showing that most patients receiving heme prophylaxis continue having acute and chronic symptoms (Sardh et al. 2017; Marsden et al. 2015). A recent study has shown that frequent administration of heme infusions can lead to a chronic inflammation of the liver that induces heme oxygenase-1, increases the degradation of heme, and preserves the overexpression of ALAS1, situation that might perpetuate and aggravate the recurrence of long-term attacks (Schmitt et al. 2018). The patient developed severe side effects due to chronic use of heme (i.e., anaphylaxis and iron overload). It has been shown that a long-term iron overload due to prolonged use of heme can be associated with liver fibrosis (Willandt et al. 2015). She underwent OLT, which produced complete clinical and biochemical remission of AIP by correcting the genetic mutation in the liver (Soonawalla et al. 2004). This was the first time this procedure was reported in Latin America (Jaramillo-Calle et al. 2018).

New drugs for prevention and treatment of attacks are currently under evaluation. A gene therapy to transport the normal HMBS gene to hepatocytes showed promising results in preclinical studies (Yasuda et al. 2010) but failed to reduce ALA and PBG levels in humans (D'Avola et al. 2016). Another therapy is a small interfering RNA against ALAS1 (Givosiran, Alnylam Pharmaceuticals) that demonstrated efficacy in reducing circulating ALAS1-mRNA and urinary ALA and PBG in humans (Sardh et al. 2016). A phase III study of Givosiran is currently underway (ClinicalTrials.gov: NCT03338816).

One limitation of this study is that case reports and series are written retrospectively from medical records, so all relevant information may not have been included. Furthermore, case reports usually present severe or unusual forms of a diseases, which could explain the high frequency of severe symptoms in this study. Despite these limitations, this is the most extensive description of AHPs in Colombia to date and provides a ground for future research.

Conclusions

During 65 years, there have been important deficiencies in availability, use, and interpretations of porphyria diagnostic tests in Colombia. Our study suggests that 12% of patients considered as having AHPs are erroneously diagnosed because the necessary tests are not performed or their results are not interpreted properly, less than 50% of attacks are confirmed by quantitative PBG tests, diagnostic tests to identify the types of porphyrias are performed in less than 2% of patients with confirmed diagnosis of AHPs, and genotype of AHPs is almost unknown. In view of these diagnostic problems, it is very likely that AHPs are widely underdiagnosed in the country. These issues will be solved through the establishment of specialized porphyria laboratories that participate in external quality assurance

schemes (Aarsand et al. 2011). On the other hand, the use of heme in Colombia has increased greatly in the last two decades. Consequently, mortality has decreased more than twofold. Finally, delayed diagnosis, severe motor neuropathy, and late administration of heme are associated with a poor prognosis. Therefore, efforts in Colombia should be directed toward making PBG tests and intravenous heme available within 24 h for symptomatic patients with known or suspected diagnosis of AHPs and attacks.

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Compliance with Ethics Guidelines

Conflict of Interest

Daniel A. Jaramillo-Calle and Daniel C. Aguirre Acevedo declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

Daniel A. Jaramillo-Calle designed the study, analyzed and interpreted the data, and drafted and reviewed the article. He is guarantor for the article.

Daniel C. Aguirre Acevedo participated in the design of the study and had input for revising the article.

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RESEARCH REPORT

Cobalamin D Deficiency Identified Through Newborn Screening

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Abstract Cobalamin D deficiency (cblD) is one of the least common cobalamin metabolism disorders. It may result in isolated homocystinuria, isolated methylmalonic aciduria, or combined methylmalonic aciduria and homocystinuria (cblD-combined). Only seven cases of the combined cblD form have been reported to date. Due to the rarity of this disorder, the presentation and symptoms are not well described. We present an eighth case of the cblD-combined subtype, who had a positive newborn screen (NBS) on day of life 3. She was symptomatic and developed lethargy and poor oral intake at 8 days of life. She was treated with 10% dextrose, folinic acid, intramuscular hydroxocobalamin, and betaine. Despite the early initiation of treatment, she developed complications of the disease and was found to have abnormal brain imaging findings at 17 days of age and macular atrophy at 3 months of age and has global developmental delay. We provide detailed description of her presentation, her treatment, and her complications to aid in the understanding of this rare disorder, which is very similar to the more common cobalamin C disorder (cblC).

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Introduction

Vitamin B12 (cobalamin) is metabolized to 5′-deoxyadenosylcobalamin (AdoCbl), a required cofactor for the mitochondrial enzyme methylmalonyl-CoA mutase and methylcobalamin (MeCbl), a required cofactor for the cytoplasmic enzyme methionine synthase (Watkins and Rosenblatt 2011). Methylmalonyl-CoA mutase is required for the conversion of methylmalonyl-CoA into succinyl-CoA, and methionine synthase is required for the remethylation of homocysteine to methionine (Watkins and Rosenblatt 2011). Remethylation disorders affect the remethylation process of homocysteine to methionine, resulting in accumulation of homocysteine (Huemer et al. 2017). Acquired or inherited disturbances of cobalamin metabolism may result in elevations of homocysteine and/or methylmalonic acid in plasma and urine.

Cobalamin D (cblD) deficiency is one of the rarest cobalamin metabolism disorders (Atkinson et al. 2014). CblD results in isolated homocystinuria (cblD-homocystinuria) due to missense mutations located in the C-terminal part of the protein, isolated methylmalonic aciduria (cblD-methylmalonic aciduria) due to mutations in the N-terminal part of the protein, or combined methylmalonic aciduria and homocystinuria (cblD-combined) due to nonsense, splice site, or truncating variants toward the C-terminal part of the protein (Coelho et al. 2008). Fewer than 20 patients with cblD deficiency have been described in the literature, only seven of these had cblD-combined (Stucki et al. 2012; Miousse et al. 2009; Suormala et al. 2004; Parini et al. 2013; Soylu Ustkoyuncu et al. 2018).

We report an eighth case of cblD-combined who presented in the neonatal period symptomatically after an abnormal newborn screen. Despite early treatment, she has developmental delay and bilateral maculopathy. We

describe her clinical, biochemical, and radiographic findings, the progression over the first months of her life, and her current status.

Case Report

The patient had a positive newborn screen, and follow-up testing was pending when she developed poor feeding and decreased alertness as an 8-day-old infant. She was born at term to consanguineous parents who are second cousins once removed (maternal grandfather and father are first cousins); she has an older brother who is healthy. The newborn screen, collected at 22 h of life, was positive for low methionine, 4 μmol/L (cutoff >8 μmol/L), and was reported on day of life (DOL) 3. C3 acylcarnitine was not flagged as the value was 5.5 μmol/L (cutoff <6.3 μmol/L); the C3/C2 ratio was elevated at 0.4 (cutoff <0.3), but this was not flagged per the California newborn screening protocol. Homocysteine and other follow-up testing were recommended after the positive newborn screening result. Homocysteine was collected on DOL 5 but had not been resulted at the time that she developed clinical symptoms. The patient presented on DOL 8 to her PCP with poor feeding and jaundice and was admitted to an outside hospital on DOL 9. The follow-up newborn screen test results were not yet available. The metabolic service was contacted on DOL 10, and urgent homocysteine and other laboratories were requested. At that time, ammonia, lactate, and pH were normal, and urine ketones were negative. The infant was receiving dextrose only due to the concern for an inborn error. Reinitiation of breast milk and the administration of intramuscular hydroxocobalamin (after the collection of laboratories) were recommended. The outside hospital homocysteine was resulted on DOL 11, along with that previously obtained, both were markedly elevated. The homocysteine collected on DOL 5 was the peak value at 299.5 μmol/L (ref. <10.4 μmol/L) and from DOL 11 was elevated at 208 μmol/L (ref. 3–10 μmol/L). On DOL 12, she was transferred to our hospital for further evaluation and management. Due to a suspected diagnosis of cblC disease, she was empirically initiated on intravenous 10% dextrose solution at 80 mL/kg/day, intramuscular hydroxocobalamin (1 mg/day), betaine (250 mg/kg/day), and folinic acid (5 mg/day). After the prolonged protein restriction at the outside hospital prior to our management, protein intake was recommended to be at DRI (dietary reference intake) of 2.0–2.2 g/kg/day temporarily during the initial presentation and neonatal crisis, while the DOL 5 and 12 methylmalonic acid levels were pending, due to a possible markedly elevated methylmalonic acid level. Diet was not restricted in protein after the initial period, as current guidelines recommend against dietary protein restriction in combined remethylation disorders. Methylmalonic acid level on admission on DOL 12 was 38 μmol/L, ref. <0.3 μmol/L, and on DOL 5 had been 97.2 μmol/L, ref. <0.318 μmol/L. Hematologic parameters included a platelet count of 24 (ref. 140–450 × $10e^9$/L), hemoglobin of 13.6 g/dL (ref. 13.5–21.5 g/dL), and white blood count of 4.7 (ref. 5–21 × $10e^9$/L) on DOL 12. At the time of admission to our hospital, she continued to be encephalopathic. Methionine on admission was 0 μmol/L (ref. 9–42 nmol/mL). After 5 days on the above treatment (betaine dose was increased to 500 mg/kg/day on DOL 14), her mental status and her tone had improved, her methionine improved to 33 nmol/mL (ref. 10–60 nmol/mL), total homocysteine came down to 53 μmol/L (ref. 3–10 μmol/L), and MMA came down to 10.24 μmol/L (ref. <0.3 μmol/L).

Ophthalmological examination including a dilated fundus examination at 18 days of age was normal with a normal macular appearance. At 3 months of age, she had developed mild nystagmus, and fundus examination showed bilateral macular atrophy. A full-field electroretinography (ERG) was performed to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards (McCulloch et al. 2015) using Burian-Allen contact lens electrodes under brief general anesthesia at 7 months of age and showed normal responses. Fundus examination at that time showed bilateral macular atrophy with a bull's eye appearance that had progressed since the examination at 3 months of age (Fig. 1). The peripheral retina was relatively normal in appearance.

Current Treatment: She continues on daily hydroxocobalamin 1 mL IM (100 mcg/kg/day once daily), betaine (Cystadane®) 300 mg/kg/day divided in three daily doses, carnitine 15 mg/kg/day divided in three daily doses, and leucovorin (folinic acid) 10 mg daily, with no dietary restrictions. Her most recent levels (at 21 months of age): MMA is 41.39 μmol/L (ref. 0.0–0.4 μmol/L), total plasma homocysteine is 45 μmol/L (ref. 3–8 μmol/L), and methionine is 23 nmol/mL (ref. 9–42 nmol/mL).

Developmental History

Despite the above therapy since less than 2 weeks of age, our patient has global developmental delay and has required early intervention services. For gross motor milestones: she sat with support at 8 months, she sat unsupported at 10 months, she crawled at 10 months, and started to walk at 16 months of age. Regarding her social and language development, at 6 months she was smiling, she was babbling and saying "mama, baba" at 10 months of age, at 14 months she was imitating words, and at 21 months of age she was saying few other words in addition to "mama, baba." She requires physical therapy, occupational therapy, oral speech and language therapy, and vision therapy.

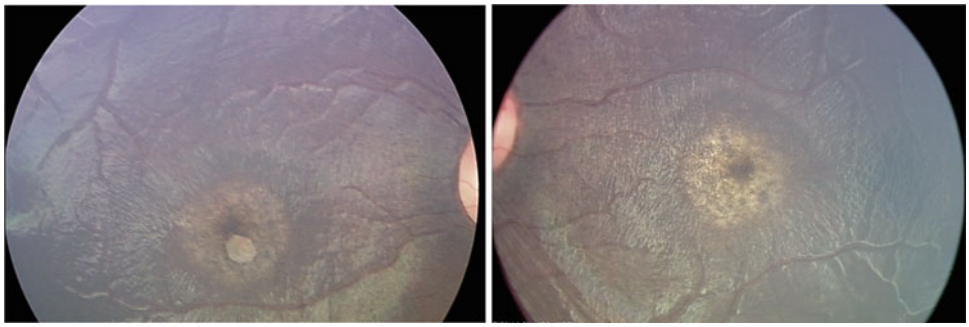

Fig. 1 Color fundus photography at 7 months of age showing bilateral macular atrophy with retinal pigment epithelium (RPE) hyperplasia beneath the anatomic fovea in each eye. There were refractile features in the region of macular RPE depigmentation, and a well-demarcated region of RPE atrophy was present inferior to the fovea in the right eye

Genetic Tests

SNP microarray identified the *MMADHC* gene to be contained within a region of homozygosity. DNA panel testing identified a homozygous pathogenic *MMADHC* mutation c.472C>T (p.Arg158Ter), confirming the diagnosis of cblD deficiency. This nonsense variant in the *MMADHC* gene is predicted to result in the loss of expression of the encoded protein. This variant was not identified in the approximately 6,500 participants in the NHLBI exome project, and gnomAD reports an allele frequency of 1/2,460,765 or 0.000004064. To our knowledge, this variant was not previously reported in other patients with cblD deficiency. Sequencing and deletion/duplication analysis of *MMACHC*, which can also cause combined methylmalonic aciduria and homocystinuria, was normal.

Imaging Studies

Brain magnetic resonance imaging at 17 days of age showed symmetric regions of abnormal T2 hyperintensity in the frontal and parietal subcortical white matter bilaterally and in the frontal periventricular white matter bilaterally. There was also delayed sulcation for age and thinning of the corpus callosum (Fig. 2). Echocardiogram at 2 weeks of age was performed due to suspected cobalamin C deficiency (cblC) and was essentially normal. Abdominal ultrasound was not performed. Upper GI with small bowel follow-through showed no evidence of malrotation. A swallow study performed at 10 months of age identified aspiration with thin liquids and penetration of nectar-thick liquids.

Discussion

Seven other patients have been reported in the literature with cblD-combined; two of these are siblings (Goodman et al. 1970). The age of presentation varied from the first 22 days of life (Coelho et al. 2008) to 14 years of age as published in the original report in 1970 (Goodman et al. 1970). The current report presents the first reported patient to have been identified through a newborn screening program, and this is the earliest reported symptomatic presentation of the described patients with cblD-combined type. This patient's mutation and her biochemical findings were consistent with the combined MMA/HC cblD diagnosis.

Overall, the combined disorders of remethylation that lead to both homocystinuria and methylmalonic aciduria may present in the neonatal period or infancy, as the reported patient presented when she was 8 days old. Usual neonatal presentations are similar to the patient's presentation; they may have lethargy, decreased oral intake, hypotonia, in addition to anemia, and thrombocytopenia.

Abnormal neurological findings have been observed in the combined remethylation disorders as well, such as microcephaly, seizures, hypotonia, and global developmental delay (Huemer et al. 2017). The current manuscript reports a patient with developmental delay who requires physical and speech therapy.

Ophthalmological findings are common in cblC disease and include macular atrophy, nystagmus, strabismus, and optic nerve atrophy (Bonafede et al. 2015; Weisfeld-Adams et al. 2015; Brooks et al. 2016). The macular changes are usually progressive and associated with abnormal rod and cone function on ERG (Brooks et al. 2016). Similar manifestations might be expected in cblD patients, but given the rarity of this disorder, the eye findings have not been well described. Ophthalmologic findings were not described in the previously reported patients with cblD-combined type. The current study presents a patient with an abnormal eye exam at 3 months of age who subsequently developed nystagmus and bilateral macular atrophy. The ocular findings in the patient described in this manuscript were similar to those that have been described in patients with cblC disease (Brooks et al. 2016; Bonafede et al. 2015; Aleman et al. 2015; Bacci et al. 2017; Traboulsi et al. 1992); the patient displayed macular atrophy with a bull's

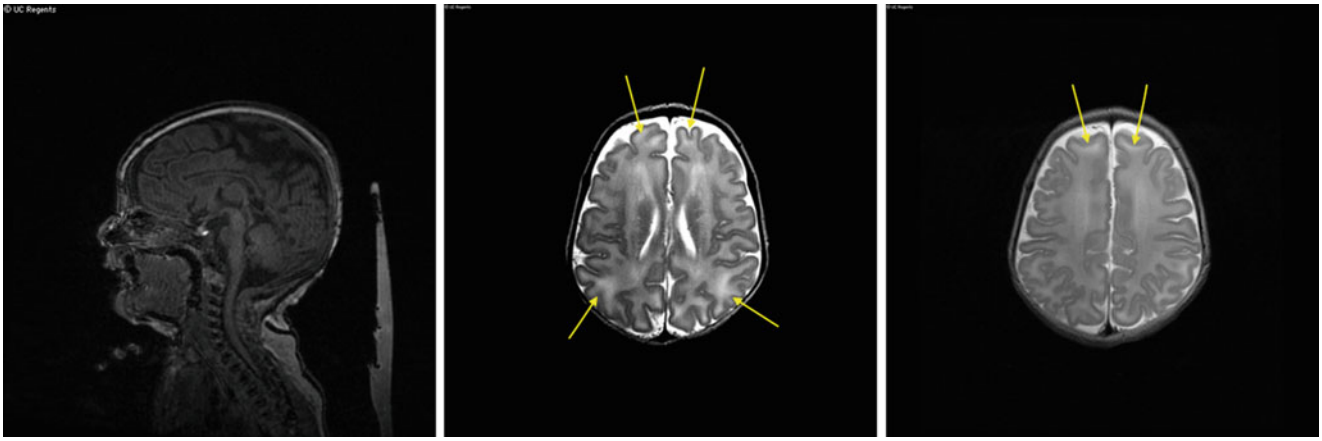

Fig. 2 Brain MRI at 17 days of life; left, midline sagittal T1 showing thin corpus callosum; middle: axial T2 showing bilateral symmetric subcortical white matter T2 hyperintensity in the frontal and parietal lobes; right, axial T2 at a slightly higher level showing bilateral symmetric subcortical frontal white matter T2 hyperintensity

eye appearance. The peripheral retina was relatively normal in appearance, and the full-field ERG demonstrated normal diffuse outer retinal function at 7 months of age; however, full-field ERG responses may become abnormal with time as progressive cone dysfunction has been reported in patients with cblC deficiency. The patient has the characteristic macular findings seen in cblC deficiency in infants, but it is possible that she will develop peripheral pigmentary retinopathy at a later stage as usually occurs in cblC deficiency (Brooks et al. 2016).

