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CASE REPORT

Diaphragmatic Eventration in Sisters with Asparagine Synthetase Deficiency: A Novel Homozygous *ASNS* Mutation and Expanded Phenotype

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Abstract *Background*: Asparagine Synthetase Deficiency (ASNSD; OMIM #615574) is a newly described rare autosomal recessive neurometabolic disorder, characterised by congenital microcephaly, severe psychomotor delay, encephalopathy and progressive cerebral atrophy. To date, seven families and seven missense mutations in the ASNSD disease causing gene, *ASNS*, have been published. *Methods*: We report two further affected infant sisters from a consanguineous Indian family, who in addition to the previously described features had diaphragmatic eventration. Both girls died within the first 6 months of life. Whole exome sequencing (WES) was performed for both sisters to identify the pathogenic mutation. The clinical and biochemical parameters of our patient are compared to previous reports. *Results*: WES demonstrated a homozygous novel missense *ASNS* mutation, c.1019G > A, resulting in substitution of the highly conserved arginine residue by histidine (R340H). *Conclusion*: This report expands the phenotypic and mutation spectrum of ASNSD, which should be considered in neonates with congenital microcephaly, seizures and profound neurodevelopmental delay. The presence of diaphragmatic eventration suggests extracranial involvement of the central nervous system in a disorder that was previously thought to exclusively affect the brain. Like all previously reported patients, these cases were diagnosed with WES, highlighting the clinical utility of next generation sequencing in the diagnosis of rare, difficult to recognise disorders.

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Please note that Jun Sun and Angela J. McGillivray contributed equally and should be shared first authors. Maya Chopra and Asan should be co-corresponding authors.

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Introduction

Asparagine Synthetase Deficiency (ASNSD) was first described in 2013 as a rare autosomal recessive inborn error of non-essential amino acid synthesis caused by homozygous or compound heterozygous mutations in the *ASNS* gene (Ruzzo et al. 2013). In the initial report of nine individuals from four families, key features included: congenital microcephaly, intellectual disability, progressive cerebral atrophy and early onset intractable seizures. Recently, four additional patients from three families were reported (Ben-Salem et al. 2015; Alfadhel et al. 2015; Palmer et al. 2015). The *ASNS* gene is highly expressed in the brain and encodes for asparagine synthetase, the enzyme which catalyses the ammonia transfer from glutamine to aspartic acid (Ruzzo et al. 2013). While CSF and plasma amino acid profiles may be abnormal in ASNSD, normal levels have been seen in some patients, making biochemical diagnosis unreliable and contributing to the challenge in diagnosing this rare disorder.

We report two sisters, born to consanguineous parents (first cousins) of Indian ancestry, with ASNSD caused by a novel missense homozygous mutation in *ASNS*. This is now the eighth family to be reported with this disorder, and the eighth pathogenic mutation. We describe the phenotypic, biochemical and molecular features of our two patients and compare them with previously reported cases.

Clinical Report

We report on two affected sisters born to first cousin Indian parents (see Fig. 1a).

Patient 1

Patient 1 was born at 38 weeks' gestation following a pregnancy complicated by intrauterine growth restriction. Her birth weight was 2,216 g, length 45.5 cm and OFC 30.5 cm (all less than 3rd percentile). She had flattened and simple ears and a prominent nasal tip. Following an episode of aspiration, she was found to have elevation of the right hemidiaphragm on chest X-ray. Airway fluoroscopy demonstrated paradoxical movement on the right side consistent with phrenic nerve palsy and eventration. She underwent two right-sided hemidiaphragmatic plications. She had progressive respiratory failure due to diaphragmatic failure followed by central respiratory failure. She became dependent on nasogastric feeds and her growth remained static. She was jittery and had early truncal hypotonia, then generalised hypertonia with clonus. There were no seizures. Cerebral MRI at 3 weeks showed mild ventriculomegaly, simplified gyral pattern, and hypoplasia of the pons and cerebellum and mild delay in myelination. At 3 months there was cortical volume loss, corpus callosum thinning and bilateral caudate atrophy. EEG at 3 weeks showed low voltage with no epileptiform activity. At 3 months EEG was very abnormal with episodic bursts of theta, delta and sharp activity with an intervening isoelectric pattern. She became dependent on ventilator support. Critical care was withdrawn at 6 months of age.

Patient 2

Patient 2 was born 7 years after Patient 1. Two healthy boys were born in the intervening period. She was born at 37 weeks and 5 days by caesarean section following reduced foetal movements and abnormal cardiotocography (CTG). Intrauterine growth restriction with severe microcephaly was noted prenatally. Her birth weight was 2,236 g, length 44.5 cm and head circumference 28.5 cm (all <3rd percentile). Respiratory insufficiency was present from birth, requiring intubation and ventilation. She had a sloping forehead, hypertelorism and a prominent nasal tip. She had hyperekplexia but no evidence of clinical seizures. Amp-integrated Brainz EEG demonstrated a premature pattern but there were no electrical seizures. She initially had hypertonia and hyperreflexia, which evolved into hypotonia and minimal gag reflex. Cerebral MRI on day 5 showed a simplified gyral pattern, mild ventriculomegaly with enlarged axial spaces, suggesting a moderate degree of atrophy and hypoplasia of the corpus callosum, pons and inferior cerebellum (see Fig. 1b–g). Myelination was age-appropriate. She developed right-sided diaphragmatic eventration (see Fig. 1h), with associated refractory lung collapse, diminishing respiratory effort and three failed extubation attempts. She had right-sided choanal stenosis. She died on day 11.

Methods

Preliminary Investigations

See supplementary information.

Homozygosity Mapping in Patient 2

The determination of long segments of chromosome homozygosity (LSCH) in Patient 2 was performed with single nucleotide polymorphism (SNP) analysis using an Illumina HuamnCore-12 v1.0 array (mean resolution 0.2 Mb).