Cardiomyopathy has been the main cardiac disease reported in patients with remethylation disorders (Huemer et al. 2017). The current study reports a patient with a normal echocardiogram performed at 2 weeks of age and no cardiac symptoms at age 21 months.

Renal complications may include thrombotic microangiopathy that may lead to atypical hemolytic-uremic syndrome with its associated complications (Huemer et al. 2017). There was no evidence of renal disease, with normal BUN/creatinine at 2 months of age and negative urine analysis on multiple occasions, but no renal imaging was performed in the current study.

Conclusion

CblD-MMA/HC can be identified on newborn screening for remethylation defects by flagging low methionine, which is not done in all states in the United States, and may be missed by newborn screening by elevated C3 acylcarnitine alone, as illustrated here. Early identification may decrease the morbidity and the mortality associated with this condition. Early hospitalization might have been avoided with a more rapid turnaround time of the newborn screen follow-up laboratories. Developmental delay, macular degeneration, and other complications may develop despite early treatment and compliance with therapy, though the progression of the disease and its complications may progress more slowly with the appropriate therapy. Careful monitoring and close follow-up are warranted.

One Sentence Summary

We report a case of cobalamin D combined subtype that was identified by the newborn screening program, with a detailed description of the patient's maculopathy and brain imaging findings.

Details of Contributions of Authors

Dr. Aya Abu-El-Haija: Wrote the manuscript, reviewed the literature, and participated in the clinical care and clinical diagnosis of the patient.

Dr. Bryce Mendelsohn: Revised the manuscript, participated in the biochemical and molecular diagnosis of the patient, and participated in the clinical care of the patient.

Dr. Anthony Moore: Revised the manuscript and participated in the evaluation of the ophthalmologic findings of the patient.

Kara Weisiger: Revised the manuscript and participated in the clinical diagnosis and clinical care of the patient.

Dr. Jacque L. Duncan: Revised the manuscript, performed and interpreted the ERG, took the ophthalmic

images, and participated in the evaluation of the ophthalmologic findings of the patient.

Dr. Orit A. Glenn: Revised the manuscript and participated in the evaluation of the radiologic findings of the brain imaging of the patient.

Dr. Renata Gallagher: Revised the manuscript, participated in the biochemical and molecular diagnosis of the patient, and participated in the clinical care of the patient.

Compliance with Ethics Guidelines

Conflict of Interest

Aya Abu-El-Haija, Bryce A. Mendelsohn, Anthony T. Moore, Jacque L. Duncan, Orit A. Glenn, Kara Weisiger, and Renata C. Gallagher declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was not obtained from the patient given that this is de-identified patient information and is a single case report.

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RESEARCH REPORT

Lathosterolosis: A Relatively Mild Case with Cataracts and Learning Difficulties

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Abstract Lathosterolosis is a rare defect of cholesterol synthesis. Only four previous cases have been reported, two of whom were siblings. We report a fifth patient, with a relatively mild phenotype. He presented at 5 years of age with bilateral posterior cataracts, which were managed with lensectomies and intraocular lens implants. He also had learning difficulties, with a full-scale IQ of 64 at 11 years of age. His head circumference is between the 0.4th and 2nd centiles, and he has mild hypotonia and subtle dysmorphism (a high-arched palate, anteverted nostrils, long philtrum and clinodactyly of toes). The diagnosis was established after sequencing a panel of genes associated with cataracts, which revealed compound heterozygous *SC5D* mutations: c.479C>G p.(Pro160Arg) and c.630C>A p.(Asp210Glu). The plasma lathosterol concentration was markedly raised at 219.8 μmol/L (control range 0.53–16.0), confirming the diagnosis. The c.630C>A p.(Asp210Glu) mutation has been reported in one previous patient, who also had a relatively mild phenotype (Ho et al., JIMD Rep 12:129–134, 2014). The mutation leads to a relatively conservative amino acid substitution, consistent with some residual enzyme activity. Our patient's family did not notice any benefit from treatment with simvastatin. In summary, milder patients with lathosterolosis may present with learning difficulties, cataracts and very subtle dysmorphism. The diagnosis will be missed unless plasma sterols are analysed or relevant genes sequenced.

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Introduction

The importance of cholesterol in the human body has long been recognised (Herman 2003). Among other roles, cholesterol is needed for the structure of cell membranes and myelin, for the synthesis of multiple hormones and bile acids and for signalling within the developing embryo, where cholesterol serves to modify hedgehog protein maturation (Herman 2003; Merkens et al. 2009). Furthermore, precursors within the cholesterol synthesis pathway may serve important roles in their own right, for instance, 7-dehydrocholesterol is the immediate precursor for vitamin D synthesis (Herman 2003). More recently, the phenotypes of defects in several stages of cholesterol biosynthesis have been identified (Herman 2003; Waterham 2006), with Smith-Lemli-Opitz syndrome being the most common and well described (Herman 2003; Merkens et al. 2009). It is

not yet clear to what extent these diseases are caused by the reduction in cholesterol function, as opposed to the effects of accumulation of precursors and by-products (Waterham 2006).

Lathosterolosis is a defect of cholesterol synthesis caused by deficiency of 3-beta-hydrocholesterol-delta-5-desaturase (*SC5D*), thereby preventing conversion of lathosterol to 7-dehydrocholesterol (Brunetti-Pierri et al. 2002). Only four affected individuals have been reported (Brunetti-Pierri et al. 2002; Krakowiak et al. 2003; Rossi et al. 2007; Ho et al. 2014), of whom one was a foetus aborted at 21 weeks' gestation and one died at 18 weeks of age. The four patients all had microcephaly, postaxial polydactyly, syndactyly and variable hepatic involvement. The live-born patients all had facial dysmorphism and cataracts, and those surviving beyond 6 months also displayed central hypotonia and a degree of global developmental delay.

We describe a fifth case of lathosterolosis, confirmed by both genetic and biochemical analyses, with a relatively mild phenotype. The patient has been mentioned briefly in papers on the use of the next-generation sequencing in children with cataracts (Gillespie et al. 2014, 2016).

Case Report

The proband is the second child of healthy non-consanguineous white English parents; his older sister is healthy with normal development. He was born at term following an uneventful antenatal course. There were no significant perinatal concerns, but the patient did have laryngomalacia and neonatal jaundice necessitating phototherapy within the first 72 h of life, with a maximum serum bilirubin level of 299 μmol/L.

The patient has always had good physical health, but his developmental milestones were delayed, and he did not walk or talk until 3 years of age. He attended mainstream primary education with some additional support, but from 11 years of age, he has attended a secondary school for children with special needs. His clinical presentation is consistent with an autistic spectrum disorder. He has deficits in social communication, with particular impairment in reciprocity, poor eye contact and difficulties matching his emotional responses to those around him. These difficulties severely affect his social functioning. He also demonstrates a restricted range of interests, and these deficits have been present from a very young age.

His parents describe poor visual attention from an early age, and he failed a routine school vision test when aged 5 years. He was found to have bilateral posterior lens opacities, as well as astigmatism and myopia. The following year, the proband underwent bilateral lensectomies and intraocular lens implants. YAG capsulotomies were performed for posterior capsular opacification when the proband was 7 years old. The patient's current vision is stable at 0.5 right and 0.3 left LogMar.

In view of the cataracts and the developmental delay, the proband was referred to the Manchester Centre for Genomic Medicine when he was 8 years old. Subtle dysmorphic features were noted (Fig. 1), including a broad forehead, prominent ears, a high-arched palate with crowding of teeth, anteversion of the nostrils, a long philtrum and clinodactyly of toes. At 11 years of age, his height was 143.8 cm (between 25th and 50th centiles), weight 34.9 kg (25th to 50th centiles) and occipitofrontal head circumference 51.1 cm (0.4th to 2nd centiles). Mild hypotonia and joint hyper-extensibility were present.

Initial investigations were unremarkable, including array comparative genomic hybridization. When the patient was 10 years old, next-generation sequencing was undertaken on a panel of 115 genes associated with congenital or childhood cataracts (Gillespie et al. 2014). This identified two heterozygous mutations in the *SC5D* gene: c.479C>G p.(Pro160Arg) and c.630C>A p.(Asp210Glu). The latter mutation has been reported in a previous patient

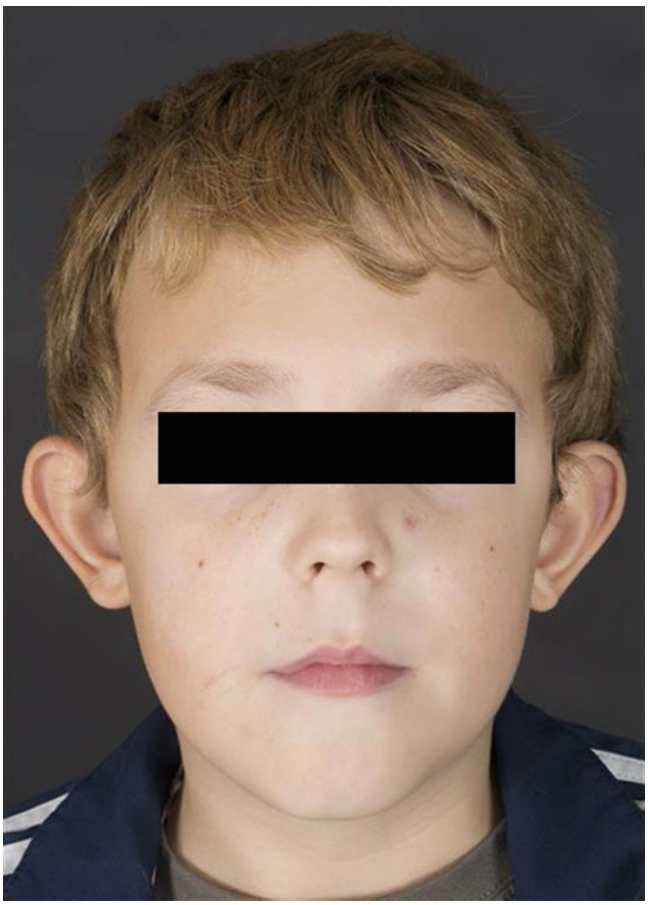

Fig. 1 Facial appearance of patient aged 11 years

(Ho et al. 2014). The parents were each heterozygous for one mutation.

The diagnosis of lathosterolosis was confirmed by plasma sterol analysis by GC-MS, which revealed a markedly elevated plasma lathosterol concentration of 219.8 μmol/L (control range 0.5–16.0 μmol/L). There was also a raised concentration of 8(9)-cholestenol, the immediate precursor of lathosterol, but other intermediates of cholesterol synthesis were normal (Table 1). The total plasma cholesterol concentration was 3.4 mmol/L (reference range 1.2–4.0 mmol/L).

Further assessment showed a mild iron deficiency anaemia (haemoglobin 105 g/L), normal plasma electrolytes, urea and creatinine, bilirubin, albumin, alanine transaminase and alkaline phosphatase, with a minimally deranged coagulation profile (PT 12.6 s, reference range 9.9–11.8 s; APTT 21.6 s, reference range 23.0–31.9 s). There was vitamin D deficiency (plasma total 25-hydroxy, vitamin D concentration 16.4 nmol/L, reference range >50 nmol/L), but other fat-soluble vitamins were normal. An abdominal ultrasound scan at 13 years of age showed no abnormalities of the liver and also excluded malformations of the renal tract.

Cognitive assessment was undertaken using the Wechsler Intelligence Scale for Children, 4th UK Edition (WISC-IV), and the "word reading" and "pseudoword decoding" subtests of the Wechsler Individual Achievement Tests, 2nd UK Edition (WIAT-II). The patient scored within the *extremely low* range of the WISC-IV (standard score = 64 = first centile), indicating developmental delay in cognitive ability. He scored within the *borderline* ranges of perceptual reasoning and processing speed indices of the WISC-IV, the "pseudoword decoding" subtest of the WIAT-II, and the *extremely low* range of ability on the "word reading" subtest. The patient demonstrated greater aptitude for tasks involving concrete visual stimuli, with marked weakness in language comprehension (Table 2).

A trial of treatment was commenced with simvastatin 40 mg daily and cholesterol 1.75 g daily. In a previous patient, treatment with simvastatin normalised the plasma lathosterol concentration and was associated with an increase in developmental quotient from 55 to 64 (Ho et al. 2014). We planned to repeat the sterol measurements and cognitive assessment after 6 months' treatment, but we found that the patient's family had discontinued treatment after 4 months, having observed no benefit, particularly in terms of behaviour.

Discussion

The main clinical features reported in lathosterolosis have been multiple malformations, learning disability, cataracts

Table 1 Plasma sterol analysis by GC-MS

	Patient sample (μmol/L)	Control range (μmol/L)
8-Dehydrocholesterol	0.53	<2.0
7-Dehdrocholesterol	0.93	0.70–1.96
Desmosterol	0.23	2.65–9.22
8(9) Cholestenol	21.61	<4.0
Lathosterol	219.79	0.53–15.98
Cholestanol	6.95	3.87–18.04
Lanosterol	0.26	0.00–1.53

Table 2 Psychometric assessment

		Standard and scaled scores (Percentile)
Wechsler Intelligence Scale for Children, 4th UK Edition		
Full-scale IQ	–	64 (1st)
Verbal comprehension index	–	69 (2nd)
Perceptual reasoning index	–	73rd (4th)
Working memory index	–	68 (2nd)
Processing speed index		73 (4th)
Wechsler Individual Achievement Test, 2nd UK Edition		
Word reading		63 (1st)
Pseudoword decoding		72 (3rd)

and liver involvement (see Table 3). Many features resemble those of Smith-Lemli-Opitz syndrome, the commonest defect of cholesterol synthesis. Smith-Lemli-Opitz syndrome is known to have a wide range of severity, from cases that are lethal in utero to others with minimal learning difficulties or dysmorphism. Lathosterolosis has generally been considered a very severe condition: of the four previous cases, one died aged 18 weeks with intractable myoclonus and respiratory failure (Krakowiak et al. 2003; Parnes et al. 1990), and one was aborted at 21 weeks' gestation due to multiple malformations (Rossi et al. 2007). All previous cases have been diagnosed by sterol analysis, undertaken because Smith-Lemli-Opitz syndrome was suspected; this may have introduced an ascertainment bias, and it is possible that a number of patients with milder forms of the condition have been missed. The patient reported here had learning difficulties and bilateral cataracts but only a few minor malformations and no liver involvement.

Microcephaly has been a universal finding in all reported cases of lathosterolosis. Our patient has learning difficulties with a full scale IQ of 64. The two previous patients who survived beyond infancy also had cognitive impairment (Brunetti-Pierri et al. 2002; Rossi et al. 2007; Ho et al. 2014). The patient described by Ho et al. had a developmental quotient of 64 at the age of 3 years 9 months (Griffiths Mental Developmental Scales), suggesting that the degree of cognitive impairment was similar to our patient's. Developmental assessments were not reported for the other patient (Brunetti-Pierri et al. 2002; Rossi et al. 2007). Our patient has autistic features, but this is not specifically mentioned in previous reports.

Cataracts were a prominent feature in our patient and were the clue that led to the diagnosis. Cataracts were also

Table 3 Summary of the current and previously reported patients

	Brunetti-Pierri et al. (2002) and Rossi et al. (2007)	Rossi et al. (2007)	Krakowiak et al. (2003) and Parnes et al. (1990)	Ho et al. (2014)	Current patient
SC5D mutations	p.R29Q & p.G211D	p.R29Q & p.G211D	homozygous p.Y46S	p.K148E & p.D210E	p.P160R & p.D210E
Lathosterol plasma level	338 µmol/l	N/A	N/A	81.6 µmol/l	219.8 µmol/l
Cholesterol level	Normal	N/A	N/A	Normal	Normal
Age at diagnosis	2 years	Postmortem (21 weeks' gestation)	Postmortem (aged 18 weeks)	22 months	10 years
Cognition	Impaired	N/A	N/A	DQ 64 at 3.8 years	IQ 64 at 11 years
Microcephaly	Yes	Yes	Yes	Yes	Yes
Cataracts	Bilateral from 6 years	–	Bilateral from birth	Bilateral dot cataracts from 4 years	Bilateral from 5 years
Liver	Liver failure & portal hypertension with abnormal biochemistry; cholestasis & cholangiolitis on histology	Extramedullary haematopoiesis & atrophy of hepatocytic laminae on histology	Hepatosplenomegaly with abnormal biochemistry; cirrhosis and vacuolated cells on histology	Normal biochemistry; mildly increased heterogeneity on ultrasound	Normal biochemistry & imaging
Dysmorphism	Epicanthus, broad nasal bridge, anteverted nares, long philtrum, micrognathia, high palate	N/A	Ptosis, short nose, micrognathia	Micrognathia, bitemporal narrowing, broad nasal tip	High palate, very mild ptosis, anteverted nares & long philtrum
Limbs	Hexadactyly & syndactyly of left foot	Hexadactyly of both hands and feet, bilateral talipes	Hexadactyly & syndactyly of both feet	Hexadactyly & syndactyly of both feet	Clinodactyly of both feet
Other malformations	Bilobed gall bladder, T8 butterfly vertebra	Lumbosacral myelomeningocele, Arnold-Chiari type 2 malformation	Ambiguous genitalia	Single umbilical artery	

N/A not available

noted in all previous patients except the aborted foetus. The cataracts were present at birth in one patient (Parnes et al. 1990; Krakowiak et al. 2003) and in the others were first noted at the age of 4 years (Ho et al. 2014), 5 years (our patient) and 6 years (Brunetti-Pierri et al. 2002) (Table 3). The cataracts were bilateral in all cases but asymmetrical in one case, only requiring extraction in one eye. One patient only had small dot cataracts at 4 years with normal vision (Ho et al. 2014).