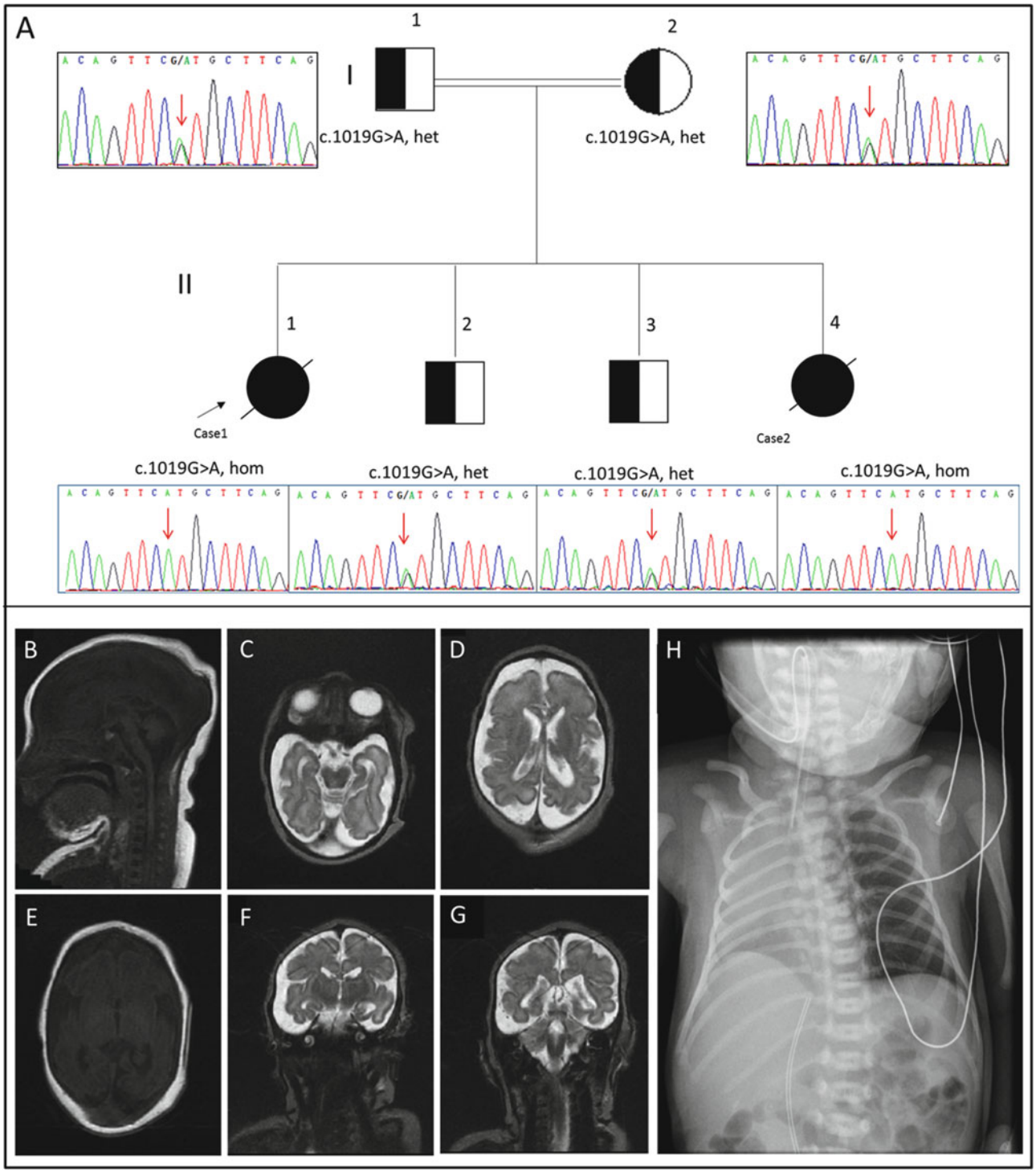

Fig. 1 (**A**) Pedigree and chromatograms of the DNA sequence changes in *ASNS*. The parents and siblings are heterozygous for the c.1019G > A variant. Patients 1 and 2 are homozygous for this variant. (**B**–**G**) MRI brain of Patient 2 at 5 days old. B Sagittal T1 demonstrates small corpus callosum, atrophic brainstem and vermis. C&D Axial T2 images show severe microcephaly and prominence of the lateral ventricles, particularly the temporal horns. E Axial T1 shows wide Sylvian fissure and prominent subarachnoid space overlying the small cerebral hemispheres. F Coronal T2 shows generalised microcephaly, with severe involvement of the temporal lobes. G Coronal T2 demonstrates enlarged trigones of both lateral ventricles. (**H**) Chest X-ray of Patient 2, showing raised right hemidiaphragm and midline mediastinal shift to the right

Exome Sequencing

Whole exome sequencing (WES) was performed on two affected subjects by BGI, Shenzhen, China (see supplementary information).

Data Analysis

Raw data was processed by the Illumina pipeline (version 1.3.4) for image analysis, error estimation, base calling and generation of the primary sequence data. A local algorithm was used to remove low-quality reads and potential adaptor contamination from the primary data. The called variants were annotated using Gaea, a BGI in-house developed annotation pipeline. The details of data analysis are described in the supplementary information.

Variant Interpretation

Given the history of consanguinity in the family, homozygosity mapping followed by exome sequencing was implemented. Variants identified by exome sequencing were filtered based on their annotated function and allele frequency in control databases. We then focussed on the subset of the filtered variants which were identified within LSCH. The associated phenotypic features of candidate genes were analysed against the patient's phenotype. The phenotype match was based on the OMIM database (http://omim.org/) and published papers.

Sanger Sequencing

The identified possible disease causing mutation in *ASNS* was confirmed as homozygous by Sanger sequencing for two patients and as heterozygous in unaffected family members (parents and two unaffected brothers).

Molecular Dynamics Simulation

A workspace of automated homology modelling, SWISS-MODEL (Biasini et al. 2014), was used to construct the 3D structure of ASNS and mutant R340H from homo sapiens (amino acid sequence NP_899199). By alignment, the structure of asparagine synthetase B (ASNB) from E. coli (Larsen et al. 2000) was selected as the template to construct the model as it shares a sequence identity of ~49% with the human ASNS sequence.

See supplementary information.

Results

Patient 1

Initial normal genetic investigations included 400 band resolution G-banding metaphase karyotype, 17p13.2 (*LIS1*) florescent in situ hybridisation and subtelomere multiligand probe amplification. Metabolic investigations showed normal urine amino and organic acids, serum ammonia, creatine kinase, orotic acid, very long chain fatty acids, cholesterol, 7-dehydrocholesterol, transferrin isoforms and cerebrospinal fluid (CSF) pyruvate, glucose, protein, lactate and amino acids. While serum and CSF amino acids were reported as normal, neither included analysis of asparagine. Serology provided no evidence for prior infection with cytomegalovirus, toxoplasmosis, rubella or syphilis.

Patient 2

Initial genetic investigations included a normal oligonucleotide chromosome array, and an SNP array which did not detect any clinically significant genomic imbalance but identified 20 long continuous stretches of homozygosity on chromosomes 2, 5, 7, 8, 9, 10, 12, 16 and 18, representing 8.6% of the genome (see supplementary information). Metabolic investigations showed normal urinary organic and amino acids and glycosaminoglycans, plasma very long chain fatty acids, 7-dehydrocholesterol and transferrin isoforms. Plasma amino acids detected low asparagine (7 μmol/L, reference range 26–76 μmol/L) and cystine levels (19 μmol/L, reference range 28–77 μmol/L). CSF amino acid analysis was not performed.

Whole Exome Sequencing

WES was performed for the two affected daughters in this family. For Patient 2, for whom an adequate DNA sample was available, 9,570 megabases of raw data was generated with a depth of 98.11-fold for the target region. For Patient 1, the quantity was insufficient for standard WES library construction. Only 4,009 megabases of raw data was generated with a depth of 32.87-fold for the target region for Patient 1 (see supplementary information).

Because of concerns of inadequate coverage in Patient 1, primary analysis focussed on Patient 2. The list of candidate genes from Patient 2 was then compared manually with Patient 1.

In total, 25,232 variants were identified in Patient 2, of which 10,217 were in regions of LSCH. After filtering out those variants which were synonymous, non-coding or present in >1% of population according to internal and public databases, 62 variants remained of which 19 were homozygous. These 19 homozygous variants were then manually compared to Patient 1. There was at least 10× coverage in Patient 1 for 18 of the 19 sites. The one variant that was not covered well in Patient 1 was in the *FMNL2* gene, which is not known to be disease causing. Of the 19 homozygous variants in Patient 2, 11 were also homozygous in Patent 1.

Identification of Pathogenic Mutation

The 19 variants remaining after filtration were interpreted manually according to phenotype, function, site conservation and software prediction (supplementary information). Of the 19 homozygous variants identified in Patient 2, two were predicted to be deleterious by PolyPhen2 software (Adzhubei et al. 2010) and Ens Condel software (Gonzalez-Perez and Lopez-Bigas 2011), and only one of these two variants, a missense variant in the *ASNS* gene (c.1019G > A), was also identified in Patient 1 in homozygous form. The c.1019G > A in the *ASNS* gene was absent in the four control databases used in this analysis. Given the consistent clinical, neuro-imaging and biochemical phenotype, this homozygous mutation in the *ASNS* gene (c.1019G > A) was determined to be the disease causing mutation in this family. We found that most reported pathogenic *ASNS* mutations are located in the same domain as the mutation identified in our patients (see Fig. 2a).

The missense mutation c.1019G > A in the *ASNS* gene results in substitution of the highly conserved arginine residue by histidine at position 340 (see Fig. 2b). Sanger validation of this mutation was performed on all family members. The parents and brothers were found to be heterozygous for the mutation (see Fig. 1), which is consistent with the autosomal recessive inheritance mode of ASNSD.

Molecular Dynamics Simulation

We constructed a protein model of human ASNS based on the structure of ASNB from *E. coli*. According to the sequence alignment, position R340 in human ASNS corresponds to R324 in *E. coli* ASNB, and this residue is highly conserved (Supplementary Fig. 1). The protein structure of human ASNS was modelled according to the structure of ASNB (Fig. 2c). The topology of the two proteins, human ASNS (brown) and *E. coli* ASNB (blue), are very similar, except for the unstable loop regions (Fig. 2c). From the structural alignment, we found that the highly conserved position 340 is very close to the catalytic activity centre. Indeed, when arginine at the corresponding position in *E. coli* was substituted by alanine, leucine or lysine, the Asn synthetase activity disappeared (Richards and Schuster 1998). It is possible that the mutation from arginine to histidine also affects the enzyme activity of ASNS in humans.

We also performed molecular dynamics (MD) simulation on wild type (WT) and mutant R340H. The details of this experiment are in the supplementary information. We demonstrated that the R340 mutation results in a protein which is less compact.

Discussion

We describe two infant sisters with congenital microcephaly, severe psychomotor retardation and cortical and posterior fossa atrophy in whom WES identified a novel pathogenic homozygous *ASNS* mutation, c.1019G > A, p.R340H. This variant was absent in public and internal databases, predicted to be pathogenic on in silico analysis and confirmed on Sanger sequencing. The parents and unaffected siblings were confirmed as heterozygous for the mutation. Furthermore, reduced plasma asparagine in Patient 2 provided further evidence of a phenotypic consequence for the mutation. With these additional two cases, there are now 15 published patients with homozygous or compound heterozygous mutations in *ASNS* resulting in ASNSD, a newly described very rare neurometabolic disorder.

The *ASNS* gene maps to chromosome 7, spans 35 kb and contains 13 exons (Zhang et al. 1989). The enzyme asparagine synthetase converts aspartate to asparagine, with glutamine as the amino donor group in an ATP-dependent reaction (Alfadhel et al. 2015; Zhang et al. 1989).

The in-silico model of the mutant ASNS protein shows that the highly conserved position 340 is adjacent to the catalytic region, therefore substitution of arginine residue by histidine at this position may have a significant effect on the enzyme reaction.

The R340H mutation is in the C-terminal domain of the ASNS enzyme, and most reported pathogenic mutations are located in the same domain. In 3D modelling, the change is near the ATP binding pocket, in common with the majority of *ASNS* mutations reported to date (Palmer et al. 2015). Mutations in these regions may alter ATP binding or hydrolysis (Palmer et al. 2015).

There is enrichment of *ASNS* expression in foetal mouse brain, in patterns similar to those seen in the primary microcephaly genes (Ruzzo et al. 2013; Bond et al. 2002; Jackson et al. 2002). The hypomorphic *Asns* mouse model

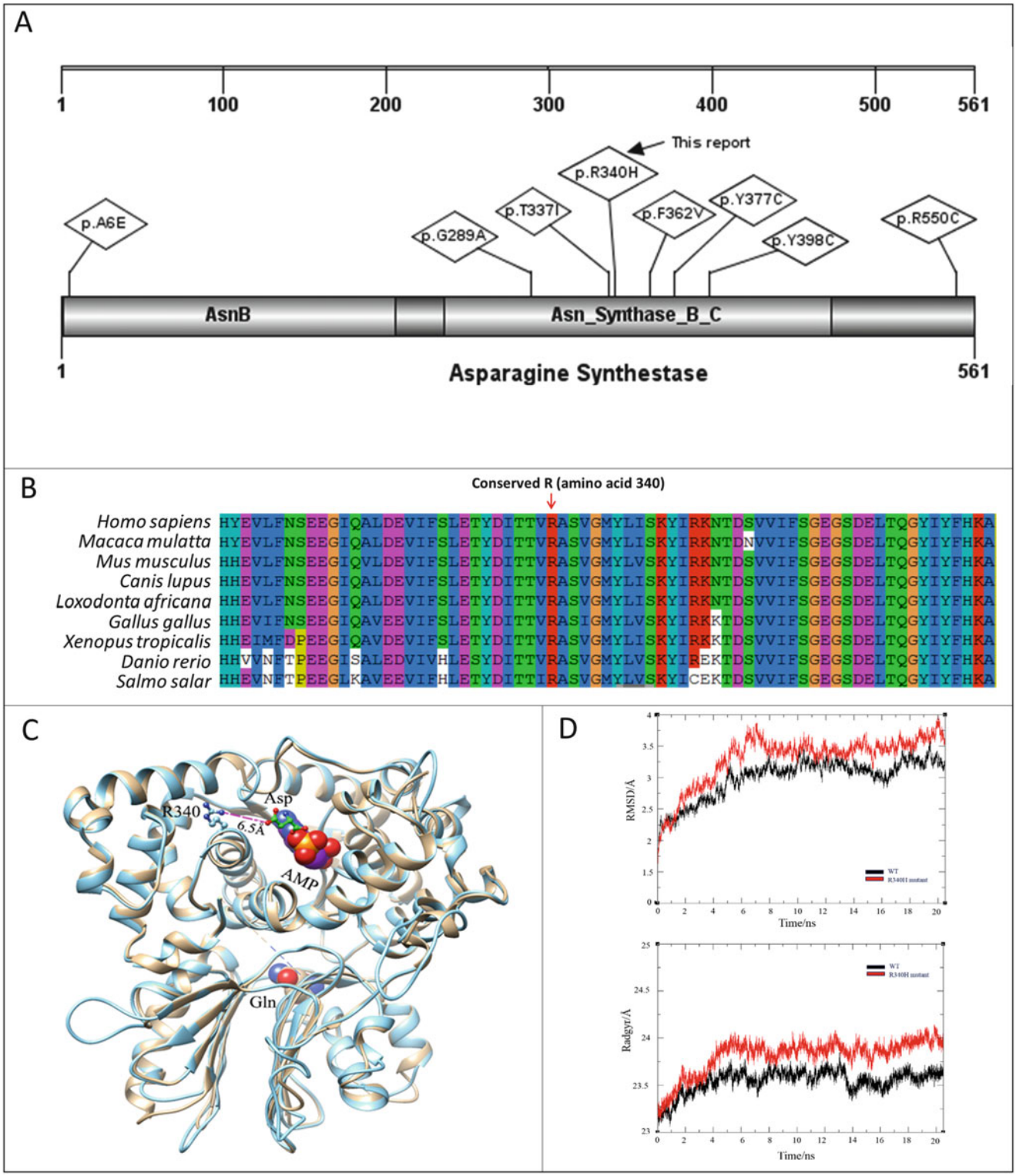

Fig. 2 (**A**) Allelic spectrum and location by functional domain of reported pathogenic mutations of the *ASNS* gene. The R340H mutation identified in this study is marked with a *black arrow*. (**B**) Evolutionary sequence conservation analysis. Multiplex sequence alignment of the ASNS protein across ten different species from *homo sapiens* to *Salmo salar* shows that Arginine (R) at codon 340 is highly conserved among species. The *red arrow* locates codon 340 of the ASNS protein. (**C**) ASNS and asnB ASNB structure. The E. coli ASNB is shown in *blue*. (**D**) Molecular dynamics simulations on wild type and mutant R340H ASNS protein. The *black curve* denotes the structural dynamics of the WT protein and the *red curve* denotes the R340H protein. The root square deviation of backbone atoms (RMSD) reflects a stable protein, allowing for comparison of structure. Radgyr (radius of gyration) reflects the volume of protein structure. The higher Radgyr value in the *red curve* suggests that the mutant protein is held in a more extended state

shows structural abnormalities which reflect the human phenotype, with enlarged ventricles and reduced cortical thickness, as well as deficits in learning and memory (Ruzzo et al. 2013).

The phenotypic consequences of deficiency of this enzyme can be attributed to deficiency of asparagine and accumulation of glutamine and aspartate. Asparagine is considered a non-essential amino acid, and nutritional availability is one of the key transcriptional regulators of ASNS (Balasubramanaian et al. 2013; Palmer et al. 2015). The predominantly neurological phenotype of the disorder is proposed to be due to the poor transport of exogenous sources of asparagine across the blood–brain barrier, suggesting that the amino acid is 'essential' in the central nervous system (Ruzzo et al. 2013; Palmer et al. 2015). In support of this proposed mechanism, Palmer et al. demonstrated a reduction in patient fibroblast proliferation when cultured in a growth medium depleted of asparagine, mimicking the environment within the blood–brain barrier. While peripheral cells compensate for *ASNS* hypofunction with dietary sources of asparagine, cells within the blood brain barrier – which have a significant requirement for this substrate – experience significant depletion (Palmer et al. 2015). Accumulation of aspartate and glutamine result in increased neuronal excitability, the likely mechanism for seizures and hyperekplexia (Ruzzo et al. 2013).

On review of all cases (see Table 1), severe microcephaly of prenatal onset and with a progressive course, severe psychomotor delay, appendicular hypertonia, hyperreflexia and progressive brain atrophy are universally present. Early onset epilepsy was present in 10/15 cases, but all had an abnormal EEG. 4/14 patients were described as having hyperekplexia, and two additional cases (Patient 1 in our report and Palmer et al. 2015) were described as having unusual jittery movements.

Aberrations in serum and CSF levels of asparagine, glutamine and aspartate are seen in some patients with ASNSD, but normal values do not rule out the diagnosis, making biochemical diagnosis unreliable and underpinning the diagnostic challenge of this disorder (see Table 1). Of the ten patients in whom plasma asparagine was measured, six were below reference range. Interestingly only two patients had CSF asparagine measured and both were below reference range. Low CSF asparagine may be a better diagnostic clue to this condition, and reflect the predominantly central effect of this disorder.

Our cases were remarkable for the presence of diaphragmatic eventration in both patients. None of the genes with homozygous variants in our patients were known to be associated with this feature. Diaphragmatic eventration is broadly described as a permanent elevation of a hemidiaphragm that is free of defects as a result of paralysis or varying degrees of atrophy of muscle fibres, with clinical manifestations ranging from asymptomatic to respiratory failure (Ghribi et al. 2015; Tiryaki et al. 2006; Yazici et al. 2003). Cases may be congenital and less commonly, acquired, most often secondary to phrenic nerve injury (Yazici et al. 2003). Given our patients' presentations, and the origin of the phrenic nerve from C3-5, we wonder if ASNSD has consequences for the extracranial central nervous system. While no cases of diaphragmatic eventration have been reported in ASND previously, patient CII.3 in Ruzzo's series was noted to have right hemidiaphragm palsy at 2 months of age (Patient 11, Table 1). The autopsy on Patient DII2 (Ruzzo et al. 2013, supplementary information) showed spinal cord hydromelia, secondary degeneration of motor neurons and reactive gliosis in an affected child, offering potential support to spinal cord involvement in this condition. Despite some functional differences, the blood–spinal cord barrier has long been considered an extension of the blood–brain barrier (Bartanusz et al. 2011). It is likely that the reliance of the brain on endogenous production of asparagine is mirrored in the spinal cord, but the basis of the vulnerability of the origin of the phrenic nerve specifically remains unclear.