Abnormalities of the digits have been the commonest malformations in previous patients with lathosterolosis, possibly due to the role of hedgehog signalling in limb development. All four previous patients have had postaxial polydactyly (on feet ± hands) and syndactyly of the toes, but our patient has neither feature, demonstrating that these are not always present in lathosterolosis. The only limb abnormality in our patient was clinodactyly of the toes.

The current patient has a high-arched palate, mildly anteverted nostrils and a long philtrum, as have two previous patients (Brunetti-Pierri et al. 2002; Krakowiak et al. 2003). He does not, however, have the other reported dysmorphic features, namely, bitemporal narrowing, epicanthic folds, a broad nasal tip, micrognathia and small chin. Malformations in previous patients have also included ambiguous genitalia (Krakowiak et al. 2003), horseshoe kidneys (Rossi et al. 2007), bilobed gall bladder (Brunetti-Pierri et al. 2002), T8 butterfly vertebra (Brunetti-Pierri et al. 2002), lumbosacral meningomyelocele and Arnold-Chiari type 2 malformation (Rossi et al. 2007) (Table 3). None of these have been found in our patient but he has not had a skeletal survey.

Variable hepatic involvement has been reported in previous cases of lathosterolosis. For the foetus aborted at 21 weeks' gestation, liver histology showed extramedullary haematopoiesis and atrophy of hepatocytic laminae (Rossi et al. 2007). Two other patients had persistently raised plasma levels of bilirubin, transaminases and alkaline phosphatase. One of these developed liver failure and portal hypertension at 7 years of age, with cholestasis and cholangiolitis on histology (Rossi et al. 2007); the other had hepatosplenomegaly, and histology showed cirrhosis and vacuolation, especially of histiocytes, with storage of lipids and mucopolysaccharides (Parnes et al. 1990). The final patient had normal plasma bilirubin, transaminases and alkaline phosphatase levels and no hepatic symptoms, but ultrasound showed mildly increased heterogeneity (Ho et al. 2014). Our patient had neonatal jaundice but no subsequent symptoms or biochemical evidence of liver disease, and liver ultrasound was normal. Though we found no evidence of liver disease, we plan to continue monitoring for cholestasis, particularly in view of the vitamin D deficiency and very mildly prolonged prothrombin time.

Like all previous cases, our patient has two missense mutations in *SC5D*. Both affect highly conserved amino acids, are predicted damaging in silico (Gillespie et al. 2014) and are extremely rare according to the reference datasets, GnomAD and ExAC. The p.(Asp210Glu) variant has previously been associated with lathosterolosis in an unrelated patient who also had a relatively mild phenotype (Ho et al. 2014), and it is possible that this mutation leaves some residual enzyme activity, accounting for the milder phenotype. It has been suggested that the phenotypic severity may correlate with the lathosterol level in cultured fibroblasts (Ho et al. 2014), but as our patient declined a skin biopsy, this analysis was not performed. His plasma lathosterol concentration was over 2.5 times that in the previous mild patient (Ho et al. 2014), but this may be influenced by the greater age of our patient. The plasma cholesterol concentration has been normal in all patients in whom it has been measured, in contrast to the mouse model (Krakowiak et al. 2003).

In conclusion, this patient displays a milder phenotype than previously described in lathosterolosis, with bilateral cataracts, microcephaly and learning difficulties as the main features. Of particular note, the polydactyly and liver involvement seen in the other cases were absent. There may be a number of patients like this, with cataracts and learning difficulties, who will only be diagnosed if plasma sterol analysis or sequencing of relevant genes is undertaken.

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Contributions of Individual Authors

Drs. Anderson and Morris managed the patient and wrote the case report. Dr. Taylor undertook the next-generation sequencing, and Professors Clayton-Smith and Clayton confirmed the diagnosis clinically and by sterol analysis, respectively. Drs Rust and Ashworth undertook psychological assessment and ophthalmological management, respectively. All authors critically reviewed the manuscript.

Dr. Andrew Morris is the corresponding author and serves as guarantor.

Competing Interest Statement

Drs. Anderson, Rust, Ashworth, Taylor and Morris and Professors Clayton-Smith and Clayton declare that they have no conflict of interest.

Consent

The patient's parents have given written consent for publication. Ethical Committee approval is not needed for this case report.

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RESEARCH REPORT

DPAGT1 Deficiency with Encephalopathy (DPAGT1-CDG): Clinical and Genetic Description of 11 New Patients

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Abstract Pathogenic mutations in *DPAGT1* cause a rare type of a congenital disorder of glycosylation termed DPAGT1-CDG or, alternatively, a milder version with only myasthenia known as DPAGT1-CMS. Fourteen disease-causing mutations in 28 patients from 10 families have previously been reported to cause the systemic form, DPAGT1-CDG. We here report on another 11 patients from 8 families and add 10 new mutations. Most patients have a very severe disease course, where common findings are pronounced muscular hypotonia, intractable epilepsy, global developmental delay/intellectual disability, and early death. We also present data on three affected females that are young adults and have a somewhat milder, stable disease. Our findings expand both the molecular and clinical knowledge of previously published data but also widen the phenotypic spectrum of DPAGT1-CDG.

Abbreviations

CDG	Congenital disorder of glycosylation
CMS	Congenital myasthenic syndrome
DPAGT1	Dolichyl-phosphate GlcNAc

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	phosphotransferase 1
EEG	Electroencephalogram
EOEE	Early-onset epileptic encephalopathy
GlcNAc	*N*-acetyl glucosamine
IEF	Isoelectric focusing
LC/MS	Liquid chromatography/mass spectrometry
MRI	Magnetic resonance imaging
N-linked	Asparagine linked
PMM2	Phosphomannomutase 2
TF	Transferrin

Background

Protein glycosylation is the most frequent posttranslational protein modification, showing an immense variation (Eklund and Freeze 2005). It can occur at different amino acids [for instance, asparagine (*N*-linked) (Stanley and Taniguchi 2015) or serine/threonine (*O*-linked)], using several different monosaccharides as the linkage sugar [e.g., glucose, mannose, or *N*-acetylglucosamine (GlcNAc)] (Seeberger 2015), and the final carbohydrate decoration may be everything from a monosaccharide to a large polysaccharide (Prestegard et al. 2015). A few percent of the genome is dedicated to these processes (Henrissat et al. 2015), and mutations in these genes constitute the basis for the congenital disorders of glycosylation (CDG). The number of genetically confirmed CDG types has increased exponentially since the first one was described in 1980 (Jaeken et al. 1980) and today includes almost 130 different entities. Almost 70 of these involve deficient *N*-glycosylation, where phosphomannomutase 2 deficiency (PMM2-CDG) is the most common type (Freeze et al. 2015).

Dolichyl-phosphate GlcNAc phosphotransferase 1 (DPAGT1) catalyzes the first step in the biosynthetic pathway of the precursor oligosaccharide for *N*-linked glycosylation: the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol phosphate, resulting in the formation of dolichol pyrophosphate GlcNAc (Wu et al. 2003). The first patient identified with mutations in *DPAGT1* was described in 2003 (Wu et al. 2003), and thereafter over 40 patients have been described in publications (Jaeken et al. 2015). Interestingly, a recently released manuscript on the preprint server for biology "bioRxiv" investigates in great detail known mutations in DPAGT1, using crystal structures of the enzyme together with either the substrate UDP-GlcNAc or the inhibitor tunicamycin (https://doi.org/10.1101/291278). Two clinically distinguishable phenotypes are seen with the first being a systemic disease with encephalopathy [DPAGT1-CDG (formerly CDG-Ij)] and the second a congenital myasthenic syndrome (DPAGT1-CMS) with tubular aggregates seen in muscle biopsies (Jaeken et al. 2015). The DPAGT1-CDG phenotype is most often a severe disease, where more than 80% of the reported patients died before 5 years of age. However, 18 of the previously reported 29 patients were from the same consanguineous family (Imtiaz et al. 2012), thus the very high mortality rate may be skewed by one specific mutation. Few patients with a milder (but systemic) disease have been described, for instance, two siblings in their 30s (Iqbal et al. 2013). All patients have shown moderate to severe intellectual disability. Epilepsy is a common feature and often debuts early. A number of symptoms typical for CDG, such as feeding difficulties, cataracts, hypertrichosis, hyporeflexia, joint contractures, elevated liver enzymes, and abnormal brain magnetic resonance imaging (MRI), have been described also in this subtype. However, findings otherwise common in CDG, such as night blindness, inverted nipples, and lipodystrophy, have only rarely been reported (Jaeken et al. 2015).

In this report, we present the genetic and clinical data available for an additional 11 patients with the systemic form of this disease, most of whom presented with a very severe phenotype.

Methods

Genetic Characterization

Exome sequencing was performed as previously described (Simon et al. 2017). Sanger sequencing was performed using standard PCR methods targeting the nine coding exons for human *DPAGT1* (NM_001382.3). Primer sequences are available upon request.

Results

Clinical Characterization

The inclusion criteria for this study was a biochemical test showing underglycosylation of transferrin (TF) and/or exome sequencing data showing likely damaging homozygous or compound heterozygous mutations in the *DPAGT1* gene.

Table 1 is a summary of mutations as well as clinical symptoms of our 11 patients from 8 families and also 13 patients previously published (Wu et al. 2003; Imtiaz et al. 2012; Iqbal et al. 2013; Yuste-Checa et al. 2017; Vuillaumier-Barrot 2005; Wurde et al. 2012; Carrera et al. 2012; Timal et al. 2012; Ganetzky et al. 2015). Another 16 patients from 1 consanguineous family were affected, all died in early infancy and showed a similar clinical phenotype, but they are not included in the following calculations as they were not formally tested and the individual clinical descriptions are lacking (Imtiaz et al. 2012). There seems to be no gender predilection for

Table 1 Patient data

Reference	Mutation 1 (protein consequence)	Mutation 2 (protein consequence)	gnomAD (no of het, homo)	Tf glycosylation	Gender	Ethnicity	BW (g)/GL (wks + days)	Percentiles (weight)	Deceased	MRI	EEG
This report; P1	c.2T>C (p.Met1?)	c.341C>G (p.Ala114Gly)	5,0/1,0	Type I pattern	F	Armenian	–/–	–	–	Normal	–
P2	c.509A>G (p.Tyr170Cys)	c.584G>C (p.Ala195Gly)	4,0/0,0	Type I pattern	M	Native Am./ Caucasian	3,390/38+0	75	–	–	–
P3	c.509A>G (p.Tyr170Cys)	c.584G>C (p.Ala195Gly) (assumed, never tested sib)	4,0/0,0	Normal (8 weeks)	M	Native Am./ Caucasian	–/–	–	5 months	–	+
P4	c.1117C>G (p.Pro373Ala)	c.1197T>A (p.Tyr399*)	0,0/0,0	Type I pattern	M	Caucasian	1,875/36 + 0	<1	–	Normal	–
P5	c.116_117delinsAA (p.Ala39Lys)	c. 380_395dup16 (p.Ser133Alafs*64)	0,0/2,0	Type I pattern	F	Caucasian	3,064/term	25	3 months	Normal	–
P6	c.419A>G (p.Tyr140Cys)	c.419A>G (p.Tyr140Cys)	1,0/1,0	Type I pattern	M	Arabic	3,350/41 + 2	40	10 months	Thin corpus callosum	Hyps
P7	c. 380_395dup16 (p.Ser133Alafs*64)	c.739C>T (p.Arg247Trp)	2,0/3,0	Type I pattern	M	–	3,239/term	40	Alive at 7 years	Increased space below cerebellum	–
P8	c. 380_395dup16 (p.Ser133Alafs*64)	c.739C>T (p.Arg247Trp) (assumed, never tested sib)	2,0/3,0	Never performed	F	–	2,670/term	10	4 years 1 month	General atrophy	General slowing
P9	c.488T>C (p.Leu163Pro)	Unidentified deletion	4,0/–	Type I pattern	F	Caucasian	–/–	–	Alive at 18 years	Normal	Hyps
P10	c.488T>C (p.Leu163Pro)	Unidentified deletion	4,0/–	Type I pattern	F	Caucasian	–/–	–	Alive at 16 years	Normal	+
P11	c.26dupT (p.Met9Ilefs80*)	c.739C>T (p.Arg247Trp)	8,0/3,0	Type I pattern	F	Caucasian	2,400/38 + 0	5	Alive at 17 years	Normal	–
Wu et al. (2003)	c.509A>G (p.Tyr170Cys)	Unknown (splice – >message decay)	4,0/–	Type I pattern	F	–	–/41 + 3	–	Alive at 21 years	Normal	Hyps
Vuillaumier-Barrot (2005)	c.890A>T (p.Ile297Phe)	c.162-8G>A (splice)	6,0/0,0	Type I pattern	–	–	–/–	–	–	–	–
	c.890A>T (p.Ile297Phe)	c.162-8G>A (splice)	6,0/0,0	Type I pattern	–	–	–/–	–	–	–	–
Imtiaz et al. (2012)[a]	c.902G>A (p.Arg301His)	c.902G>A (p.Arg301His)	5,0/5,0	Type I pattern	M	Arabic	–/term	–	5 years	Less myelin	+
	c.902G>A (p.Arg301His)	c.902G>A (p.Arg301His)	5,0/5,0	Type I pattern	M	Arabic	–/–	–	–	–	–
Wurde et al. (2012)	c.341C>G (p.Ala114Gly)	c.341C>G (p.Ala114Gly)	1,0/1,0	Type I pattern	F	Turkish	2,610/38 + 3	10	8 months	Global atrophy	Hyps
	c.341C>G (p.Ala114Gly)	c.341C>G (p.Ala114Gly)	1,0/1,0	Type I pattern	F	Turkish	2,600/38+6	5	1 year	–	+
Carrera et al. (2012)	c.901C>T (p.Arg301Cys)	c.1054T>G (p.Leu385Arg)	4,0/7,0	Type I pattern	M	Spanish	3,095/term	25	6 weeks	Normal	–
Timal et al. (2012)/Adamowicz et al. (2011) (abstr)	c.206T>A (p.Ile69Asn)	c.161+5G>A (splice)	1,0/2,0	Type I pattern	M	–	1,410/–	<1	2.5 years	–	+
Iqbal et al. (2013)	c.85A>T (p.Ile29Phe)	c.503T>C (p.Leu168Pro)	26,0/0,0	Type I pattern	F	Pakistani	–/–	–	Alive at 34 years	–	–
	c.85A>T (p.Ile29Phe)	c.503T>C (p.Leu168Pro)	26,0/0,0	Type I pattern	M	Pakistani	–/–	–	Alive at 32 years	–	–
Ganetzky et al. (2015)	- (p.Leu118Val)	- (p.Leu118Val)	0,0/0,0	Type I pattern	F	–	5th centile/ 40 + 3	5	1.5 months	Cerebellar hypoplasia	–
Yuste-Checa et al. (2017)	c.329T>C (p.Phe110Ser)	c.902G>A (p.Arg301His)	0,0/5,0	–	–	–	–	–	–	–	–

ID/DD	Epilepsy	Other neurological	Eye/retina	Skin	Liver	Heart	Muscles	Skeletal	Other
–	–	Microcephaly	Strabismus	Hypertrichosis, lipodystrophy	Elevated transaminases	Long QT	Axial hypotonia, extremity hypertonia	–	Low AT-III, PLE
–	–	Intracranial hemorrhage	–	–	–	–	Congenital hypertonia	Contractures	FTT
++	+	Intracranial hemorrhage	–	–	–	–	–	–	FTT
–	–	Arthrogryposis	Congenital cataract	Anasarca	Normal transaminases	Normal	Fetal and newborn akinesia	Contractures	Lung hypoplasia
++	No	Microcephaly	–	Loose (due to underweight)	Elevated transaminases	–	Hypotonia	–	FTT, retained umb cord
–	ES ->EE	Tremor	Congenital cataract	Hypertrichosis	Enlarged, elevated transaminases	Normal	Hypotonia	No dysplasia	FTT, low prot S/AT-III
++	+	Non-ambulatory, non-verbal	Astigmatism, ocular melanosis	Normal	Elevated transaminases	Long QT	Hypotonia, exotropia	Normal	FTT, repeat pneumonias
++	+	Rocking, head banging	Optic nerve atrophy, nystagmus	Normal	Elevated transaminases	Normal	Hypotonia, fetal akinesia	Normal	–
++	ES ->ME	Stroke-like episodes, autism	Visual range 2 m, strabismus	Normal	Normal transaminases	Normal	Hypotonia, areflexia	Scoliosis, osteoporosis	High protein S, spont menarche
++	Focal ep	Autism	Strabismus	Normal	Normal transaminases	Normal	Hypotonia, areflexia	Scoliosis	High protein S, spont menarche
++	No	Microcephaly, ataxia	RP, optic nerve atrophy	Normal	Normal transaminases	Normal	Hypotonia	Severe scoliosis	Induced puberty
++	ES ->EE-> no sz	Microcephaly	Exotropia	Dimples	Normal transaminases	Normal	Hypotonia	Clinodactyly	Spont menarche
–	–	–	–	Normal	Normal transaminases	–	–	NK	–
–	–	–	–	Normal	Normal transaminases	–	–	NK	–
++	+	–	–	–	Elevated transaminases	–	Hypotonia	–	Repeat aspirations
–	–	Fetal hypokinesia	–	–	–	–	–	–	–
++	ES ->EE	Hyperexcitability, microcephaly	Congenital cataract, nystagmus	Hypertrichosis, inverted nipples	Hepatomegaly	–	Hypotonia, strabismus	–	–
++	EE	Hyperexcitability, microcephaly	Congenital cataract		–	–	–	–	–
–	–	Akinesia	Papillar atrophy	Thick skin, hypertrichosis	–	–	Hypotonia	Camptodactyly, contractures	–
–	Tonic seizures	–	Congenital cataract	–	Elevated transaminases	–	Hypertonia	Joint contractures	FTT, low AT-III, chronic anemia
+	+	Aggressiveness, speech delay	Night blindness	–	–	–	Hypotonia	–	–
+	+	Aggressiveness, speech delay	–	–	–	–	Hypotonia	–	–
–	–	Arthrogryposis, fetal akinesia	Congenital cataract	Normal	–	–	Hypotonia	Arachnodactyly	–
+	–	Hypoacusia	–	–	–	–	Hypotonia, weakness	–	–

- = not known/performed or not reported, no = not present/negated, + = pathological finding not further specified, ++ = severe finding not further specified, gnomAD = number of hetero- and homozygotes reported in the database per allele

BW birth weight, *GL* gestational length, *hyps* hypsarrhythmia, *ID/DD* intellectual disability/developmental delay, *ES* epileptic spasms (West syndrome), *EE* epileptic encephalopathy, *ME* myoclonic epilepsy, *FTT* failure to thrive, *RP* retinitis pigmentosa, *AT-III* antithrombin III

[a] The paper indicates 16 additional members of the family being affected, all died in early infancy, similar clinical phenotype

DPAGT1-CDG, out of the previously published patients where the gender is reported, 5/10 patients were female, and in our cohort 6/11 are female. The lengths of the pregnancies were reported in 13/24 of the cases, and in these, delivery was at term in 12/13. However, the babies generally have a birth weight in the lower spectrum, ranging from <1 to 40th percentile in all but one case. The phenotype is usually very severe, and including our patients, 10 patients have been confirmed deceased before the age of 5 years (6 died at or before 12 months of age; data is missing on 7). Neurologically these patients are often severely afflicted: All patients eligible for testing have shown signs of developmental delay/cognitive impairment of at least moderate severity. 13/24 patients were reported to have epilepsy, mostly of early-onset types, where four had West syndrome with hypsarrhythmia on electroencephalogram (EEG) and clinical spasms at debut. So far, only 3/24 described patients have been negated to have epilepsy. Sixteen patients were described as hypotonic, whereas another three were described as hypertonic. Other common neurological findings include microcephaly (6/24), arthrogryposis/fetal akinesia (5/24), and strabismus/squint (6/24) (Table 1). Ten patients have had an MRI scan of their brains reported, but no consistent pathological findings were seen. In 6/10 patients, the scans were considered as normal; others had findings such as global atrophy, cerebellar hypoplasia, and thin corpus callosum. Cataract is a common finding in this CDG type, described in 7/24 patients, and 4/24 patients have a confirmed retinal pathology. Hypertrichosis has been noted in 4/20 patients. Lab investigations available from 22 out of 24 patients show that all but one patient had a type I TF pattern using isoelectric focusing (IEF) or liquid chromatography/mass spectrometry (LC/MS) analysis. 6/14 patients reported an elevation in their liver transaminases (data missing on 10 patients). Five patients have a reported deficiency in protein S and/or antithrombin III.