ASNSD is the third of three recently recognised disorders of synthesis of non-essential amino acids, all of which are characterised by severe encephalopathy and microcephaly (Ruzzo et al. 2013), following glutamine synthetase deficiency (Haberle et al. 2012) and the serine biosynthesis disorders (de Koning and Klomp 2004). While supplementation has resulted in some therapeutic benefits for these two disorders (van der Crabben et al. 2013; Haberle et al. 2012), the prenatal presentation of ASNSD, transport into the blood–brain barrier and potential for competition with other amino acids have been raised as potential issues that need to be addressed when considering the use of asparagine in patients with ASNSD (Palmer et al. 2015).

With this report, there are now 15 patients with ASNSD. All cases to date have been diagnosed with WES, highlighting the utility of next generation sequencing in diagnosing rare, newly described disorders. The presence of diaphragmatic eventration in our patients suggests that the consequences of this disorder are not confined to the brain, but extend to the extracranial central nervous system.

Table 1 Overview of our patients compared with 13 others previously reported

	Family 1		Family 2	Family 3		Family 4	Family 5	Family 6		Family 7			Family 8			Feature present/total
	This report		Palmer et al. 2015	Alfadhel et al. 2015		Ben-Salem et al. 2015	Ruzzo et al. 2013									
Ethnicity	Indian		Chinese/ Brunei	Saudi Arabian		Emirati	Iranian Jews	Iranian Jews		Bangladeshi			French Canadian			
ASNS mutation[a]	Homo c. 1019G > A, p.R340H		CP c. 866G > C/ 1010C > T, p.G289A, T377I	Homo c. 1160A > G, Y377C		Homo c. 1193A > C, p.Y398C	Homo. c. 1084T > G, p.F362V	Homo c. 1084T > G, p.F362V		Homo c. 1648C > T, p.R550C			CP c. 1648C > T/c. 17C > A, p.A6E/p.R550C			
Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Gender	F	F	M	M	F	M	M	M	F	M	M	M	M	M	M	
Age	6 months[b]	11 day[b]	7 years	5 years	4 years	5 years	14 years	14 years	12 years	4 months[b]	3 months[b]	6 months[b]	9 days[b]	11 month[b].	12 monthsb.	
Birth HC (cm)/ percentile (Villar et al. 2014)	30.5/<3rd	28.5/<3rd	32.5/3rd–10th	29.5 <3rd	26.5/<3rd	29.5/<3rd	31.5/<3rd	31/<3rd	31/<3rd	31.5/<3rd	33/10th	32/<3rd	31.5/<3rd	31/<3rd	28.5/<3rd	
Birth weight (kg)/ percentile (Villar et al. 2014)	2.22/<3rd	2.24/<3rd	3.34/50th	3.1/10–25th	2.65/<10th	3.1/<10th	NA	NA	NA	3.4kg/50th	3.52/50th	3.23/50th	NA	NA	2.160/50–75th	
Neurological																
Severe psychomotor delay	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15/15
Axial hypotonia	+	+	+	+	+	NA	–	–	–	–	+	+	+	+	+	10/14
Peripheral hypertonia	+	+	+	+	+	NA	+	+	+	+	+	+	+	+	+	14/14
Hyperreflexia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15/15
Epilepsy/age of onset	–	–	+/1 month	+/1 day	+/1 day	+/1 day	+/1 month	+/2 weeks	+/3 weeks	–	–	–	+/ 4 days	+/9 months	+/8 days	10/15
Hyperekplexia	–	+	–	–	–	–	–	–	–	+	+	+	–	–	–	4/15
Abnormal EEG	+	–	+	+	+	+	+	+	+	+	+	+	NA	+	+	
Progressive microcephaly	+	NA	+	+	+	+	+	+	+	+	+	+	NA	+	+	13/13
Extra-CNS features																
Diaphragmatic eventration and/or phrenic nerve palsy	R + L eventration	R eventration	–	–	–	–	–	–	–	–	Right hemidia-phragmatic palsy	–	–	–	–	3/15
Brain MRI																
Decreased cerebral volume	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15/15
Decreased size pons	+	+	+	–	–	+	–	–	–	+	+	+	+	+	+	10/15
Gyral simplification	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	12/15
Biochemistry																
Low plasma asparagine	NA	+	+	+	+	–	NA	–	–	NA	+	NA	+	–	NA	6/10
High plasma glutamine	–	–	NA	–	–	–	NA	+	+	NA	NA	NA	–	–	NA	2/9
Low CSF asparagine	NA	NA	NA	+	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2/2
High CSF glutamine	–	NA	NA	+	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1/3

[a] *Homo* homozygous, *CP* compound heterozygous
[b] Deceased
NA not available

Concise Synopsis

A novel homozygous mutation in *ASNS* causes asparagine synthetase deficiency in two sisters with microcephaly, severe psychomotor delay, cerebral atrophy and diaphragmatic hernia.

Authors' Contributions

Jun Sun was responsible for the NGS experiment, interpretation of mutation data and drafting the relevant part of manuscript.

Angela J McGillivray, **Jason Pinner**, **Drago Bratkovic and Elizabeth Thompson** drafted the relevant parts of manuscript and critically revised the paper.

Fengxia Liu performed the bioinformatic analysis of the NGS data.

Zhihui Yan and **Huifeng Jiang** performed and interpreted the protein simulation experiment.

Xiuxiu Wei performed and interpreted the Sanger sequencing experiment.

Asan was responsible for the experiment resign and reviewed the manuscript.

Maya Chopra was responsible for the study conception and design, the drafting of the manuscript.

Guarantor

Asan.

Disclosure

Maya Chopra has received a reimbursement from BGI as an invited speaker to a conference sponsored by BGI.

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Compliance with Ethics Guidelines

The parents of the patients have consented to take part in this study. They have consented to the publication of this paper. Consent for photographs was declined. There are no photographs in this paper.

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RESEARCH REPORT

Measurement of Elevated Concentrations of Urine Keratan Sulfate by UPLC-MSMS in Lysosomal Storage Disorders (LSDs): Comparison of Urine Keratan Sulfate Levels in MPS IVA Versus Other LSDs

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Abstract Keratan sulfate (KS) is commonly elevated in urine samples from patients with mucopolysaccharidosis type IVA (MPS IVA) and is considered pathognomonic for the condition. Recently, a new method has been described by Martell et al. to detect and measure urinary KS utilizing LC-MS/MS. As a part of the validation of this method in our laboratory, we studied the sensitivity and specificity of elevated urine KS levels using 25 samples from 15 MPS IVA patients, and 138 samples from 102 patients with other lysosomal storage disorders, including MPS I ($n = 9$), MPS II ($n = 13$), MPS III ($n = 23$), MPS VI ($n = 7$), beta-galactosidase deficiency ($n = 7$), mucolipidosis (ML) type II, II/III and III ($n = 51$), alpha-mannosidosis ($n = 11$), fucosidosis ($n = 4$), sialidosis ($n = 5$), Pompe disease ($n = 3$), aspartylglucosaminuria ($n = 4$), and galactosialidosis ($n = 1$). As expected, urine KS values were significantly higher (fivefold average increase) than age-matched controls in all MPS IVA patients. Urine KS levels were also significantly elevated (threefold to fourfold increase) in patients with GM-1 gangliosidosis, MPS IVB, ML II and ML II/III, and fucosidosis. Urine KS was also elevated to a smaller degree (1.1-fold to 1.7-fold average increase) in patients with MPS I, MPS II, and ML III. These findings suggest that while the UPLC-MS/MS urine KS method is 100% sensitive for the detection of patients with MPS IVA, elevated urine KS is not specific for this condition. Therefore, caution is advised when interpreting urinary keratan sulfate results.

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Introduction

Keratan sulfate (KS) is a complex glycosaminoglycan composed of repeating disaccharides of D-galactose and N-acetyl-D-glucosamine (Zhang et al. 2005). Keratan sulfate proteoglycans have been found in the cornea, cartilage, and bones. Degradation of KS occurs in the lysosomes by a stepwise series of enzymatic reactions (Wraith 1995). Deficiency of one of the enzymes involved in this process results in storage of partially degraded KS causing cellular and organ dysfunction (Wraith 1995).

Morquio syndrome (MPS IVA; OMIM # 253000) is an autosomal recessive lysosomal storage disorder (LSD) resulting from an inability to degrade KS and chondroitin-6-sulfate. Patients typically present with short stature, kyphosis, genu valgum, enlarged organs such as liver or spleen, ocular abnormalities, and the radiological finding of dysostosis multiplex. Neurologic function is typically not affected. The age of onset is often in early childhood but milder variants may present in adolescence (Mendelsohn et al. 2013). Recently, enzyme replacement therapy with recombinant human N-acetyl-galactosamine-6-sulfatase (rhGALNS, elosulfase alfa) for the treatment of MPS IVA (Lyseng-Williamson 2014) has been approved by the Food and Drug Administration. Elevation of urinary KS is associated with MPS IVA and measurement of total urine glycosaminoglycans (GAGs) or qualitative GAG analysis to detect KS are commonly used clinical screening tests (Wood et al. 2013). However, a high false negative rate has

been reported for these assays and false negative results are more common in mildly affected patients (Whitley et al. 1989; Piraud et al. 1993; Gray et al. 2007).