Mutational Spectrum

We combined exome sequencing and Sanger sequencing to identify 12 different *DPAGT1* mutations in 11 patients, of which only 2 [c.341C>G; p.(Ala114Gly); c.509A>G p.(Tyr170Cys)] were previously reported to cause DPAGT1-CDG (Wu et al. 2003; Wurde et al. 2012) (Table 1). We then searched the gnomAD (Genome Aggregation Database) (gnomad.broadinstitute.org) [gnomADr2.0.2, accessed 03.01.2018], containing data from 123,136 exome sequences and 15,496 whole-genome sequences of unrelated individuals, and found that 15 of the mutations were found at low frequency in heterozygous carriers, whereas 8 were previously unreported. None of the mutations occurred in a homozygous state (Table 1). The most prevalent heterozygous mutation was c.85A>T; p.(Ile29Phe), with a total of 26 reported carriers and a carrier frequency of 0.00084 in a South Asian population.

In the previously published patients, 13 different pathological alleles have been described: 2 affecting a splice site and the rest being missense mutations (Table 1). We add one stop mutation, two frameshift mutations, six missense mutations, and one mutation of the initiating methionine. In two siblings the amino acid exchange p.(Leu163Pro) seems to be heterozygous based on genomic DNA analysis, but homozygous based on cDNA, suggesting an undetected deletion. Only one parent (the father) was a carrier. The pathogenicity of the mutations were tested in silico, and based on our previous experience (Ng et al. 2016), we used the in silico pathogenicity determining program Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/).

Discussion

We present data on 11 unreported patients with DPAGT1-CDG, increasing the known patient population by one third. Most of the mutations are missense, and no new splice mutations were found. Combining the novel mutations and the already published ones, they occur throughout the gene without locus-specific accumulation (Fig. 1). However, 14/20 missense mutations are within or very proximal to a membrane-spanning section of the protein (Fig. 1). The full-blown phenotype of this CDG type is very severe, showing early-onset epileptic encephalopathy (EOEE), pronounced muscular hypotonia, severely delayed development, and early death (Jaeken et al. 2015). In our cohort this was confirmed as 30% of the cases died before the age of one, and only three lived to be teenagers. This makes DPAGT1-CDG one of the most severe CDG types. Mortality in, for instance, the most common type, PMM2-CDG, is estimated to 20% during the first year in the severe cases, after which it stabilizes. Epilepsy is a common finding in patients with glycosylation deficiencies, with a wide spectrum of severity and semiology (Freeze et al. 2015). Severe epilepsy, often beginning as Ohtahara syndrome (early infantile epileptic encephalopathy) or West syndrome (hypsarrhythmia, clinical spasms, and developmental arrest) and sometimes developing into multifocal hard-to-treat epilepsy, has been reported for many CDG types, including ALG1-CDG (Fiumara et al. 2016; Barba et al. 2016), ALG3-CDG (Fiumara et al. 2016; Barba et al. 2016), ALG6-CDG (Fiumara et al. 2016), ALG13-CDG (Hamici et al. 2017), ALG14-CDG (Schorling et al. 2017), DPM2-CDG (Fiumara et al. 2016), DOLK-CDG (Helander et al. 2013), RFT1-CDG (Barba et al. 2016), SLC35A2-CDG (Ng et al. 2013), and SLC35A3-CDG (Marini et al. 2017). In the published cohort of DPAGT1-CDG patients

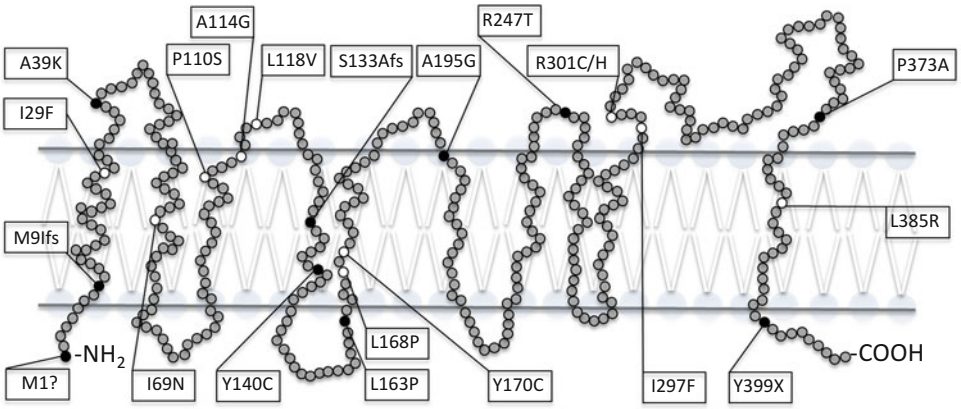

Fig. 1 A depiction of the predicted structure of the DPAGT1 protein. There are ten membrane-spanning domains with the N- and C-termini both on the luminal side. All known missense mutations, a stop codon-introducing mutation, a mutation of the initiating methionine, and two frameshift mutations are indicated with boxes. Gray circles indicate amino acids not affected in DPAGT1-CDG so far, white circles depict amino acids that have previously been published to be exchanged in DPAGT1-CDG, and black circles indicate amino acids changes described for the first time in this paper

including this report, 4/24 patients have had the diagnosis West syndrome, and 9 more have a definitive diagnosis of epilepsy, whereas only 3 were negated to have epilepsy. This strengthens the notion that one should consider CDG as a cause of EOEE, when its etiology is unknown. Why incorrect glycosylation causes epilepsy is not fully known, but it must be multifactorial since deficiencies in many different glycosylation pathways cause epilepsy (Freeze et al. 2012, 2015). Many *N*- and *O*-glycoproteins are involved in CNS development and function, including NCAMs, voltage-gated channels, and neurexins (UniProt Consortium 2015). Deficient glycosylation of these types of proteins may well cause epilepsy, but there is very little literature on the subject readily available. A recent report (Izquierdo-Serra et al. 2018) showed nicely that hypoglycosylation of the $Ca_V2.1$ channel (a neuronal pore-forming voltage-gated calcium channel) might be the molecular explanation to a phenomenon known as stroke-like episodes, a feared complication in CDG. These episodes involve confusion, hemiparesis, and sometimes seizures and may last several days. There is no standard, widely accepted, treatment; however, antiepileptic drugs seem to have a place in the treatment arsenal (Izquierdo-Serra et al. 2018). Also, deletions in the glycoprotein neurexin 1 (NRXN1) gene cause epilepsy (Perez-Palma et al. 2017) and autistic features (Kasem et al. 2018), both common symptoms in many CDG types.

In many CDG types, female teenagers do not enter puberty spontaneously, and puberty therefore needs to be induced using estrogens and progesterone (de Zegher and Jaeken 1995; Miller and Freeze 2003). In our cohort, two young women have gone through puberty without the aid of exogenous hormones and continue to menstruate regularly, whereas puberty in the third girl was recently induced with hormonal therapy. Furthermore, the clinical data on the first DPAGT1-CDG patient published (Wu et al. 2003) was updated during the finalization of this paper (Table 1), and she is also regularly menstruating without the aid of hormones (Freeze, HH, pers. comm.). In the only previously published description of an adult female with DPAGT1-CDG, there was no information on her sexual development (Iqbal et al. 2013). Thus, females with DPAGT1-CDG seem to have a fair chance of developing normal menstruations, and it may be clinically relevant to await spontaneous menarche.

The general suggestion in the literature is to avoid surgery in CDG patients due to coagulation abnormalities (Linssen et al. 2013). A problem noted in all our adult patients, however, was severe scoliosis, having an immense impact on their quality of life. We therefore decided to perform spinal surgery on three of the adolescent patients (P9-11), closely monitoring the coagulation parameters. The procedures were well tolerated and radically improved their quality of life.

In conclusion we describe 11 new patients with DPAGT1-CDG and confirm the previous notion that this is generally a severe subtype. However, attenuated forms exist. Here, patients survive into the adulthood presenting a rather static encephalopathic phenotype with other problem such as scoliosis. With the more general introduction of massive parallel sequencing in the diagnostic arsenal of cognitive impairment and epilepsy, we certainly expect more patients with attenuated types of CDG to be presented in the literature within the near future.

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Synopsis

A thorough analysis of the genetic and clinical features of 11 novel DPAGT1-CDG patients.

Details of the Contributions of Individual Authors

BGN, HHF, and EAE designed the study. HRU, LP, XZ, CAS, KDJ, and EAE provided and analyzed clinical information. BGN, JMR, SG, AM, MK, DAN, KJB, JS, and MJB provided and analyzed genetic data. PB provided and analyzed biochemical data. EAE wrote the initial manuscript; all authors revised the manuscript and approved the final version.

Competing Interest Statement

Bobby G. Ng, Hunter R. Underhill, Lars Palm, Per Bengtson, Jean-Michel Rozet, Sylvie Gerber, Arnold Munnich, Xavier Zanlonghi, Cathy A. Stevens, Martin Kircher, Deborah A. Nickerson, Kati J. Buckingham, Kevin D Josephson, Jay Shendure, Michael J. Bamshad, Hudson H. Freeze, and Erik A. Eklund declare that they have no conflict of interest.

Compliance with Ethic Guidelines

The research was performed under a Sanford Burnham Prebys Medical Discovery Institute IRB protocol. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Patient Consent Statement

Families included in this research study provided written informed consent. Proof that informed consent was obtained is available upon request.

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RESEARCH REPORT

Enzyme Replacement Therapy During Pregnancy in Fabry Patients

Review of Published Cases of Live Births and a New Case of a Severely Affected Female with Fabry Disease and Pre-eclampsia Complicating Pregnancy

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Abstract Fabry disease (FD) is an X-linked, lysosomal storage disease. Mutations in the gene coding for alpha-galactosidase A lead to globotriaosylceramide (Gb-3) accumulation in lysosomes and in placenta and umbilical cord. Impact of FD and treatment with enzyme replacement (ERT) on foetal development is undisclosed.

A 38-year-old primigravida with FD (G85N) is reported. She has 50% reduced alpha-galactosidase A activity and elevated plasma and urine-Gb-3. She was severely affected with ischaemic stroke at age 23, hypertension, albuminuria and moderately reduced renal function. ERT was initiated at age 23 years in 2001 and continued during spontaneous pregnancy at age 38. In third trimester she developed moderate-to-severe pre-eclampsia, successfully managed by methyldopa. Chorion villus sampling revealed a male foetus without the maternal gene mutation. Planned Caesarean section was performed without complications at gestational age week 38 + 6, delivering a healthy boy. Histopathological placental examination showed no sign of Gb-3 accumulation. Literature survey disclosed a total of 12 cases, 8 were treated with ERT during pregnancy and 5 infants inherited the family mutation. All outcomes were successful. In the six cases with available placental histopathological examination, Gb-3 accumulation was only seen on the foetal side if the foetus had the inherited mutation.

In conclusion, the present case, describing the first data from a severely affected FD patient receiving ERT during pregnancy complicated by pre-eclampsia, together with all other published cases, has emphasized that ERT is safe during pregnancy and resulting in successful foetal outcome; despite this, ERT is by the health authorities advised against during pregnancy.

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Introduction

Fabry disease (FD, OMIM # 301500) is an X-linked, lysosomal storage disease. Mutations in the galactosidase alpha gene cause missing or reduced activity of the alpha-galactosidase A enzyme, leading to storage of globotriaosylceramide (Gb-3) and related glycosphingolipids in lysosomes (Brady 1967; Kint 1970; Bishop et al. 1988). In female patients with FD, phenotypic expression is variable due to X-chromosome inactivation and residual enzyme activity (Echevarria et al. 2016), varying from nearly unaffected to full phenotypic disease similar to males (Macdermot et al. 2001; Whybra et al. 2001; Wilcox et al. 2008). Gb-3 accumulation has been found in the placenta and umbilical cord during pregnancy with a male foetus with a mutation in the α-galactosidase A gene (Thurberg and Politei 2012), leading to considerations of potential impact on foetal well-being during pregnancy. No

randomized clinical trials have been performed in pregnant patients with FD, and the efficacy and safety of enzyme replacement therapy (ERT) on pregnancy and foetal development have not been established. The present case describes the first data from a severely affected, ERT-receiving FD patient during pregnancy complicated by pre-eclampsia. The present paper also provides a review of the literature, since cases of patients with FD during pregnancy are rare, and currently treatment is done on individual, experimental basis. Controlled clinical trials or even large single-centre cohort presentations are unlikely to be performed on this rare topic; hence, documentation of treatment and outcome in all individual patients with FD during pregnancy is important to gather global information.

Case

Clinical Findings

We present a case of a 38-year-old primigravida with FD, diagnosed at age 2 years, with a missense mutation G85N, reduced alpha-galactosidase A activity to 50% of normal lower limit (10 nmol/h/mg protein, reference range: 20–65) and elevated plasma-Gb-3 and urine-Gb-3 of 6.5 μmol/L (reference range, 1.6–3.3) and 13.9 mol Gb-3/mol sphingomyelin (reference range, <0.3) in 2001 before ERT, respectively. Phenotypically, she was severely affected with ischaemic stroke, hypertension, albuminuria and moderately reduced renal function at age 23 years and transient ischaemic attack at age 32. She has been treated with ERT since 2001 (as the first patient in Denmark) as well as acetylsalicylic acid 75 mg/day and enalapril 10 mg/day. Cardiac involvement is limited to short PQ interval.

At age 37, the patient initiated in vitro fertilization with donor sperm and planned pre-implantation genetic diagnosis. However, spontaneous pregnancy was achieved at age 38. The patient consented to chorion villus sampling at gestational age of 10 + 5 weeks and genetic analysis revealed that the male foetus had not inherited the maternal gene mutation.

During pregnancy, enalapril was switched to labetalol 100 mg × 3/day, while acetylic acid (75 mg/day) and ERT (agalsidase-beta, 1 mg/kg, every other week) were continued. In the third trimester, blood pressure and albuminuria increased while renal function decreased, consistent with moderate-to-severe pre-eclampsia (Fig. 1). The condition was successfully managed by methyldopa (initially 250 mg × 3/day increasing to 500 mg × 3/day). Eclampsia did not develop. Mild haemolysis, slightly elevated liver enzymes and platelets low in the normal range were observed; however, criteria for HELLP syndrome (i.e. haemolysis, elevated liver enzymes and low platelet count) were not met.

Planned Caesarean section without complications was performed at gestational age week 38 + 6, delivering a healthy boy without FD. Weight, length and head circumference at birth were 2,675 g, 47 cm and 34 cm, respectively. Apgar score was 10/1, 10/5 and 10/10.

One year after delivery the renal function and proteinuria was unchanged compared to pre-pregnancy values.

Material and Methods

Placenta

Tissues: Term placenta was obtained from an uncomplicated normal pregnancy from a non-Fabry person to compare with the placenta from the Fabry patient. Tissue for immunohistochemical analyses was fixed in 4% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) by immersion for 2 h followed by further processing.

Antibodies: Polyclonal rabbit antihuman α-Gal A was kindly provided by Genzyme Corp. (Framingham, MA, USA). For immunohistochemical light microscopy analysis, visualization was performed using horse radish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (Dako, P044801-2, Glostrup, Denmark) (1:200).