Oguma et al. (2001) followed by Martell et al. (2011) reported LC-MS/MS assays that measure two specific KS disaccharides after enzymatic digestion of the GAG polymer with keratanase II. These disaccharides, Gal-1-4GlcNAc(6S) (1S) and Gal(6S)-1-4GlcNAc(6S) (2S), were found to be increased in all of the MPS IVA patients suggesting this assay would be highly sensitive for the detection of patients with this condition. Additionally, using an ELISA assay, Tomatsu et al. (2005) showed that plasma KS was increased in patients with mucolipidosis type II (I cell disease; OMIM#252500) or III (OMIM#252600) as well as in patients with MPS I (OMIM#607014), II (OMIM#309900), III (OMIM#A:252900,B:252920, C:252930), VI (OMIM#253200), and VII (OMIM#253220). Elevation of urine KS has also been previously observed in patients with fucosidosis (OMIM#230000) (Greiling et al. 1978).

Here, we expand on the previous studies by analyzing 25 additional urine samples from Morquio A patients. Urinary KS was also measured in 138 urine samples from 102 patients with various LSDs: MPS I ($n = 9$), MPS II ($n = 13$), MPS III ($n = 23$), MPS VI ($n = 7$), beta-galactosidase deficiency (OMIM#230650 and 253010) ($n = 7$), mucolipidosis (ML) type II, II/III ($n = 12$), and III ($n = 39$), alpha-mannosidosis (OMIM#248500) ($n = 11$), fucosidosis ($n = 4$), sialidosis (OMIM#256550) ($n = 5$), Pompe disease (OMIM#232300) ($n = 3$), aspartylglucosaminuria (OMIM#208400) ($n = 4$), and galactosialidosis (OMIM#256540) ($n = 1$), as well as 105 controls. The clinical sensitivity and specificity of urine KS for the detection of MPS IVA were examined, and showed that urine KS is a more sensitive diagnostic marker than total urine GAGs for MPS IVA. Also, evaluation of urine KS levels in younger (<3 years) unaffected individuals is described to better delineate age-dependent reference ranges for this biomarker compared to previous studies. Finally, clinical laboratories should be cautioned that patients with other LSDs may show elevations in urinary KS. Therefore, while urine KS is a sensitive biomarker for the detection of MPS IVA patients, its accumulation is not specific to this condition.

Materials and Methods

Patient Samples

Samples were submitted to the Greenwood Genetic Center biochemical diagnostic laboratory for diagnostic evaluation or as part of the Longitudinal Studies of the Glycoproteinoses (NCT01891422). Analyses were performed on randomly collected urine samples from patients with mucopolysaccharidosis (MPS) I ($n = 9$), II ($n = 13$), III ($n = 23$), IVA ($n = 18$), and VI ($n = 7$), as well as from patients with other LSDs: beta-galactosidase deficiency ($n = 7$), mucolipidosis II ($n = 4$), II/III ($n = 8$), and III ($n = 39$), alpha-mannosidosis ($n = 11$), fucosidosis ($n = 4$), sialidosis ($n = 3$), Pompe disease ($n = 3$), aspartylglucosaminuria ($n = 4$), and galactosialidosis ($n = 1$), in whom the diagnosis was confirmed by enzyme and/or mutation analysis. Control urine samples were collected from healthy volunteers ($n = 25$; age: 12 months – 63 years) and patients with other, unrelated metabolic disorders ($n = 80$; age: 20 days to 36 years), and used to establish age-dependent, normal reference ranges. All the samples were stored at −20°C prior to use.

Urine Keratan Sulfate Measurements

Urine keratan sulfate disaccharides Galβ1-4GlcNAc(6S) (predominant species) and Gal(6S)β1-4GlcNAc(6S) were produced by keratanase II enzymatic digestion of keratan sulfate (KS) as described previously (Oguma et al. 2001; Martell et al. 2011). The internal standards (heavy-isotope-labeled Galβ1-4GlcNAc(6S) $^{13}C_6$, and Gal(6S)β1-4GlcNAc (6S) $^{13}C_6$), the reference standards (Galβ1-4GlcNAc(6S) and Gal(6S)β1-4GlcNAc(6S)), KS (Bovine cornea, Na salt), and keratanase II (*Bacillus* sp.) were all obtained from GlycoSyn, Lower Hutt, New Zealand. Urine keratan sulfate was detected via stable isotope dilution using ultra performance liquid chromatography (UPLC), utilizing a Thermo Hypercarb 5 µm 50 × 2.1 mm column, and tandem mass spectrometry (MS/MS), utilizing an Acquity/Xevo TQD instrument (Waters Corporation, Milford, MA). Transitions (Galβ1-4GlcNAc(6S): 462.05 → 97.01, $^{13}C_6$-Galβ1-4GlcNAc(6S): 467.78 → 97.01, Gal(6S)β1-4GlcNAc(6S): 563.87 → 462.69) $^{13}C_6$-Gal(6S)β1-4GlcNAc(6S): 569.75 → 468.81) were monitored by multiple reaction monitoring in negative ion mode. The monosulfated and the disulfated disaccharides of KS were calculated by means of separate standard curves, and the final KS concentration was reported as the sum of both. The standard curves for both KS disaccharides included concentrations of 0.103, 0.313, 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 µg/ml. The average inter-day precision in five samples analyzed was 9.1% and 8% for monosulfated KS and disulfated KS, respectively. The average intra-day precision in five samples analyzed was 4.9% and 5.9% for monosulfated KS and disuflated KS, respectively. The lower limit of quantification (LLOQ) is 0.75 ug/ml. The average percent deviation at the LLOQ is −10.87% (for monosulfated KS) and −4.74% (for disulfated KS), and the

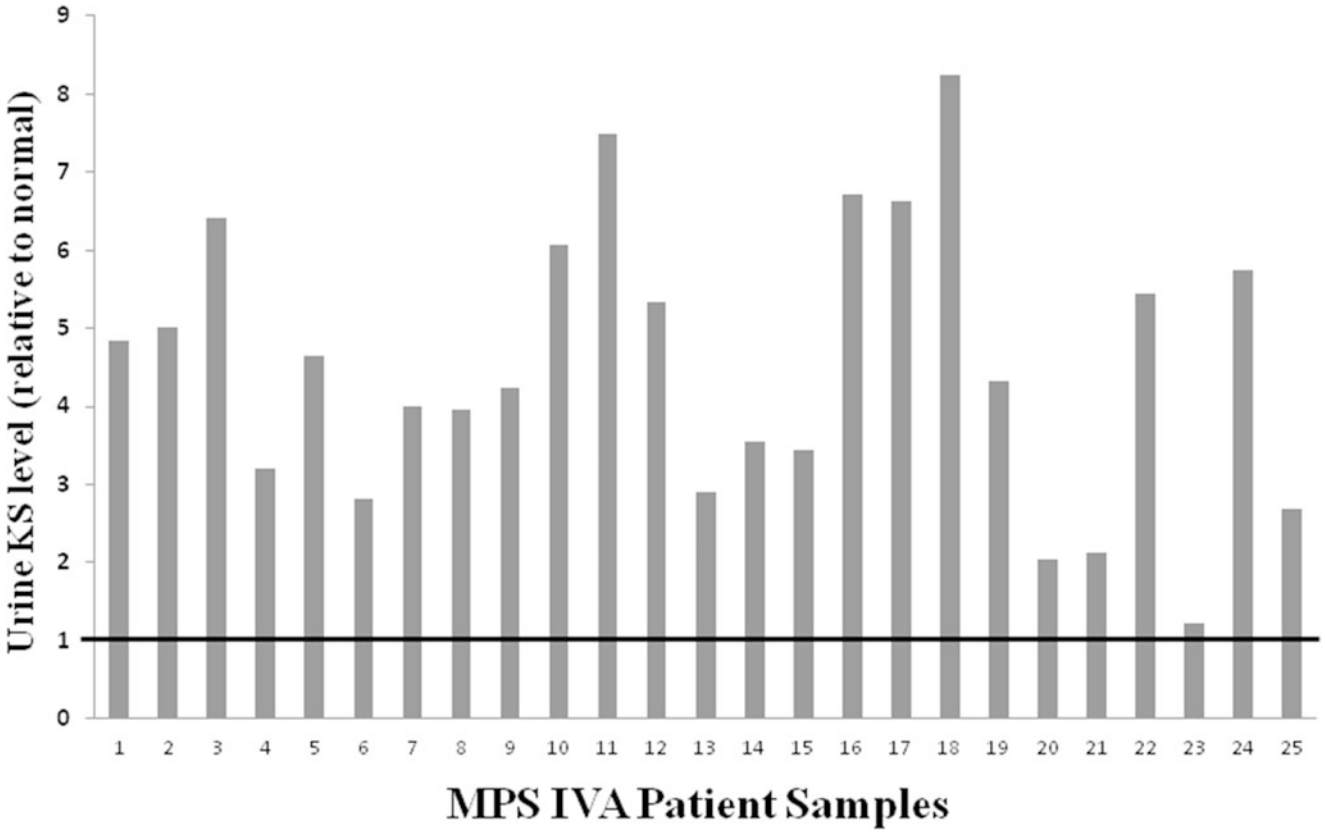

Fig. 1 Urine keratan sulfate is elevated in all MPS IVA patients. Urine keratan sulfate level in 25 MPS IVA patient samples is shown relative to the upper limit of the appropriate age-specific reference range (indicated by the *bold black line*) such that the *Y* axis value represents the fold elevation of urine KS in each patient

intra-day variability at the LLOQ is 9.08% (for monosulfated KS) and 14.91% (for disulfated KS).

Urine Total Glycosaminoglycans Analysis

GAGs were measured in 25 samples from 15 MPS IVA patients using the Blyscan kit (1,9-dimethyl-methylene blue staining (DMB)) (Blyscan Sulfated Glycosaminoglycan Assay Biocolor Ltd. Northern Ireland, UK) and comparing absorbance at 656 nm to that of a standard curve. Age-related reference ranges were developed previously in the laboratory.

Statistical Analysis

KS values in urine samples from patients with MPS IVA or other LSDs were normalized to the upper limit of the appropriate age-specific normal reference range. An unpaired *t* test was used to compare normalized KS levels in the samples from patients with MPS IVA with those from control samples, and from patients with other LSDs. Values were considered statistically significant at $p < 0.05$.

Results

Establishing Normal Reference Ranges

In order to establish age-dependent urine KS normal reference ranges, urine KS was measured in 105 unaffected controls (20 days – 63 years of age). It was observed that urine KS levels decrease with age (Supplementary Fig. 1), as has been previously observed by Martell et al. (2011). This phenomenon was also observed for GAGs (de Jong et al. 1989; Whitley et al. 1989; Piraud et al. 1993; Gray et al. 2007; Wood et al. 2012). Therefore, it was important to establish age-dependent reference ranges to accurately interpret KS levels in patient samples. Decrease in urine KS was especially evident in patients under 3 years of age. Therefore, this patient population was divided further into three age groups: 0–4 months of age ($n = 14$), 5–18 months of age ($n = 16$), and 19–36 months of age ($n = 14$). Urine KS values observed in these age groups were 8.9–28.5; 5.6–17.0; and 4.8–9.6 μg urine KS/mg creatinine, respectively. Further age-dependent normal ranges included 3–5 years of age (1.7–9.6 μg urine KS/

Table 1 Urine keratan sulfate (KS) and glycosaminoglycan (GAG) measurements from 25 MPS IVA patients

Age (Yr)	Total urine GAGs (mg/mmol crnn)	Urine KS (μg/mg crnn)
2	27.21 (<24)	46.51 (<9.6)
2	30.39 (<24)	48.14 (<9.6)
2	28.32 (<24)	61.51 (<9.6)
3	33.80 (<16)	30.68 (<9.6)
3	46.02 (<16)	44.62 (<9.6)
4	16.70 (<16)	25.83 (<9.6)
5	19.75 (<16)	27.13 (<9.6)
5	26.45 (<16)	38.36 (<9.6)
5	**13.68 (<16)**	38.03 (<9.6)
6	22.02 (<12)	26.32 (<6.2)
6	30.89 (<12)	37.66 (<6.2)
6	**10.71 (<12)**	46.48 (<6.2)
6	20.08 (<12)	33.07 (<6.2)
6	25.35 (<12)	18.00 (<6.2)
6	25.33 (<12)	21.98 (<6.2)
7	**11.01 (<12)**	21.31 (<6.2)
7	21.51 (<12)	41.67 (<6.2)
7	24.42 (<12)	41.13 (<6.2)
7	25.89 (<12)	51.04 (<6.2)
9	26.55 (<12)	26.79 (<6.2)
10	12.44 (<12)	12.64 (<6.2)
10	**9.57 (<12)**	13.14 (<6.2)
10	20.92 (<12)	33.79 (<6.2)
14	7.57 (<6.5)	7.09 (<5.8)
>15	12.12 (<6.5)	12.34 (<2.2)

Values in parentheses are the appropriate age-specific upper limit of the reference range and patient values within the normal range are in bold font. Total GAGs were measured by DMB incorporation and keratan sulfate by LC-MS/MS. All 25 samples had elevated urine KS but only 21/25 (83%) had elevated total GAGs

mg creatinine), 6–10 years of age (2.5–6.2 μg urine KS/mg creatinine), 11–14 years of age (1.5–5.8 μg urine KS/mg creatinine), and >14 years of age (0.8–2.1 μg urine KS/mg creatinine). The cut-off values (or the upper limit of normal) referred to in this manuscript represent the highest values observed in each of the age-groups, excluding any outliers. In order to compare individual patient results, urine keratan sulfate values were normalized to the upper limit of the appropriate age-specific normal reference range.

Urine KS in MPS IVA Patients

Urine KS levels were measured in 25 samples from 15 Morquio syndrome type A patients (Fig. 1). As expected, all of the MPS IVA patient samples had elevated urine keratan sulfate levels that ranged from a 1.22–8.27 fold

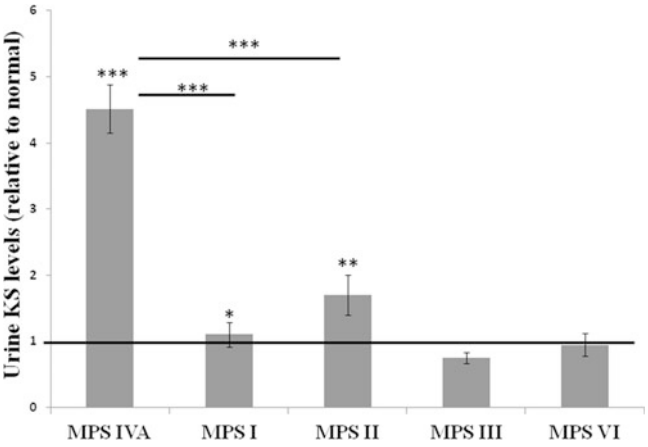

Fig. 2 Mild elevations of urine KS observed in other MPS disorders. Average urine keratan sulfate level for patients with five different MPS disorders is shown relative to the upper limit of the appropriate age-specific reference range (indicated by the *bold black line*). The *Y* axis represents the fold elevation of urine KS in each patient population. Patients with MPS I and MPS II on average had higher urine KS levels than controls, but significantly lower levels than MPS IVA patients. Error bars represent standard error of the means. MPS I-VI = mucopolysaccharidosis I-VI. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Statistical analysis performed by *T*-test

increase (average 4.51-fold increase), when normalized to the upper limit of the age-appropriate normal range.

The sensitivity of urine KS analysis versus standard total urine GAG analysis was compared for the detection of patients with MPS IVA (Table 1). Whereas urine KS was elevated in all 25 MPS IVA samples (100% sensitivity), total urine GAGs were elevated in only 20 of the 25 MPS IVA samples (83% sensitivity), when compared to the appropriate age-specific reference range. The degree to which urine KS levels were elevated (average 4.51-fold) was also significantly higher than that for total urine GAGs (average 1.63-fold; $P < 10^{-8}$). This demonstrates that the quantification of urine KS is a more sensitive biomarker than total urine GAGs for the diagnosis of MPS IVA.

Urine KS in Patients with Other Lysosomal Storage Disorders

In order to assess the clinical specificity of elevated urine KS levels and the clinical utility of urine KS as a screening biomarker for MPS IVA, urine KS was measured in samples from patients diagnosed with LSDs other than MPS IVA, including selected mucopolysaccharidoses, mucolipidosis types II, II/III, III and other glycoproteinoses, Pompe disease, and beta-galactosidase deficiency.