Immunohistochemistry: Placental tissue for light microscopic investigations was dehydrated in graded alcohols and embedded in paraffin. Paraffin sections of 2 μm were cut on a Leica RM 2165 microtome (Leica, Ballerup, Denmark) and processed as previously described (Vinge et al. 2010). Briefly, sections were heated and placed in xylene overnight, prior to rehydration in graded alcohols. Rehydrated sections were heated in Tris-EGTA buffer for antigen retrieval in a microwave oven for approximately 20 min, cooled and permeabilized with 0.05% saponin (1% bovine serum albumin (BSA), 0.2% gelatine, 0.05% saponin in 0.01 M PBS) and blocked for endogenous peroxidase activity before incubation with primary antibodies. Sections were incubated with the primary antibody in 0.01 M PBS, 0.1% BSA and 0.02 M NaN_3, followed by incubation with HRP-conjugated secondary antibody. Peroxidase labelling was visualized by incubation with diaminobenzidine and 0.03% H_2O_2 for 10 min. Sections were counterstained with Meier's haematoxylin stain and examined in a Leica DMR (Leica, Wetzlar, Germany) microscope equipped with a Leica DFC320 camera (Leica, Wetzlar, Germany) and processed using Adobe Photoshop 8.0 software.

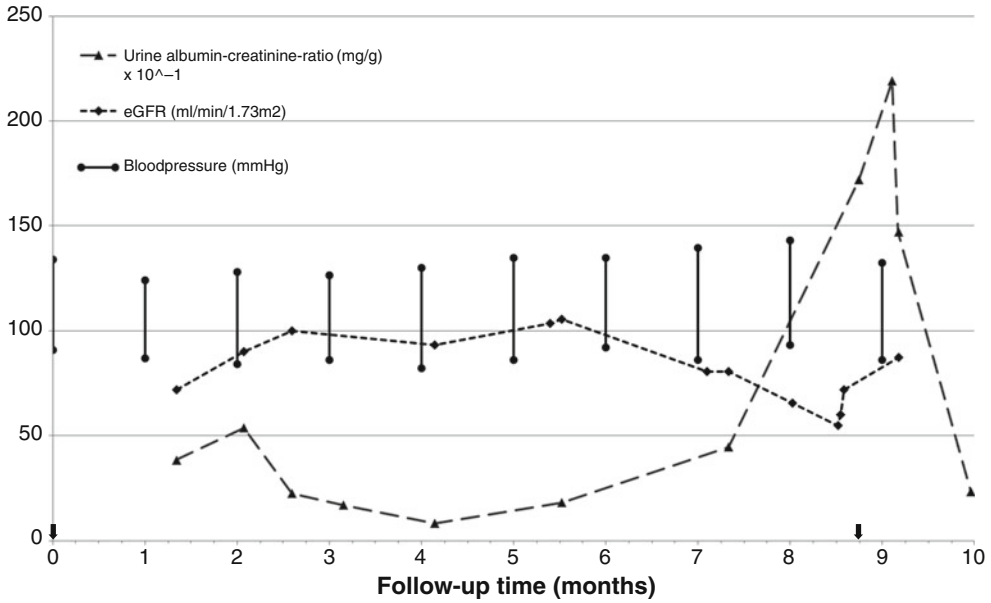

Fig. 1 Blood pressure, urine albumin-creatinine ratio and eGFR during pregnancy and 1 month after delivery; Systolic blood pressure (mmHg) (upper black dots), diastolic blood pressure (mmHg) (lower black dots); eGFR (mL/min/1.73 m^2) (punctuated black line); Urine albumin-to-creatinine ratio (mg/g) (punctuated black line with triangles); Arrows indicate start and end of pregnancy, respectively

Review of Literature

A literature search was done in PubMed using search criteria Fabry disease *AND* pregnancy *AND* live birth *AND*/*OR* placental histology and including only full papers (i.e. not abstracts) written in English language.

Results

Immunohistochemical detection of alpha-galactosidase A in human placenta showed a distinct granular labelling for α-galactosidase A in the foetal placenta including syncytiotrophoblast cells, in endothelial cells and in interstitial cells. There was no difference in labelling between the Fabry patient (Fig. 2a) and the control (Fig. 2b).

The literature review, and including our case, documented 13 cases of Fabry pregnancies resulting in live births, 6 of which had been treated with agalsidase-beta, 3 with agalsidase-alpha, and 4 without ERT (Tables 1, 2 and 3). All pregnancy outcomes were positive independent of ERT and genetic affection of the foetus. Histopathological examination of the placental tissue disclosed Gb-3 accumulation in the maternal side of the placenta in both ERT-treated and untreated mothers, while Gb-3 accumulation could be seen on both maternal and foetal side in two cases where the foetus had inherited the family mutation.

Discussion

ERT with agalsidase-alpha or agalsidase-beta is recommended for female patients with verified FD and symptoms. All ERT-treated females were symptomatic before pregnancy in the included cases. The pregnant FD females have followed general guidelines for treatment during pregnancy, although a closer monitoring has been preferred. ERT dosage of either agalsidase-alpha or agalsidase-beta has remained unchanged during pregnancy. Included cases have not presented complications related to ERT or negative effect of ERT on foetal outcome. However, in one ERT-treated case (8%), a foetus showed signs of growth restrain the last 2 weeks before birth, amniorrhexis occurred in week 36, and due to pathological cardiotocographic monitoring, acute Caesarean section was performed, delivering a healthy boy.

There are no cases describing progression of FD during pregnancy of patients receiving ERT. Contrarily, in one case (Kalkum et al. 2009, Case 1) there were described increasing acroparaesthesias and fatigue after discontinuation of ERT early in pregnancy, why ERT was re-introduced at week 14 (Kalkum et al. 2009). In another case (Kalkum et al. 2009, Case 2) the patient was treated solely with ERT, while carbamazepine and gabapentin (for neuropathic pain) and irbesartan (for hypertension) were discontinued according to general guidelines. Interestingly blood pressure

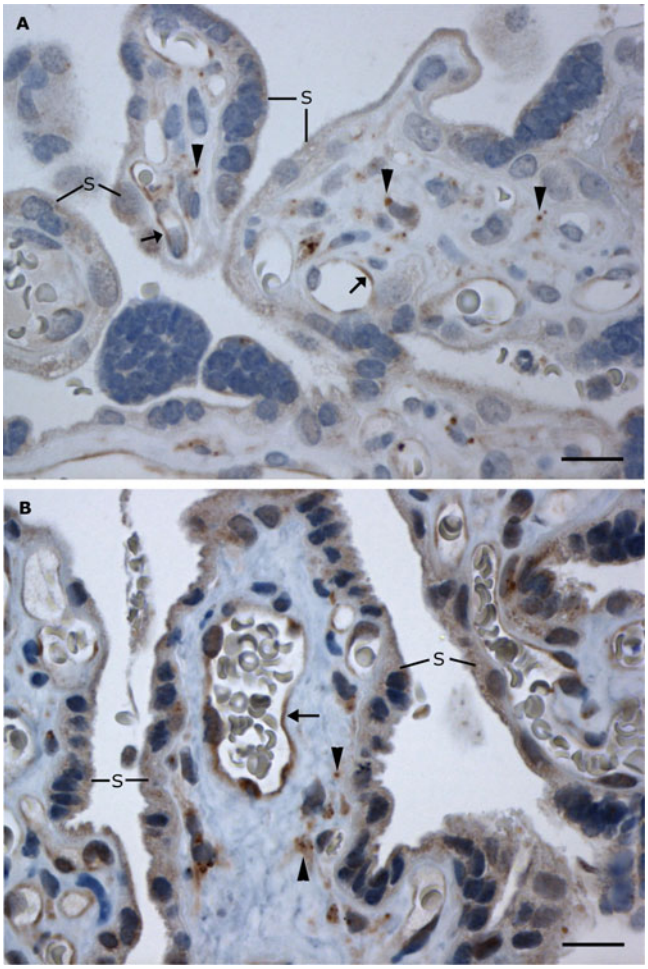

Fig. 2 Immunohistochemical labelling for α-galactosidase A in term placenta from the Fabry patient (**a**) and from control (**b**). Labelling (brown colour) was seen in vascular endothelial cells (arrows), in interstitial cells (arrowheads) and in syncytiotrophoblast cells (S). Bars, 20 μm

remained stable during pregnancy, and the patient did not experience neuropathic pain (Kalkum et al. 2009). In a case by Germain et al. (2010), the pregnant female had severe proteinuria and massive Gb-3 storage in podocytes as well as focal/segmental glomerulosclerosis prior to pregnancy. Treatment with ACE and ARB was discontinued as recommended, and patient continued ERT as monotherapy without worsening of proteinuria during pregnancy (Germain et al. 2010).

Data on complications during pregnancy of FD patients is scarce. One retrospective survey study reported data from 41 women (102 pregnancies) and found no life-threatening complications but a higher rate of proteinuria and hypertension, while pre-eclampsia, gestational diabetes, premature births, miscarriage and intrauterine death were not found more commonly in FD patients vs the general population (Holmes and Laney 2015). In the study 10% (4/41) of females were treated with ERT, and none had complications (Holmes and Laney 2015). Gestational diabetes is seen in 2–10% of the general population, and pre-eclampsia is seen in 3% of the general population (Holmes and Laney 2015). In the present study, a numerically higher percentage of pre-eclampsia was observed 8%, but sample size is small (1/13), and in a larger series with 102 FD pregnancies, 5% had pre-eclampsia (not significantly different from the general population). Holmes and Laney did not discuss severity and treatment of complications during pregnancy. The current case was treated with enalapril due to proteinuria prior to the pregnancy. As guidelines suggest, ACE inhibitors are to be avoided during pregnancy (Vest and Cho 2012), and the patient was successfully switched to labetalol. Furthermore, as pre-eclampsia developed, the current case was uptitrated in methyldopa according to guidelines of non-Fabry patients and was well-managed. The present review did not disclose life-threatening complications during pregnancy in FD patients.

Four published cases present data for FD pregnancies without ERT. In the first case (Bouwman et al. 2010, Case A), the patient was asymptomatic at age 39, with an uneventful pregnancy which resulted in a healthy boy without the family mutation and without any accumulation of Gb-3 in the placenta (Bouwman et al. 2010). In the second case (Vedder et al. 2006, Case A; Bouwman et al. 2010, Case C), a mildly affected 23-year-old female with acroparaesthesias as only symptom was not receiving ERT. Pregnancy and birth disclosed no complications, but the male infant inherited the family mutation, and there was seen Gb-3 accumulation in both the maternal and placental side of the placenta (Vedder et al. 2006; Bouwman et al. 2010). Parent et al. (2010) presented a symptomatic patient at age 36, with acroparaesthesias, abdominal cramp, diarrhoea, hypohidrosis, hearing loss and tinnitus. ERT was discontinued at gestational week 4 due to concerns of potential risks. Pregnancy and birth were uncomplicated, giving a healthy boy without the family mutation. Placental examination showed Gb-3 storage inclusion, predominantly on the maternal side. Interestingly, the patient reported diminished acroparaesthesias and gastrointestinal symptoms during pregnancy, which returned to pre-pregnancy intensity 6 weeks after delivery (Parent et al. 2010). In a case with a non-Fabry mother (having a child with a hemizygote FD father) giving an obligate carrier infant girl, pregnancy was uncomplicated. Birth was complicated by foetal distress leading to vacuum extraction, but a healthy girl was delivered. No Gb-3 storage was seen in the placenta (Vedder et al. 2006).

In summary, in non-ERT patients ($n = 4$), no progression or complications were observed during pregnancy, while the ERT patients ($n = 9$) did not disclose progression of FD, but two pregnancies were complicated by gestational diabetes and hyperthyroidism and pre-eclampsia.

Table 1 Literature review of cases of live births from pregnancies in Fabry disease, related to treatment with enzyme replacement therapy, severity of disease in the mother, genotype, pregnancy and foetal outcome and histopathological examination of the placenta – according to gene mutation inheritance – foetus without Fabry disease

Author	Age (years)	ERT before and during pregnancy	Fabry signs and symptoms	Maternal mutation	Disease progression and/or complications during pregnancy	Delivery/foetal outcome	Foetal gene mutation status and enzyme activity	Placental histopathological examination
Wendt et al. (2005)	34	18 months before and throughout pregnancy[a]	Acroparaesthesias Abdominal cramps Diarrhoea Fatigue Proteinuria (mild; 0.5 g/day mg/day)	ND	ND	Vaginal delivery (week 37) Healthy boy	No mutation Normal enzyme activity	ND
Germain et al. (2010)	21	2 months before and throughout pregnancy[b]	Neuropathic pain Diarrhoea Anhidrosis Lower limb oedema Proteinuria (+++; 4.87 g/day) Severe kidney involvement	C52R	No progression Angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers were discontinued	Vaginal delivery (week 38) Healthy boy	No mutation Normal enzyme activity	ND
Bouwman et al. (2010) (Case A)	39	None	None	F18S	No progression	Vaginal delivery Healthy boy	No mutation Enzyme activity ND	No Gb-3 accumulation
Parent et al. (2010)	36	2 years before[b] Discontinued at week 4	Acroparaesthesias Abdominal cramps Diarrhoea Hypohidrosis Tinnitus Hearing loss	C56X	No progression Diminished acroparaesthesias and gastrointestinal symptoms during pregnancy. Intensity of symptoms returned to pre-pregnancy state 6 weeks after delivery	Vaginal delivery (week 38) Healthy boy	No mutation Enzyme activity ND	Inclusions in vascular smooth muscle, endothelial, pericytes of umbilical arteries and veins, chorionic villi mainly of maternal side
Present case	38	15 years before and throughout pregnancy[b]	Hypertension Ischemic stroke (age 23) Proteinuria (0.5 g/day) Mild reduction of renal function (mGFR 60 mL/min/1.73 cm^2)	G85N	No progression of FD Developed moderately-severe pre-eclampsia in third trimester Developed mild haemolysis, slightly elevated liver enzymes and platelets low in the normal range Enalapril (for hypertension) was switched to labetalol 100 mg × 3/day Acetylic acid (75 mg/day for stroke prophylaxis) was continued	Caesarean section (week 38) Healthy boy	No mutation Enzyme activity ND	No Gb-3 accumulation

ND not defined
[a] Agalsidase-alpha
[b] Agalsidase-beta

Table 2 Literature review of cases of live births from pregnancies in Fabry disease, related to treatment with enzyme replacement therapy, severity of disease in the mother, genotype, pregnancy and foetal outcome and histopathological examination of the placenta – according to gene mutation inheritance – foetus with Fabry disease

Author	Age (years)	ERT before and during pregnancy	Fabry signs and symptoms	Maternal mutation	Disease progression and/or complications during pregnancy	Delivery/foetal outcome	Foetal gene mutation status and enzyme activity	Placental histopathological examination
Vedder et al. (2006) (Case A) Bouwman et al. (2010) (Case C)	23	None	Acroparaesthesias	Y134M	No progression	Vaginal delivery Healthy boy	Inherited mutation Abnormal enzyme activity	Maternal- and foetal placental and umbilical cord Gb-3 accumulation
Vedder et al. (2006) (Case B)	ND	None	None	None (paternal mutation, hemizygote, N298S)	Uneventful	Vaginal delivery, assisted by vacuum extraction due to foetal distress Healthy girl	Obligate carrier Abnormal enzyme activity	No Gb-3 accumulation
Kalkum et al. (2009) (Case 2)	38	4 years before and throughout pregancy[a]	Acroparaesthesias Abdominal pain	N320I	No progression Discontinuation of carbamazepine and gabapentin (for neuropathic pain) with reoccurrence of pain, except headache at week 19, which was treated with paracetamol Discontinuation of irbesartan (for hypertension); blood pressure remained stabile	Vaginal delivery (week 40) Healthy girl with small haemangioma over medial spine	Inherited mutation Enzyme activity ND	ND
Bouwman et al. (2010) (Case B)	24	3 years before and throughout pregnancy[b]	Acroparaesthesias Proteinuria (+; 0.7 g/day) Multiple white matter lesions	R310X	No progression	Vaginal delivery Healthy girl	Inherited mutation Enzyme activity ND	No Gb-3 accumulation Smooth muscles of umbilical cord with typical inclusion bodies
Politei (2010) and Thurberg and Politei (2012)	37	2 years before and throughout pregnancy[b]	Acroparaesthesias Angiokeratomas Cornea verticillata Syncopes Fatigue	L415P	No progression	Vaginal delivery (week 38) Healthy boy	Inherited mutation Enzyme activity ND	Maternal- and foetal placental Gb-3 accumulation (extensive) in many cell types

ND not defined
[a] Agalsidase-alpha
[b] Agalsidase-beta

Table 3 Literature review of cases of live births from pregnancies in Fabry disease, related to treatment with enzyme replacement therapy, severity of disease in the mother, genotype, pregnancy and foetal outcome and histopathological examination of the placenta – according to gene mutation inheritance – genetic testing not performed

Author	Age (years)	ERT before and during pregnancy	Fabry signs and symptoms	Maternal mutation	Disease progression and/or complications during pregnancy	Delivery/foetal outcome	Foetal gene mutation status and enzyme activity	Placental histopathological examination
Kalkum et al. (2009) (Case 1)	33	3 years before Paused during first trimester. Resumed at week 14[a] due to recurrence of acroparaesthesias and fatigue	Acroparaesthesias Hypohydrosis Angiokeratoma Migraine Tinnitus depression Proteinuria	A143T	Pregnancy complicated by hyperthyroidism and gestational diabetes (insulin treated)	Caesarean section (week 36) due to amniorrhexis and pathological cardiotocographic monitoring Healthy boy	Mutation ND Normal enzyme activity	ND
Tasci and Bicik (2015) (Case 1)	26	No ERT before. Initiated at week 8[b]	Cornea verticillata Carotic stenosis Proteinuria (0.1 g/day)	L275F	No progression Regression of proteinuria	Vaginal delivery (week 40) Healthy girl	Mutation ND Enzyme activity ND	ND
Tasci and Bicik (2015) (Case 2)	29	2 months before and throughout pregnancy[b]	Angiokeratoma Acroparaesthesias Fatigue Proteinuria (0.3 g/day)	L275F	No progression	Vaginal delivery (week 40) Healthy girl	Mutation ND Enzyme activity ND	ND

ND not defined
[a] Agalsidase-alpha
[b] Agalsidase-beta

Direct comparison between ERT- and non-ERT-treated females will be difficult as there is bias by indication as ERT-treated females per indication are (more) symptomatic. Gb-3 storage was seen in the placenta of one female receiving ERT on both the maternal and foetal side (the foetus had inherited the family mutation). In pregnancy of FD females without ERT, Gb-3 storage was seen in the placenta, at least in the two symptomatic patients, and storage on the foetal side was seen if the foetus had inherited the family mutation. In the placenta of the obligate carrier female, no storage was seen which might be due to residual endogenous enzyme production.

That Gb-3 accumulation can occur prenatally was shown in 1985 in a 22-week foetus (Tsutsumi et al. 1985), where the cornea was examined biochemically and histopathologically. The α-galactosidase activity in the cornea was very low compared with that of the normal control. Histopathological examination demonstrated presence of intracytoplasmic lamellar bodies surrounded by a single membrane in the epithelial cells. The lamellar bodies were thought to result from abnormal accumulation of ceramide trihexoside. It was concluded that ceramide trihexoside had already begun to accumulate in the epithelial cells in mid-trimester gestation (Tsutsumi et al. 1985). As discussed by Bouwman et al. (2010), the accumulation of Gb-3 in placental tissue differs as is seen in the included cases. The pathophysiology is still not fully elaborated, but accumulation seems to depend on both maternal and foetal characteristics, e.g. disease status, severity, enzyme activity levels and ERT (Bouwman et al. 2010). It is still unknown if ERT can cross the placental barrier and clear foetal placental tissue from Gb-3. Accumulation of Gb-3 has been described in both maternal and foetal placental tissue despite ERT treatment in one case (Thurberg and Politei 2012). The present case cannot contribute to answer this question as the foetus was unaffected and therefore had endogenous enzyme production.

The present case represents the sixth case of agalsidase-beta treatment during pregnancy and brings the total of ERT-treated pregnancies to nine and confirms as shown earlier (Bouwman et al. 2010; Germain et al. 2010; Politei 2010; Tasci and Bicik 2015; Kalkum et al. 2009; Parent et al. 2010) that the treatment seems to be safe for mother and foetus throughout pregnancy.

ERT is used in comparable rare lysosomal storage disorders, e.g. Pompe disease, mucopolysaccharidoses (MPS) and Gaucher disease. However, as in FD, ERT is not approved during pregnancy due to insufficient data. Kłos et al. (2017) published a review on ERT during pregnancy of Pompe disease patients ($n = 5$) and found it to be seemingly safe with no complications for neither mother nor infant. Contrarily, in one patient there was observed disease progression, while ERT was withheld during pregnancy (Kłos et al. 2017). A recent case series of MPS patients during pregnancy showed a general tendency for both pregnancy and delivery being of high-risk (Stewart et al. 2016). ERT was given in four cases with no adverse effect. All infants developed normally regardless of ERT status during pregnancy (Stewart et al. 2016). In Gaucher disease, ERT during pregnancy has not yet been found associated with any adverse effects (Elstein et al. 2004). Outcomes were similar for ERT-treated and untreated females, and all included infants were healthy and developed according to expectations. The study emphasized that Gaucher females who need ERT should be continued during pregnancy (Elstein et al. 2004).

The present review is the first to put together all clinical information on published Fabry pregnancies. Our case has emphasized that even in a severely affected female with FD combined with severe complication to pregnancy, safe and successful outcome is possible. However, strict monitoring is recommended. Furthermore, neither maternal disease severity nor treatment with ERT seems to affect foetal outcome if the patient is well-managed. General guidelines of treating pre-eclampsia and other pregnancy-related complications should be followed.

Regulatory authorities advise against ERT during pregnancy in FD as well as other rare lysosomal storage diseases. Controlled clinical trials or even large single-centre cohort presentations are unlikely to be performed on this rare topic; hence, documentation of treatment and outcome in all individual patients with FD during pregnancy is important. Interestingly, the medical community seems to have chosen ERT during pregnancy despite the warning from regulatory authorities. It seems to be time to reconsider whether it is prudent and due time to change the advice against ERT in Fabry pregnancies, since no adverse pregnancy outcomes have been published.

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Contribution of Individual Authors

EIC, UFR and CVM were involved in the conception and design of the paper. Data collection, analysis and interpretation

were performed by EIC, RN, HM and CVM. The manuscript was drafted by EIC, UFR and CVM, while all authors have performed critical revision for important intellectual content.

Take-Home Message

ERT was continued during pregnancy in a severely affected female with Fabry disease and complications during pregnancy, in keeping with nine cases in the literature, resulting in a live birth: why ERT during pregnancy in Fabry disease should be considered safe and recommendations, which currently advise against ERT, should be changed.

Conflicts of Interest

CVM has received an unrestricted research grant from Genzyme. UF-R has received speaker honoraria, unrestricted research grants and appeared in advisory boards of Genzyme, Shire and Amicus and ad hoc advice to Protalix and Freeline. Erik Christensen, Rikke Nielsen, Helle Mogensen and Åse Rasmussen declare that they have no conflict of interest.

Details of Ethics Approval and Patient Statement of Consent

All material was collected with informed patient consent and was approved by the Danish National Committee on Health Research Ethics (approval no. KF 20060063). Informed consent for publication of data has been given by the patient.

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RESEARCH REPORT

Hyperornithinemia, Hyperammonemia, and Homocitrullinuria Syndrome Causing Severe Neonatal Hyperammonemia

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Abstract Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (OMIM 238970) is an autosomal recessive disorder that is caused by a deficiency of mitochondrial ornithine transporter 1, resulting in dysfunction of the urea cycle. HHH is the rarest of the urea cycle disorders, reported in fewer than 100 patients. It is characterized by extreme phenotypic variability, including diverse ages of onset and severity of phenotype. We report the first confirmed instance of HHH syndrome in a premature infant (31 2/7 weeks) with severe hyperammonemia (1,300 μmol/L).

This case highlights the importance of considering HHH in the differential diagnosis for neonatal hyperammonemia. Because HHH is not detected by newborn screening, and the characteristic biochemical triad may be subtle or even absent, it has the potential to be underdiagnosed; however, making the diagnosis has critical therapeutic implications as treatment is distinct from other urea cycle defects. For instance, lysine supplementation is a beneficial treatment unique to HHH. Therefore, we present here a review of previously reported cases in order to demonstrate the full spectrum of the disease and highlight potentially diagnostic features.

Abbreviations

ASA	Argininosuccinate
CPS I	Carbamoyl phosphate synthetase I
CSF	Cerebrospinal fluid
DOL	Day of life
HHH	Hyperornithinemia-hyperammonemia-homocitrullinuria
INR	International normalized ratio
LP	Lumbar puncture
OTC	Ornithine transcarbamylase
TPN	Total parenteral nutrition

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Introduction

The urea cycle, which converts ammonia to urea, is the major pathway for disposal of waste nitrogen. Functioning of the cycle involves the tightly regulated action of five enzymatic steps as well as membrane transporters which assure smooth integration of the mitochondrial and cytoplasmic components of the cycle. Mutations in either the constituent enzymes or the transporters can result in urea cycle defects, the clinical severity of which depends in part upon the completeness of the functional alteration. Urea cycle defects can present at virtually any age. Often an environmental stressor (typically infection) evokes hyperammonemia and clinical symptomatology by overwhelming an already compromised urea cycle (Lee et al. 2014).

Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome (mitochondrial ornithine transporter 1 deficiency) is an autosomal recessive disorder characterized by a failure of mitochondrial ornithine uptake (ORNT1/SLC25A15) and consequent inadequate supply of this amino acid to the mitochondrial ornithine transcarbamylase (OTC) reaction. The result is hyperammonemia and the accumulation of ornithine and lysine in the cytoplasm (Kim et al. 2012). Homocitrulline is thought to form from carbamylation of the increased cytoplasmic lysine pool.

Patients manifest protein intolerance, episodic vomiting, growth failure, hepatomegaly, liver failure, and neurologic manifestations, including altered consciousness, seizures, pyramidal tract signs, and a variable degree of cognitive impairment with or without behavior problems. However, the clinical presentation can be highly variable (Lee et al. 2014; Lemay et al. 1992; Al-Hassnan et al. 2008; Gatfield et al. 1975).

Clinical Case Report

We present a male neonate born to a 27-year-old G2P0011 mother. Delivery was via Cesarean section at 31 2/7 weeks gestation due to maternal preeclampsia. Birth weight was 1.385 kg and the APGAR scores were 8 and 9. The mother received regular prenatal care. The patient was born to healthy non-consanguineous parents. The mother had electively terminated a prior pregnancy when she was 18 years. Family history was unremarkable with regard to intellectual disability, metabolic disease, dietary protein intolerance, or miscarriages. Both the mother and maternal grandmother described migraine headaches in association with menstruation. Prenatal laboratory tests and fetal ultrasounds were normal. Following delivery, he manifested periodic breathing that was treated with oxygen via nasal cannula and continuous positive airway pressure (CPAP) 6. He received a single dose of surfactant. On day of life (DOL) 4, worsening apnea and lethargy obliged intubation and controlled ventilation. A septic workup, including lumbar puncture, was performed because of deteriorating mental status and worsening apnea. Antibiotic therapy was initiated. Blood, urine, and CSF cultures were all negative. He initially received parenteral nutrition, but this was stopped when hyperammonemia (1,300 μmol/L) was discovered on DOL 5. The next day he was transferred to the Children's Hospital of Philadelphia, where the initial blood ammonia was 623 μmol/L and treatment was started with intravenous acylation therapy (sodium phenylbutyrate and sodium benzoate; Ammonul) and intravenous arginine. Hyperammonemia rapidly resolved, and by DOL 9 he was successfully transitioned to oral therapy with arginine and sodium phenylbutyrate.

Although the patient's ammonia levels quickly normalized with medical management, he remained critically ill and required dopamine and norepinephrine infusions for approximately 2 weeks. He had severe hepatic dysfunction and a coagulopathy reflected in an international normalized ratio (INR) that peaked at 2.31, prompting daily administration of vitamin K as well as multiple transfusions of fresh plasma, packed red cells, platelets, and cryoprecipitate for the first week of life. Neither multiple transfusions nor treatment with parenteral infusions of protein evoked hyperammonemia in our patient after his ammonia normalized. He also briefly required hydrocortisone for cardiovascular support, but steroid therapy did not cause recurrent hyperammonemia. Protein (0.25 g/day) was reintroduced into his TPN on DOL 9. His clinical picture was also notable for findings not associated with OTC deficiency such as small bilateral pleural effusions, a pericardial effusion, ascites, and generalized edema.

The Pennsylvania state newborn screening test was normal; detailed evaluation showed slight increases of phenylalanine and methionine. Urine organic acid quantitation and the blood acylcarnitine profile were normal. He never became acidotic. Blood lactate and pyruvate were not significantly elevated. Carbohydrate-deficient transferrin analysis was not suggestive of a congenital disorder of glycosylation.

Plasma amino acid quantitation, initially obtained while the baby received parenteral nutrition, showed increased glutamine, ornithine, and citrulline. The glutamine/citrulline ratio was approximately 20 – in the expected range for OTC deficiency. The elevated ornithine was originally attributed to arginine supplementation; however, a repeat blood aminogram showed even more highly elevated ornithine (315 μmol/L; normal 26–164 μmol/L) and low blood lysine (56 μmol/L; normal 67–230 μmol/L) and relatively low citrulline (5.8 μmol/L; normal 0–35 μmol/L) (Fig. 1). Enteral lysine supplementation was initiated, and the infant was transitioned from arginine to citrulline. Urine orotic acid was initially (DOL 7) high at 12.5 mmol/mol creatinine (normal <4 mmol/mol creatinine); however this declined to a normal concentration after a few days (Table 1). Urine amino acid quantitation initially showed a generalized aminoaciduria, consistent with prematurity, but repeat analysis showed a disproportionate increase of urine ornithine. Urine homocitrulline was qualitatively present on retrospective analysis, but the concentration was within the "noise" of the test so was not initially remarked upon.

Single nucleotide polymorphism (SNP) chromosomal microarray was not consistent with the Xp11.4 gene deletion syndrome that includes a loss of ornithine transcarbamylase (Deardorff et al. 2008). The karyotype was a normal male, 46 XY without regions of homozygosity.

A next-generation sequencing urea cycle disorder panel (GeneDx, Maryland) showed two heterozygous pathogenic variants in the *SLC25A15* gene (c.22 C>T with variant p.Gln8Ter (Q8X) and c.337 G>A with variant p.Gly113Ser (G113S)), which encodes the mitochondrial ornithine transporter, consistent with HHH syndrome. No other mutations were found.

Methods

For all standard runs, amino acids were analyzed using ultra-performance liquid chromatography, as described

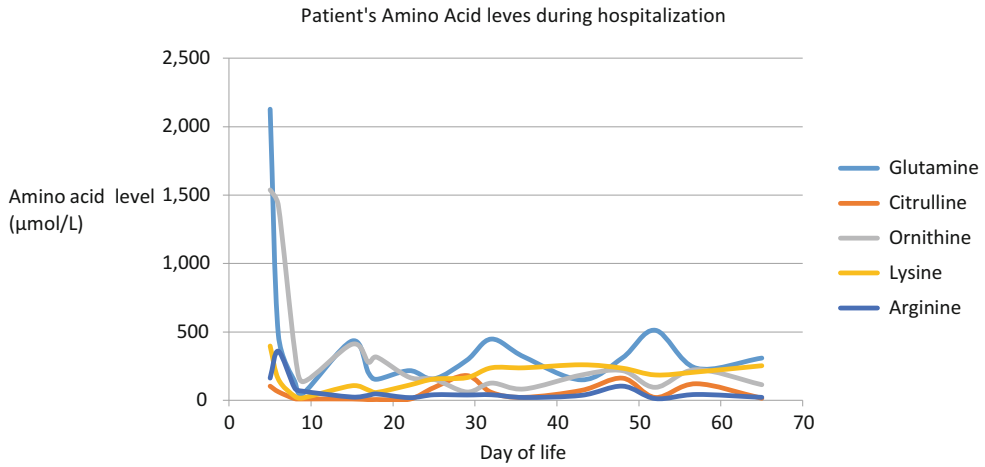

Fig. 1 Patient's plasma amino acid levels

Table 1 Patients orotic acid levels

Amino acid	Day of life	Level	Lab range
Orotic acid	DOL 2	12.49 mmol/mol Cr	2.27–4.13 mmol/mol Cr
Orotic acid	DOL 17	1.75 mmol/mol Cr	1.15–3.09 mmol/mol Cr

previously (Narayan et al. 2011). A standard column temperature of 43°C was used.

Discussion

HHH syndrome is an autosomal recessive disorder characterized by reduced ammonia clearance through the urea cycle due to mitochondrial ornithine deficiency. The basic defect is at the level of the mitochondrial ornithine transporter (ORNT1/SLC25A15), which has been mapped to chromosome 13q14. The clinical phenotype comprises protein intolerance, episodic vomiting, growth failure, hepatomegaly, and neurologic manifestations, including altered consciousness, seizures, pyramidal tract signs, and a variable degree of cognitive impairment with or without aberrant behaviors (Kim et al. 2012).

Impaired ornithine transport across the mitochondrial membrane causes ornithine accumulation in the cytoplasm and intramitochondrial ornithine deficiency. In four cases reported by Kim et al., patients all had elevated ornithine (200–1,400 µmol/L) and ammonia levels (up to 844 µmol/L) in blood and homocitrulline (~200 µmol/L) in urine (Kim et al. 2012). Our patient's initial presentation was more severe with a blood ammonia of >1,300 µmol/L. He also had hepatic dysfunction that improved in tandem with resolution of the hyperammonemia. Our patient's presentation is compared to other patients with HHH who presented in the neonatal period in Table 2 below.

Since the initial description by Shih et al., fewer than 100 patients with HHH syndrome have been reported (Shih et al. 1969; Sokoro et al. 2010). Most ethnic groups are represented, and there is no common mutation apart from a founder effect in the French-Canadian population of Quebec (Gatfield et al. 1975; Tessa et al. 2009).

The initial presentation and time of diagnosis in HHH is highly variable. In a retrospective review of 54 patients, there were 12 patients with a neonatal presentation (birth to 1 month), 13 with an infantile presentation (>1 month to 1 year), 24 with a childhood onset (>1 year to 12 years), and 5 with an adolescent to adult onset (>12 years). Only one patient was identified by newborn screen. Patients typically present acutely with intermittent hyperammonemia accompanied by vomiting, ataxia, lethargy, confusion, and coma, findings common to other urea cycle disorders. Some HHH patients have presented with fulminant liver failure (Fecarotta et al. 2006; Martinelli et al. 2015).