Selected Mucopolysaccharidoses

Urinary KS was measured in samples from patients with MPS I ($n = 9$), MPS II ($n = 13$), MPS III ($n = 23$), and

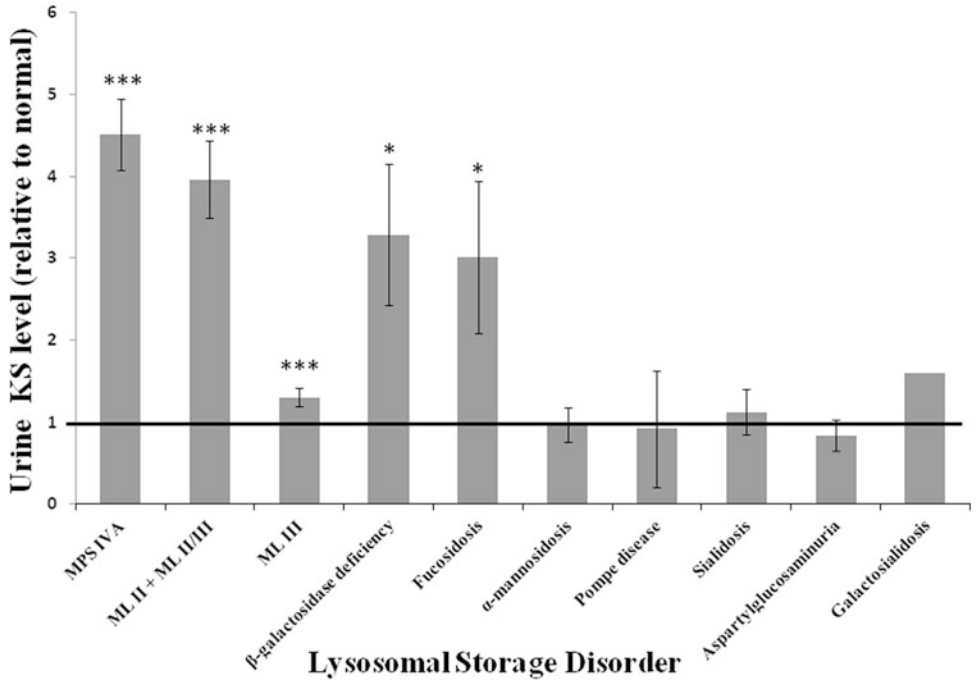

Fig. 3 Urine KS in patients with various lysosomal storage disorders. The *Y* axis represents the average fold elevation relative to the upper limit of the age-specific reference range of urine KS (indicated by the *bold black line*) in each patient population. Patients with ML, fucosidosis, and beta-galactosidase deficiency have significantly higher urine KS levels than controls. No significant difference was observed between MPSIVA and ML II + II/III, fucosidosis, and beta-galactosidase deficiency patient samples. Error bars represent standard error of the means. * = $P < 0.05$; *** = $P < 0.001$; $^{###} = P < 0.001$. Statistical analysis performed by *T*-test

MPS VI ($n = 7$) (Fig. 2). Urine KS levels in patients with MPS I and MPS II were on average 1.1 and 1.7 fold elevated, respectively, relative to the appropriate age-specific normal range. Specifically 3/9 MPS I and 9/13 MPS II patient samples had elevated urine KS. These elevations, though mild, were still statistically significant (MPS I, $P < 0.05$, and MPS II, $P < 0.005$); however these elevations were significantly lower than in patients with MPS IVA ($P < 10^{-7}$). On average MPS III and MPS VI patients were found to have normal average urine KS levels; however, 3/23 MPS III and 1/7 MPS VI patient samples had elevated urine KS levels (1.11-fold to 2.33-fold, and 1.9-fold, respectively).

GNPTAB: Associated Mucolipidoses

Based on the previous report by Tomatsu et al. (2005) that KS could also be elevated in patients with mucolipidoses (ML), as well as the fact that our laboratory found total urinary GAGs to be elevated in a subset of ML II, II/III, and III patients (data not published), urine KS levels were evaluated in a large cohort of samples from patients with ML II ($n = 4$), II/III ($n = 8$), and III ($n = 39$). We observed that patients with a severe skeletal phenotype, specifically individuals with ML II and ML II/III, had significantly higher fold elevations of urine KS compared to unaffected individuals ($P < 10^{-5}$) (Fig. 3). Moreover, in these patients, urine KS levels were as elevated (approximately fourfold) as those observed in patients with MPS IVA ($P = 0.13$). Patients with ML III, who present with a milder skeletal phenotype as compared to ML II and ML II/III patients, also had significantly elevated urine KS levels, as compared to unaffected patients ($P < 10^{-5}$); however, 16/39 ML III patients had normal urine KS. Urine KS level in ML III patient samples was significantly lower (on average 1.3-fold elevated) than urine KS levels observed in ML II and ML II/III ($P < 10^{-5}$), or MPS IVA patients ($P < 10^{-7}$) (Fig. 3).

Other Lysosomal Storage Disorders

Urine KS levels were also measured in a cohort of patients with various glycoproteinoses, including alpha-mannosidosis ($n = 10$), fucosidosis ($n = 2$; four samples), sialidosis ($n = 4$; five samples), aspartylglucosaminuria ($n = 3$; four samples), and galactosialidosis ($n = 1$), as well as in patients with beta-galactosidase deficiency ($n = 6$; seven samples) and Pompe disease ($n = 3$). Urine KS levels varied depending on the disorder, with the highest urine KS levels present in samples from patients with fucosidosis and beta-galactosidase deficiency (Fig. 3). These patients' urine KS levels were significantly elevated (approximately

threefold), as compared to age-appropriate normal controls (fucosidosis $P = 0.04$; beta-galactosidase deficiency $P = 0.01$), and there was no significant difference between urine KS levels in these patients compared to those in patients with MPS IVA ($P = 0.10$ and $P = 0.11$, respectively). One sample from a patient with galactosialidosis demonstrated 1.6-fold elevated urine KS level compared to the age-appropriate reference range. Average urine KS levels in patients with alpha-mannosidosis, Pompe disease, sialidosis, and aspartylglucosaminuria were essentially normal.

Discussion

Elevated keratan sulfate is a well-established biochemical feature of MPS IVA, in which N-acetyl-galactosamine-6-sulfatase deficiency results in impaired catabolism of this glycosaminoglycan (Glossl and Kresse 1978; Yutaka et al. 1982). In the present study, an ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was utilized to examine the clinical sensitivity and specificity of urine KS analysis for patients with not only MPS IVA, but also for patients with other LSDs. We also, for the first time, delineated urine KS ranges for unaffected individuals under the age of 3 years.

Previous reports have documented the age-dependent decrease in keratan sulfate levels (de Jong et al. 1989; Whitley et al. 1989; Piraud et al. 1993; Gray et al. 2007; Martell et al. 2011; Wood et al. 2012). However, these studies had a limited number of samples for patients under the age of 3 years. Our data demonstrate that patients within this age group ($n = 43$) display the most dramatic age-dependent decrease in keratan sulfate (Supplementary Fig. 1). Therefore, three distinct normal ranges within this cohort of patients were created: 8.9–28.5 μg/mg creatinine (0–4 months), 5.6–17 μg/mg creatinine (5–18 months), and 4.8–9.6 μg/mg creatinine (19–36 months). Having proper age-specific normal ranges is imperative for the accurate interpretation of urine keratan sulfate results, especially in young children.

Urine KS levels were elevated in all MPS IVA patient samples analyzed ($n = 25$) (Fig. 1). However, GAGs were elevated in only 83% of the same MPS IVA patient samples (Table 1). False negative results have been well documented for patients with MPS IVA using either the DMB incorporation assay for the quantitative measurement of total urine GAGs or the qualitative separation of individual GAG species by electrophoresis or thin layer chromatography (Whitley et al. 1989; Piraud et al. 1993; Gray et al. 2007). Quantitative measurement of urine KS via UPLC-MS/MS offers increased sensitivity for the detection of patients with MPS IVA compared to the assays commonly used by most laboratories today.

To evaluate the specificity of the urine KS assay, samples obtained from patients with other LSDs were analyzed. Urine KS levels were at least mildly elevated in patients with several other LSDs: MPS I, MPS II, fucosidosis, beta-galactosidase deficiency, and mucolipidosis II, II/III, and III (Figs. 2 and 3). Beta-galactosidase hydrolyzes terminal beta-linked galactose residues from GM1 gangliosides, glycoproteins, and from keratan sulfate (Okada and O'Brien 1968; O'Brien et al. 1976). A subset of patients with beta-galactosidase deficiency are classified as having Morquio syndrome type B (O'Brien et al. 1976); therefore, it is not surprising that patients with this enzyme deficiency have elevated urine keratan sulfate levels.

Elevated urine KS levels have previously been reported by Tomatsu et al. in patients with Mucolipidosis II or III (Tomatsu et al. 2005). Patients with these conditions have a deficiency of N-acetylglucosaminyl-1-phosphotransferase, which prevents the import of most lysosomal hydrolases into the lysosome. This leads to the accumulation of various GAG species in different tissues (Leroy et al. 1972; Thomas et al. 1973; Reitman et al. 1981), and likely explains the accumulation of urine keratan sulfate observed in these patients. However, the Tomatsu et al. study only included 11 samples, and the type of ML, or clinical severity, was not indicated. We analyzed urine KS in 51 samples from ML patients with a well-defined clinical phenotype and a confirmed diagnosis of either ML II, ML II/III, or ML III (Leroy et al. 2014; Lyseng-Williamson 2014). Urine KS was elevated in all samples from patients with ML II or ML II/III and the average urine KS level in these patients (fourfold increase relative to age-matched controls) was approximately the same as that in MPS IVA patients (4.7-fold increase). Alternatively, although on average patients with ML III have elevated urine KS levels compared to controls ($p < 10^{-5}$), 16/39 of these patient had normal urine KS levels. Furthermore, the urine KS levels in ML III patients were significantly lower than those in ML II and ML II/III patients ($p < 10^{-5}$). Therefore, urine KS appears to correlate with the severity of skeletal involvement, as patients with MPS IVA and ML II or ML II/III have the highest urine KS levels of all patients analyzed in this study. A correlation between urine KS level and clinical severity has already been reported in MPS IVA patients (Tomatsu et al. 2004).