In most instances, HHH syndrome involves a chronic, progressive disease with protein intolerance, coagulation abnormalities, hypotonia, developmental delay, progressive encephalopathy with mental regression, and early signs of motor dysfunction. The vast majority of patients respond well to dietary and pharmacological therapy with a protein-limited diet and chronic ammonia scavenging therapy. The disorder is rarely lethal, with about 95% of patients surviving after diagnosis. By early adulthood, most patients develop signs of pyramidal tract dysfunction, often

Table 2 Comparison of patients with HHH who presented in the neonatal period

Age at presentation	Sex	Lethargy	Seizures	Elevated, AST/ALT	Coagulopathy	Ammonia (μmol/L)	Ornithine (μmol/L)	Mutation(s)
DOL 4[a]	M	+	–	+	+	1,300	1,538	p.Q8Ter, p.G113S
1 month[b]	M	+	–	NA	+	400	370	p.S175fsX192, p.L283F
2 months[b]	M	+	–	+	–	NA	NA	p.L71Q, p.L71Q
0–2 years[c]	F	+	+	–	–	NA	689	NA
Birth[c]	F	+	–	+	+	NA	618	p.G27R, p.G27R
Birth[c]	M	+	+	+	–	NA	NA	p.S90, p.S90
Birth[c,d]	M	+	NA	+	NA	NA	1915	NA
Birth[c]	M	+	+	–	–	NA	595	p.G27R, p.Y55
Birth[c]	NA	+	NA	NA	NA	NA	NA	p.A15E, p.A15E
Birth[c]	M	+	+	NA	NA	NA	NA	p.F188del, p.G190D
Birth[c]	M	NA	NA	NA	NA	NA	616	NA
Birth[c,d]	F	NA	–	–	–	NA	NA	p.L71Q, p.L71Q
Birth[c]	M	NA	–	–	–	NA	887	p.K245, p.K245
Birth[c]	F	+	+	–	+	NA	509	p.R179, p.R179
Birth[c]	F	+	–	–	–	NA	290	p.R179, p.R179
Birth[c]	F	+	NA	–	+	NA	370	p.S175fsX192, p.L283F

M male, *F* female, *NA* not available
[a] Our patient
[b] Tessa et al. (2009)
[c] Martinelli et al. (2015)
[d] Deceased

evolving into frank spastic paraparesis and lower limb stiffness. However, cognitive function is relatively preserved. Waisbren et al. found that in seven patients with HHH, IQs ranged 70–107 (Waisbren et al. 2016). Virtually all survivors had developmental disabilities that correlated with the number, severity, and duration of episodes of hyperammonemia (Msall et al. 1984, 1988).

Because HHH is a relatively rare urea cycle disorder that can be missed on newborn screen and has developmental outcomes that correlate with the number, severity, and duration of episodes of hyperammonemia, it is important to consider it in a differential diagnosis of any patient with hyperammonemia of unknown etiology. HHH also has the potential to be underdiagnosed since the classic biochemical triad may also be subtle or even absent. To our knowledge, this is the first instance of HHH in a premature neonate and illustrates the variability of the syndrome, including severe cardiovascular instability.

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Details of Ethics Approval

Ethics approval was not required for this study.

Conflicts of Interest

None of the authors have any conflicts of interest to disclose.

Synopsis

Hyperornithinemia-hyperammonemia-homocitrullinuria is an important consideration in the evaluation of a neonate with hyperammonemia, even if the characteristic biochemical triad is not yet apparent.

Compliance with Ethics Guidelines

Conflict of Interest

The authors each declare that they have no conflict of interest.

Details of the contributions of individual authors: KTW performed clinical and biochemical evaluation of the patient and conceived and wrote the manuscript. RDG performed clinical and biochemical evaluation of the patient, conceived the manuscript, and provided oversight. MY performed clinical and biochemical evaluation of the patient and edited and conceived the manuscript. LIC performed clinical evaluation of the patient and edited and conceived the manuscript.

Ethics approval was not required for this study.

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RESEARCH REPORT

Screening for Niemann-Pick Type C Disease in a Memory Clinic Cohort

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Abstract Niemann-Pick type C disease (NPC) is a neurovisceral lysosomal storage disorder with a heterogeneous phenotype including ataxia, cognitive impairment, impairment of vertical saccades, and psychiatric symptoms, among many others. Based on clinical, genetic, and biomarker findings, recent guidelines put forward a screening for atypical and oligosymptomatic forms of NPC in clinical niches with an increased risk. Here, we report methods and results of a negative screening study in the niche of a memory clinic. We retrospectively and prospectively identified 83 patients with unclassified cognitive impairment (15 dementia, 46 mild cognitive impairment, and 22 progressive subjective cognitive decline) before 60 years of age (82 patients between 41 and 60 years). We explored the prevalence of clinical features compatible with NPC and measured plasma levels of chitotriosidase and cholestantriol. The NPC suspicion index indicated high probability for NPC in 3 and moderate probability in 16 patients. Prevalent (>5%) neurological and psychiatric features were depression, seizures, ataxia, dysarthria, and psychotic symptoms. Vertical gaze palsy without parkinsonism was observed in one patient. Cholestantriol levels were only abnormal in one patient. Chitotriosidase levels were susceptible to slight elevations that were reproducible in only two of five patients. Our study does not exclude NPC among memory clinic patients. Instead, we suggest conducting prospective screening studies in younger cohorts that include a focused neurological examination. Excluding minor cognitive impairment and discarding depression as an independent disease symptom probably further improve screening effectivity but may delay or miss therapeutic options in early or mild disease.

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Introduction

Niemann-Pick disease type C (NPC) is a rare autosomal recessive lysosomal lipid storage disorder caused by mutations in NPC1 (OMIM #257220) or NPC2 (OMIM #607625) in 95% and about 4% of cases, respectively (Patterson et al. 2017). The spectrum of this neurovisceral disease ranges from organomegaly and a developmental delay in infants to a neurodegenerative phenotype with adult onset (Vanier 2010). Among other symptoms, especially ataxia and impaired vertical saccades, the latter includes psychiatric symptoms in more than 40% and progressive cognitive impairment in more than 60% of cases (Sevin et al. 2007). Although none of these symptoms is specific, the assessment of patient history and clinical signs, for example, by means of the NPC suspicion index (NPC-SI), may help to identify clinical patterns suggestive of classical NPC (Wijburg et al. 2012).

Recently, large exome datasets indicated that NPC prevalence could be as high as 1:19,000 because of the frequency of NPC1 gene variants that can cause late-onset disease with oligosymptomatic phenotypes (Wassif et al. 2016).

Therefore, patients with NPC may present without characteristic patterns of symptoms, and probably later than the classical onset before 40 years of age. The detection of these patients has been specifically addressed in updated guidelines (Patterson et al. 2017). Based on progress in the development of biochemical biomarkers and genetic tests, these guidelines recommend a biomarker or genetic screening among patients in clinical niches that may be associated with an increased risk of NPC (Vanier et al. 2016). This has, for example, identified patients with NPC in a cohort with unexplained ataxia (Schicks et al. 2013).

In the present study, we hypothesized that NPC may be more common than expected in the niche of patients with young onset dementia, which is remarkably prevalent (50–60:100,000) and diagnostically challenging (Rossor et al. 2010). We prospectively and retrospectively screened patients up to 60 years of age that presented to an academic memory clinic with variable degrees of unexplained cognitive decline. Although no patient was identified, we share our experience including the prevalence of symptoms of the NPC spectrum and the results of biomarker measurements in such a cohort. Importantly, we discuss optimized screening strategies that will probably facilitate the detection of NPC in this clinical niche in future studies.

Methods

From a local registry with 1,811 patients that presented to the memory clinic of the University Hospital of Bonn, Germany, between January 2011 and December 2015, we retrospectively identified 350 patients up to 60 years of age without classified diagnosis. With approval of the local ethics committee (279/10), these patients were contacted with a letter that informed about rare metabolic causes of cognitive impairment and offered further information and diagnostic work-up upon request. Via telephone interview with 144 patients (41%) that responded, a physician obtained a detailed history, family history, and necessary information to determine the NPC-SI in conjunction with data and a neurological examination extracted from the patient record (Wijburg et al. 2012). Testing for plasma chitotriosidase and cholestantriol (cholestane-3β,5α,6β-triol, gas chromatography-mass spectrometry method) levels was performed when NPC was considered possible based on clinical grounds (Reunert et al. 2015). This included 45 patients with dementia or mild cognitive impairment of unknown cause, mild cognitive impairment with concomitant depression (refractory to treatment or without apparent extrinsic factors), and progressive subjective cognitive decline. All degrees of memory complaint were rated as regression, i.e., presenile cognitive decline in the NPC-SI. Based on the same criteria, we prospectively identified and tested 38 patients with possible NPC between January and July 2016. All patients with mild cognitive impairment or dementia had at least routine blood tests (including blood count, creatinine, liver enzymes, folic acid, vitamin B12, and thyroid stimulating hormone), neuropsychological screening tests (Mini-Mental State Examination (MMSE) or others), and brain imaging. Genetic testing for NPC was performed in patients with elevated biomarker levels in two consecutive samples.

Results

A diagnosis of NPC was considered in 83 patients (36 male), including 45 and 38 patients in the retrospective and prospective group, respectively. The mean age of the cohort was 53.3 years; all except one patient (27 years) was 41 years or older. Twenty-two patients (27%) had subjective cognitive decline, 46 (55%) had mild cognitive impairment, and 15 patients (18%) were diagnosed with dementia of unknown cause. Data of MMSE was available in 75 patients (90%) and was, on average, 29.1, 26.0, and 19.7, respectively.

Beside cognitive impairment, we observed both neurological and psychiatric features of the NPC phenotype. Of note, 39 patients (47%) had at least mild depression. Other psychiatric diagnoses were psychosis (hallucinations or delusions), adult attention deficit hyperactivity disorder (ADHD), anxiety disorder, and bipolar disorder (Table 1). Neurological signs or symptoms were seizures, ataxia, dysarthria, and myoclonus. One case each was identified with vertical gaze palsy (without parkinsonism), dystonia, or spasticity.

Table 2 provides a summary of patients with either positive plasma biomarkers or at least moderate disease probability according to the NPC-SI. With a range between 20 and 80, the NPC-SI indicated low, moderate, and high probability (<40, 40–69, ≥ 70) in 64, 16, and 3 patients, respectively, when all degrees of cognitive impairment were rated as presenile cognitive decline. Regarding biomarkers, levels of cholestantriol were elevated in two consecutive measurements in one patient with a high-probability NPC-SI. Levels of chitotriosidase were elevated in five patients but confirmed in a second measurement in only two patients with an NPC-SI indicating low probability. Genetic testing for NPC was performed, but negative for both alleles in all three patients with elevated biomarker levels. Based on our cohort of 83 patients, the 95% confidence interval of the prevalence estimate for NPC was 0–4.4/100 patients (Wilson interval).

Clinical follow-up of more than 12 months was available in only two of five patients with high-probability NPC-SI and/or elevated plasma biomarkers. Chronic valproate intoxication was diagnosed in the patient with negative biomarkers, but the highest NPC-SI. In one patient

Table 1 Prevalence of features related to the NPC phenotype

Clinical feature	*N* (%)
Depression	39 (47)
Seizures	6 (7)
Ataxia	5 (6)
Dysarthria	4 (5)
Psychosis	4 (5)
Adult ADHD	4 (5)
Anxiety	3 (4)
Myoclonus	2 (2)
Vertical gaze palsy	1 (1)
Bipolar disorder	1 (1)
Dystonia	1 (1)
Spasticity	1 (1)

ADHD attention deficit hyperactivity disorder

Table 2 Summary of patients with at least intermediate risk NPC-SI or elevated biomarkers

Age	Sex	COG	MMSE	NPC-SI	CHO	CHI	Additional clinical features
41	M	MCI	n.a.	81	–	–	Seizures, vertical gaze palsy
60	M	DEM	28	80	–	–	Vertical gaze palsy without parkinsonism
60	F	MCI	26	80	+	–	Ataxia, dysarthria
60	M	MCI	29	67	–	(+)	Ataxia, depression, developmental delay, psychosis
44	F	SCD	30	65	–	(+)	Ataxia, depression, anxiety
54	M	DEM	26	65	–	–	Ataxia, dysarthria, depression
42	F	MCI	n.a.	57	–	–	Ataxia, depression, developmental delay
57	M	MCI	28	55	–	–	Ataxia, depressions
43	F	MCI	n.a.	55	–	–	Spasticity, depression
57	F	DEM	n.a.	51	–	–	Dysarthria, myoclonus
58	M	DEM	14	51	–	–	Ataxia, seizures
49	F	SCD	29	50	–	–	Dystonia, depression
54	M	SCD	29	50	–	–	Dysarthria, depression
49	F	MCI	27	50	–	–	Dysarthria, depression
48	F	DEM	19	47	–	–	Developmental delay, seizures, depression
56	F	SCD	27	46	–	–	Seizures, depression
59	M	MCI	29	46	–	–	Seizures, bipolar disorder
54	M	MCI	29	46	–	–	Seizures, depression
57	F	DEM	n.a.	41	–	–	Myoclonus
57	M	MCI	29	25	–	+	Depression
60	M	MCI	26	20	–	+	
55	M	MCI	21	20	–	(+)	

CHI chitotriosidase, *CHO* cholestantriol, *DEM* dementia, *MCI* mild cognitive impairment, *n.a.* not available, *NPC-SI* Niemann-Pick type C suspicion index, *SCD* subjective cognitive decline, (+) negative in second sample

with elevated chitotriosidase, but low-probability NPC-SI, cognitive impairment was related to severe depression he developed over the course of 3 years.

Discussion

Following recommendations by recent guidelines, the present study screened for NPC in the clinical niche of patients with unclassified cognitive impairment. Although our screening was negative, several aspects are important and may inform future studies.

As a major finding, our study provides estimates of clinical features in memory clinic cohorts that are part of the NPC spectrum and assessed upon risk stratification by the NPC-SI (Wijburg et al. 2012). In general, the prevalence of about 15% of patients with unclassified or uncertain diagnosis is consistent with previous data (Hejl et al. 2002). This indicates that our study population is probably representative of other memory clinic settings. A comparable 40% prevalence of depression has also been observed by others (Knapskog et al. 2014). To our knowledge, there are no specific estimates for ataxia or dysarthria in memory clinics. Of note, their presence in 5% of our patients was found based on nontargeted neurological examinations by both neurologists and psychiatrists. Therefore, their actual prevalence may be higher. Nontargeted neurological examinations could also be the reason for the low frequency of vertical supranuclear gaze palsy, which requires assessment of both smooth-pursuit eye movements and saccades, not only in NPC (Salsano et al. 2012). Although this may be the correct estimate in patients below 60 years of age, we expected a higher prevalence because of definite impairment of vertical, especially downward gaze in patients above that age (Oguro et al. 2004). Importantly, the current study shows that because of the presence of all of these features, the NPC-SI may indicate high probability of NPC in about 4%, and at least moderate probability in more than 20% of patients in memory clinics. This rate of false positives will probably be lower in an updated version of the NPC-SI (Hendriksz et al. 2015). This version only puts special weight on combinations of more specific neurological and psychiatric symptoms, specifically presenile cognitive decline, psychotic symptoms, vertical supranuclear gaze palsy, and gelastic cataplexy.

Regarding biomarkers, the present study cannot draw any conclusion about sensitivity, but it provides data on the specificity of chitotriosidase and cholestantriol in a memory clinic cohort. Although chitotriosidase is a widely applied biomarker for lysosomal storage disorders, levels can be elevated in common disorders such as diabetes mellitus (Żurawska-Płaksej et al. 2016) and, importantly, increase with age (Bouzas et al. 2003). This is probably the reason why five patients in this study had at least one measurement with elevated plasma levels. Because levels may also be normal in NPC with late onset (Vanier et al. 2016), we conclude that chitotriosidase may be a suboptimal biomarker for a screening in aged memory clinic cohorts. Levels of cholestantriol were recently shown to be independent of age and 98% specific for NPC in a large sample of 1,902 patients (Reunert et al. 2016). With only one false-positive patient, this is in consistency with the specificity observed in our study and makes cholestantriol a promising biochemical marker to screen for late-onset NPC. Nevertheless, the present study also shows that biomarkers (and clinical features) with limited sensitivity and specificity yield more than 1% false positives when screening populations with low prior disease probabilities (Geberhiwot et al. 2018). Although cholestantriol levels can be elevated in 25% of heterozygous carriers (Jiang et al. 2011; Reunert et al. 2016), this was not observed in the present study. Inversely, because levels in 75% of carriers are normal, we cannot exclude NPC1 or NPC2 heterozygotes among those in our cohort not genetically screened. Such NPC1 and NPC2 heterozygosity has recently been found in 4 out of 50 patients with a dementia plus syndrome (Cupidi et al. 2017).

The negative outcome of this study possibly indicates that NPC is not found in late-adult patients with cognitive impairment unless there are clinically predominant other features of NPC (Patterson et al. 2013). However, our results do not support the definitive conclusion that patients with NPC do not present to memory clinics. Statistically, the upper limit of our prevalence estimate suggests that the study cohort may just have been too small. Beyond that, the sensitivity of cholestantriol levels for NPC is only about 92–97% (Jiang et al. 2011; Reunert et al. 2016). Although unlikely, direct genetic testing of the entire study cohort could thus have identified NPC cases. Nevertheless, the negative screening raises the question whether its inclusion criteria have been adequate. Because of reported onsets of neurological symptoms as late as 56 years of age, the present study included patients up to 60 years of age (Vanier 2010). Due to referral practice, almost all patients were in fact older than 40 years of age. For comparison, recent guidelines specify at-risk groups with onsets before 40 years of age (Patterson et al. 2017). We therefore suggest that future screening studies should first target memory clinics with younger patients. A second important factor was probably the inclusion of patients with quantitatively and qualitatively different cognitive impairment. The present study not only included patients with dementia but also with mild cognitive impairment and subjective cognitive decline because potentially reversible causes of cognitive impairment, including metabolic diseases, are more prevalent in the latter two categories than in the

dementia stage (Hejl et al. 2002). Moreover, patients with manifest NPC may have MMSE results in the range of mild cognitive impairment (Bauer et al. 2013). However, the inclusion of patients with minor degrees of memory impairment may as well have lowered the prior probability for a severe organic disease. Qualitatively, we included patients with any pattern of cognitive impairment in neuropsychological assessments or with abnormal screening tests alone and excluded patients with a classified diagnosis. According to recent guidelines, however, it might also be reasonable to actively screen for NPC in patients with a specific pattern of executive problems and deficits of working memory and verbal fluency suggestive of frontotemporal dementia, especially in conjunction with behavioral alterations (Klarner et al. 2007; Patterson et al. 2017). Finally, the present study included patients with depression, which can be either an independent symptom of NPC (or other neurodegenerative diseases), but also the primary cause of cognitive impairment, especially in younger patients (Patterson et al. 2012; Richard et al. 2013). Because of the prevalence of the latter, and consistent with the updated NPC-SI, future screening efforts should probably not prioritize patients with cognitive impairment and depression unless additional symptoms of NPC are present.

In conclusion, the present study cannot exclude the presence of NPC in the clinical niche of memory clinic patients. We suggest conducting prospective screening studies in younger and larger cohorts that include a focused neurological examination and measurements of plasma cholestantriol as a biomarker. Excluding minor cognitive impairment and discarding depression as an independent disease symptom probably further improve screening effectivity but may delay or miss therapeutic options in early or mild disease.

Synopsis

Niemann-Pick type C disease was not detected among memory clinic patients despite cases with abnormal suspicion index or plasma biomarkers, but future screenings may be positive in larger and younger cohorts with disease characteristics in addition to cognitive impairment and depression.

Compliance with Ethics Guidelines

Conflicts of Interest

A.T. has received travel fees by Actelion Pharmaceuticals.

M.T.H. declares that his Clinical Neuroscience unit at the Department of Neurology has received funding for this clinical study.

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Informed Consent

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

Author Contributions

Andreas Traschütz acquired the data, performed the analysis, and wrote the manuscript.

Michael T. Heneka planned the study and wrote the manuscript.

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RESEARCH REPORT

Reversible Cerebral White Matter Abnormalities in Homocystinuria

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Abstract Striking MRI brain changes resembling leukoencephalopathy are rarely seen in classical homocystinuria. Our case suggests that reversible white matter changes (WMC) are linked to elevated plasma methionine levels arising during treatment.

A 6-year-old boy with learning difficulties and a normal MRI brain scan was diagnosed with homocystinuria (initial total homocysteine 344 μmol/L and methionine 64 μmol/L). At the age of 6.5 years, he developed superior sagittal sinus (SSS) thrombosis. Antithrombotic and homocysteine-lowering treatments were started. Due to poor dietary compliance and betaine treatment, his methionine level reached 1,285 μmol/L, and left side weakness developed. Repeat MRI scan revealed new confluent WMC in previously myelinated brain areas. Further 3-month treatment with tighter dietary control significantly dropped his methionine level (233 μmol/L) with resolution of his neurological deficit and of radiological changes.

We suggest a reversible toxicity from hypermethioninaemia as a possible source of cerebral WMC (secondary to a demyelinating process) in patients with homocystinuria. It highlights the importance of homocysteine-lowering treatment as a prevention and complete resolution of neurological complications. It also demonstrates the need to consider homocystinuria in a differential diagnosis of paediatric leukoencephalopathy.

Abbreviations

CBS	Cystathionine beta-synthase
ICP	Intracranial pressure
MTHFR	5,10-Methylenetetrahydrofolate reductase
SSS	Superior sagittal sinus
WMC	White matter changes

Introduction

Classical homocystinuria is an autosomal recessive disorder of methionine metabolism secondary to deficiency of cystathionine beta-synthase (CBS) resulting in multisystemic impairment (Mudd et al. 1985). There are a wide range of neurological presentations, but the most common are psychomotor retardation and focal neurological deficit secondary to infarction. Brain imaging may demonstrate cerebral infarction, atrophy or venous occlusion (Van den Knaap and Valk 2005). Rarely, patients with abnormalities of methionine metabolism have presented with striking cerebral WMC on MR brain imaging resembling a leukoencephalopathy. A variety of different mechanisms have been proposed based on the methionine metabolic pathway. However, myelinopathy caused by hypermethioninaemia in children with homocystinuria has been sparsely reported in the literature. We demonstrate a case, where white matter abnormalities are likely linked to elevated plasma methionine levels, arising during the course of treatment, with subsequent resolution.

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Case

A 6-year-old boy was born in good condition by a normal delivery to healthy non-consanguineous Caucasian parents. He had unremarkable family and postnatal history. His early development was normal, but deterioration of speech and language skills was noted from the age of 2 years requiring educational support at school. On examination at age of 6 years and 4 months, the child was tall and had hypermetropia, a conductive hearing loss, pectus excavatum, large ears, high forehead, and a mild scoliosis. A detailed neurological assessment was limited due to his attention and coordination difficulties. He had impaired gross and fine motor skills (but no focal neurological deficit) together with delayed speech and language, reminiscent of a child <2-year-old.

In a month, the patient underwent a complete investigation for his developmental delay. His biochemical investigations revealed grossly elevated total homocysteine (344 μmol/L, normal range 0–15 μmol/L) and a mildly raised methionine levels (64 μmol/L, normal range 10–53 μmol/L), consistent with homocystinuria later confirmed by detecting a CBS gene mutation (heterozygous for the pathogenic mutation c.919G>A; p.G307S). His MRI brain scan was normal. He was also a carrier for MTHFR 677CT variant. Due to detected homocysteinaemia, his serum vitamin B12 levels (42 pmol/L, normal range 200–500 pmol/L) and folate levels (>20.0 ng/mL, normal range 2–20 ng/mL) were checked. Shortly before his next hospital presentation, he was commenced on pyridoxine, folic acid and a methionine-restricted diet.

Three weeks later, at the age of 6.5 years, he developed symptoms and signs of a raised intracranial pressure (ICP) caused by an acute SSS thrombosis confirmed on MRV/MRI scans with normal brain appearances and myelinated white matter elsewhere (Fig. 1a, c). At that time, his total homocysteine levels slightly reduced (339 μmol/L) and methionine levels increased (647 μmol/L). He was rehydrated and treated with subcutaneous dalteparin. As dietary compliance was suboptimal, he was also commenced on betaine hydrochloride adjuvant to his diet. Following 1 month of this treatment, his total homocysteine levels

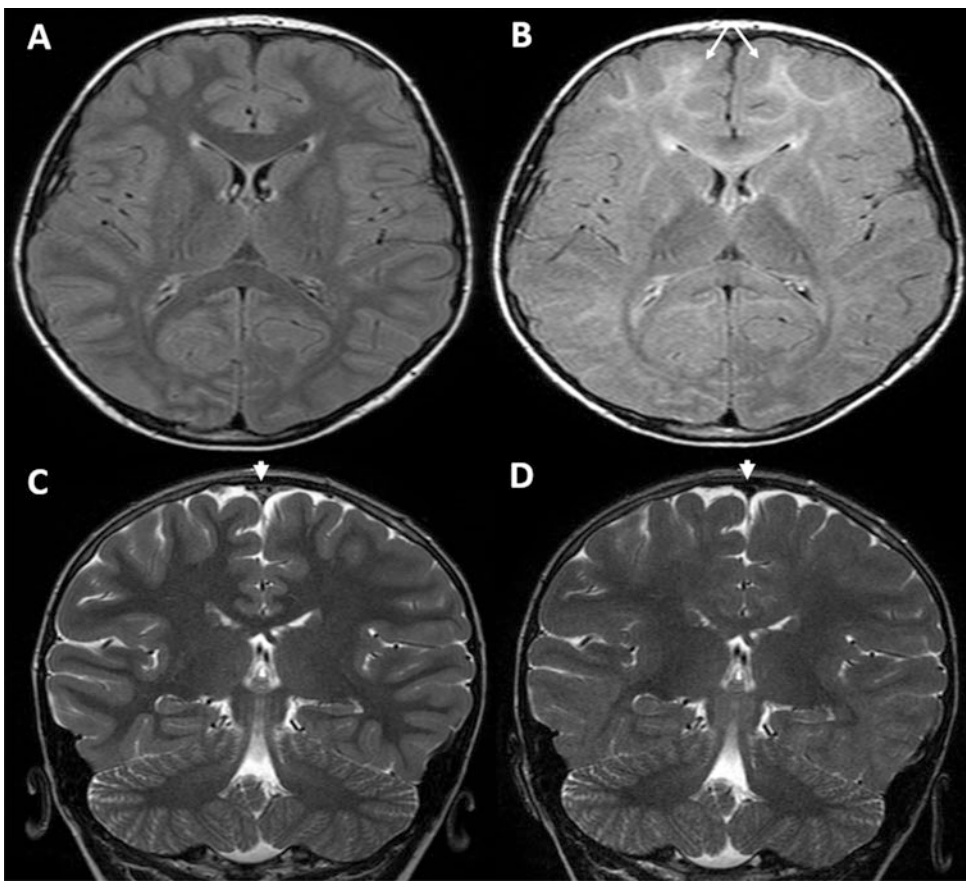

Fig. 1 Axial FLAIR images (**a**, **b**): (**a**) showed normal brain structures in November 2013; (**b**) detected diffuse white matter changes with predominance in anterior areas in February 2014. Coronal T2-weighted images (**c**, **d**): (**c**) a thrombus in the anterior and mid-portions of the superior sagittal sinus detected in November 2013; (**d**) the thrombus later started to recanalise in February 2014

rapidly reduced to <100 μmol/L. However, his plasma methionine levels reached 1,285 μmol/L and his development did not improve. At this time, he developed left-sided weakness with reduced muscle power (MRC score 4/5) but no evidence of other neurological signs and symptoms. A follow-up MRI scan revealed new confluent widespread T2 hyperintensity in previously myelinated white matter prominent in the anterior supratentorial regions with involvement of deep juxta cortical, subcortical, periventricular, capsular and brainstem areas including central tegmental tracts. The SSS thrombosis had started to recanalise (Fig. 1b, d). Within further 3-month treatment and tighter dietary control, there was a significant drop in methionine levels (233 μmol/L), resolution of his neurological deficit and improvement in his cognition, speech and motor skills. At that time, there was resolution of previous WMC and significant recanalisation of the SSS thrombosis. Following further treatment, his plasma homocysteine and methionine levels continued to fall, and our patient remained clinically well and made good developmental progress.

In the view of his diagnosis, his asymptomatic 3-year-old sister with a normal development was screened for homocysteinuria and found to have hypermethioninaemia (669 μmol/L) and homocysteinaemia (180 μmol/L). She was subsequently started on a low-methionine diet and on homocysteine-lowering treatment but became symptomatic (paroxysmal episodes of falls, leg pains, clumsiness and urinary incontinence) once betaine was commenced. However, she required extra-educational support for difficulties in speech and fine motor skills at 4 years. Her brain scan was normal even though her hypermethioninaemia peaked at 917 μmol/L. This was possibly due to much tighter control of her biochemical results, timely modifications of betaine doses and compliance with low-methionine diet.

Discussion

Our paediatric case demonstrates reversible diffuse brain WMC in classical homocystinuria with a significant hypermethioninaemia probably secondary to both betaine treatment and poor dietary compliance. It also highlights the importance of screening children with learning difficulties for amino acid disorders. These MRI changes were correlated with left-sided weakness and coincided with high methionine and mildly raised plasma homocysteine levels following homocysteine-lowering treatment. Clinical and radiological abnormalities resolved after a significant drop in plasma methionine levels.

In homocystinuria, a CBS deficiency impacts the transsulfuration pathway causing hyperhomocysteinaemia. This leads to hypermethioninaemia secondary to activation of the remethylation pathway (Fig. 2). Successful remethylation process requires betaine or 5-methyltetrahydrofolate to

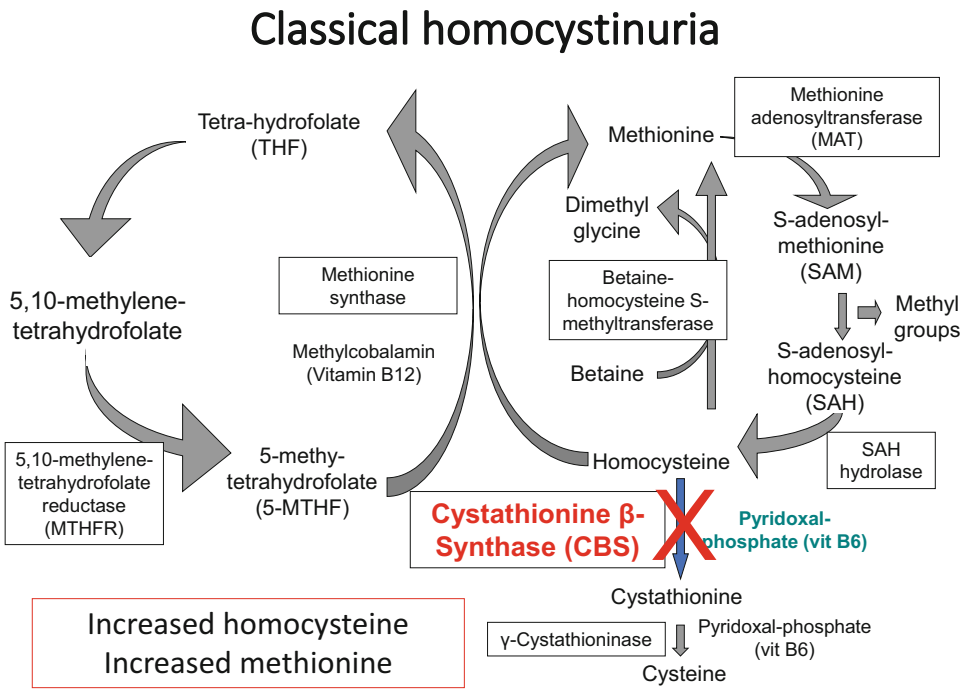

Fig. 2 Transsulfuration pathway

donate a methyl group to homocysteine causing an elevation of plasma methionine levels. Our patient, therefore, had a dramatic increase in methionine and a significant drop in homocysteine plasma levels once betaine treatment was initiated. Brenton et al. (2014) reported similar changes with progressive unilateral weakness, developmental delay and a marked increase of homocysteine and methionine but prior to homocystinuria treatment. By contrast, our patient developed a focal neurological deficit together with WMC on MRI scan on the background of poor dietary compliance and betaine treatment resulting in hypermethioninaemia.

Cerebral WMC are uncommon findings in classical homocystinuria, which were associated with hypermethioninaemia. However, Vatanavicharn et al. (2008) demonstrated an adult case with diffused reversible white matter changes in the brain and neurological deficits. Similar to our case, these radiological findings and clinical deterioration were correlated with highly elevated levels of methionine (1,282 μmol/L) and total homocysteine (266.3 μmol/L). They resolved following normalisation of methionine levels. The authors proposed that observed leukoencephalopathy was due to demyelinating process secondary to hyperhomocysteinaemia and hypermethioninaemia.

Sasai et al. (2015) also presented another adult case with reversible diffuse WMC on the background of elevated levels of plasma methionine (904.2 μmol/L) and total homocysteine (166.5 μmol/L) but with no associated neurological abnormalities. The authors proposed that treatment with betaine and elevated plasma methionine could increase a risk of the cerebral WMC. Interestingly, in patients with MAT I/MAT III deficiency, Chien et al. (2015) suggested that betaine works as an intracellular osmolyte. It causes a development of brain oedema in its high plasma levels.

Yaghmai et al. (2002) and Devlin et al. (2004) also demonstrated widespread WMC with a cerebral oedema and acute symptoms of ICP in two cases with marked hypermethioninaemia (2,272–3,037 μmol/L) and hyperhomocysteinaemia (1,190–1,205 μmol/L). These changes were detected with commencing betaine treatment and resolved following its discontinuation. Devlin et al. (2004) justified these changes by high brain intracellular concentrations of betaine secondary to its treatment whereas Yaghmai et al. to methionine toxicity (Yaghmai et al. 2002). In contrast, our case did not show any evidence for a cerebral oedema and had significantly lower levels of amino acids. Moreover, there was no evidence of betaine toxicity as hypomethioninaemia, and clinical improvement was achieved with perseverance with betaine treatment and a low-methionine diet. We, therefore, suggest that significant hypermethioninaemia secondary to betaine treatment in homocystinuria contributed to the evolution of the brain WMC. Betaine remains a beneficial medicine for homocystinuria treatment. However, with growing evidence in the literature of its neurological complications and our patient's sibling example, we support Devlin et al.'s suggestion to control plasma methionine levels targeting <1,000 μmol/L by both modifying the dietary intake and judicial use of betaine (Devlin et al. 2004). Moreover, a current recommendation in the management of cystathionine beta-synthase deficiency in patients treated with betaine is to avoid plasma methionine levels >1,000 μmol/L (Morris et al. 2017).

In patients with MAT I/III deficiency, mean methionine values >800 μmol/L had central nervous system (CNS) presentation, whereas <800 μmol/L were associated with no CNS signs or symptoms (Chien et al. 2015). Moreover, patients with >800 μmol/L methionine levels became free from CNS abnormalities after initiation of a methionine-restricted diet. The authors explained neurological deficits with a brain oedema secondary to hypermethioninaemia (>800 μmol/L) while on betaine therapy.

This toxicity from hypermethioninaemia could be explained by methionine competition for "large neutral amino acid" (LNAA) system (Chien et al. 2015). Since methionine is transported by the LNAA transporter through the blood-brain barrier, it would compete with other amino acids and inhibit their transport to the brain.

In conclusion, our case contributes to the scarce literature on the aetiology of cerebral WMC in patients with abnormal methionine metabolism and homocystinuria. It identifies a reversible toxicity from hypermethioninaemia as the likely source of demyelinating process. Our report also underlines the importance of monitoring the dose of betaine together with a tight methionine-restricted diet. This should normalise plasma methionine levels with the aim of preventing or complete resolution of neurological complications. Importantly, this case highlights the need to consider homocystinuria in a differential diagnosis of paediatric leukoencephalopathy and to screen patients with developmental delay for amino acid disorders.

Synopsis

A reversible toxicity from hypermethioninaemia is a possible source of cerebral WMC in patients with homocystinuria.

Conflict of Interest

Naila Ismayilova, Andrew D. MacKinnon, Helen Mundy and Penny Fallon declare that they have no conflict of interest.

Compliance with Ethics Guidelines

Informed consent was obtained from a patient's parent before submitting this manuscript.

Naila Ismayilova – Contributed pertinent aspects of the planning, conduct and reporting of the work described in the article *Guarantor*

Andrew D. MacKinnon – Contributor in analysis and interpretation of data and revising it critically for the important intellectual content

Helen Mundy – Contributor in analysis and interpretation of data

Penny Fallon – Contributor in analysis and interpretation of data and revising it critically for the important intellectual content revising it critically for the important intellectual content

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