The explanation for elevated urine KS levels in patients with MPS I, MPS II, and fucosidosis is less clear. The enzymes deficient in these disorders are not directly involved in keratan sulfate catabolism. Tomatsu et al. (2005) have reported that keratan sulfate is elevated in both plasma and urine samples from patients with all mucopolysaccharidosis disorders (types I, II, III, IV, VI, and VII) compared to age-matched controls. It was speculated that elevated heparan sulfate directly inhibits GALNS enzyme activity, resulting

in a secondary elevation of keratan sulfate (Rowan et al. 2013; Tomatsu et al. 2014). This could explain the mild elevation of urine KS that was observed in MPS I and MPS II patients (Fig. 2). However, urine KS was only slightly elevated in 3/23 samples from MPS III patients, in whom heparan sulfate is the primary biomarker. Therefore, other explanations for the mild increase of urine KS in these patients should be explored. Greiling et al. demonstrated that patients with fucosidosis have elevated urine KS levels (Greiling et al. 1978). They speculated that the impaired removal of alpha-fucose residues from keratan sulfate due to alpha-fucosidase deficiency could lead to KS accumulation in these patients. However, further studies are needed to follow up on these findings.

Our study demonstrates that the quantitative measurement of urine KS via UPLC-MS/MS is a highly sensitive test for the detection and diagnosis of patients with MPS IVA. However, elevated urine KS levels are not specific to patients with this condition. Patients with other LSDs such as beta-galactosidase deficiency (including MPS IVB), Mucolipidosis II, II/III, or III, MPS I, MPS II, and fucosidosis may also excrete higher levels of urine KS than age-matched controls, in some cases to the same degree as patients with MPS IVA. Therefore, caution is advised when interpreting elevated urine KS results. The results should be interpreted within the context of the patient's clinical features, and an abnormal urine KS result should always be followed by enzyme analysis to make a definitive diagnosis.

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Conflict of Interest
The authors received funding from BioMarin Pharmaceuticals to support development of the urinary KS assay.

Synopsis

Urine keratan sulfate elevations are not specific to MPS IVA and may be found in other LSDs.

Compliance with Ethics Guidelines

Conflict of Interest

Tim Wood, Laura Pollard, and Katarzyna Ellsworth received support from BioMarin Pharmaceuticals to develop/validate the urine KS assay. Additionally they received speaker honoraria from BioMarin Pharmaceuticals. Sara Cathey declares no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included as part of the Longitudinal Studies of the Glycoproteinoses (NCT01891422). The remaining samples were sent to the clinical biochemical laboratory at the Greenwood Genetic Center for clinical testing. These samples were de-identified prior to use.

Animal Rights

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

Details of the Contributions of Individual Authors

Katarzyna Ellsworth performed the laboratory work for this study as well as the review of data. She was involved in the writing of the manuscript and preparation of figures.

Laura Pollard was involved in data analysis, manuscript editing, and the preparation of figures.

Tim Wood was involved in data analysis, manuscript editing, and the preparation of figures.

Sara Cathey was involved in data analysis, manuscript editing, and the preparation of figures. Dr. Cathey also provided clinical information about specific patients.

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RESEARCH REPORT

The Spectrum of PAH Mutations and Increase of Milder Forms of Phenylketonuria in Sweden During 1965–2014

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Abstract Newborn screening (NBS) for phenylketonuria (PKU) which has a continuum of disease severities has been performed for more than 50 years. The screening method has undergone a continuous development with not only improvements of the positive predictive value but also identification of milder forms of the disease. With the introduction of genetic testing the confirmation of the diagnosis has improved. The Swedish NBS is centralized to one laboratory, which also performs confirmatory testing.

Here we present the results of NBS for PKU in Sweden during 1965–2014 describing an increase in diagnosed patients and a shift in the spectrum of phenylalanine hydroxylase (*PAH*) mutations towards an increasing heterogeneity. Milder mutations common in southern Europe and the Middle East together with lowering of the recall level for phenylalanine (Phe) have led to a shift towards milder phenotypes among the patients identified by the screening program. The inclusion of a Phe and tyrosine (Tyr) ratio as an additional marker has improved the positive predictive value to the present 0.92. Also discussed is what impact earlier sampling has had on the prediction of disease severity, concluding that the shift of age at sampling from 72 to 48 h does not increase the risk of missing patients in need of treatment.

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Introduction

Phenylketonuria (PKU; OMIM #261600) is caused by a deficiency of the liver enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1), which catalyses the conversion of phenylalanine (Phe) to tyrosine (Tyr) and requires tetrahydrobiopterin (BH_4) as cofactor. Cofactor deficiencies constitute approximately 2% of patients with elevated Phe and need different treatment regimens (Blau et al. 2010).

Treatment of PKU from early infancy with a diet low in Phe and supplemented with Phe free amino acid mixture, vitamins and other trace elements results in normal development. Without early treatment neurological damage will occur. Neonatal screening for PKU is thus an important and successful intervention (Guthrie and Susi 1963).

The disease is inherited as an autosomal recessive and to date, 866 different mutations in the *PAH* gene have been described in the Human Gene Mutation Database (HGMD Professional 2016.1). Several of these cause less severe enzyme deficiency resulting in a continuum of severities of clinical disease.

The aim of the study here is to present the results of newborn screening (NBS) for PKU in Sweden 1965–2014. Since 1990 there has been an increase in the number of diagnosed patients and a shift of the mutation spectrum in the *PAH* gene due to a high rate of immigrants from non-Nordic countries which today represent 85% of Sweden's 1,600,000 immigrants (http://www.scb.se).

Materials and Methods

Screening Methods and Cut Off

NBS for PKU started in Sweden in 1965, shortly after the first NBS programme was implemented in Massachusetts, USA (Guthrie and Susi 1963). The programme is voluntary and the coverage in Sweden has been >98% since 1972, and near 100% during the last four decades.

The Swedish neonatal screening is centralized to Centre for Inherited Metabolic Diseases, Karolinska University Hospital and the birth rate has been between 90,000 and 120,000 annually, presently being 116,000 infants per year. The confirmatory testing is performed by the same laboratory or at Sahlgrenska University Hospital, Gothenburg and Skånes University Hospital, Lund.

Blood specimens have been collected on filter paper cards Whatman™ 903 or Ahlstrom grade 226. From 1965 to 2008 the samples were collected at age 3–6 days. In 2008 new guidelines were implemented in preparation for extended tandem mass spectrometry (MS/MS) screening, resulting in sampling as soon as possible after 48 h of age.

The screening methods used in Sweden for elevated Phe levels have changed continuously since the Guthrie bacterial inhibition assay was introduced in 1965. At the same time the cut off value for Phe has been lowered and the ratio of Phe to Tyr has been included as a second marker (Table 1).

The project was approved by the Regional Ethical Committee of Stockholm.

Patients

Until December 31, 2014, a total of 4,969,207 newborns have been screened for PKU and 314 patients, 139 females and 175 males have been diagnosed, giving an incidence of 1/15,800 (Table 1). Four cases of BH_4-deficiencies have also been identified; two with 6-pyruvoyl-tetrahydropetrin synthase deficiency, one with dihydropteridin reductase deficiency and one with GTP cyclohydrolase I deficiency. All four, except the one with GTP cyclohydrolase I deficiency, were caught in the screening programme. The patient with GTP cyclohydrolase I deficiency had a Phe value of 240 μmol/L in the screening sample taken on day four and the recall level was at that time 250 μmol/L.

The mutation study enrolled index cases from 279 families, 125 females and 154 males. Among these, 217 patients (193 index patients) were diagnosed when the recall level was Phe ≥360 μmol/L (1965–1975) or ≥250 μmol/L (1976–2002) and 97 patients (86 index patients) from 2003 when the recall level had been lowered to ≥180 μmol/L. From 2006 the ratio of Phe to Tyr ≥2.0 was an additional requirement. Of the 279 families, 32 have more than one child with PKU.

Table 1 Incidences of PKU and MHP in relation to different cut offs and screening methods

Year	Screening method	Second tier	Cut off Phe recall	Ratio Phe/Tyr	Screened newborns	PKU	MHP	Incidence PKU/MHP
1965–1975	Guthrie bacterial inhibition assay (BIA)		≥360 μmol/L (6 mg%)	–	945,523	50	6	1/16,900
1976–1996	Guthrie bacterial inhibition assay (BIA)	Ion exchange chromatography from 1978 to 1998	≥250 μmol/L (4 mg%)	–	2,717,978	128	33	1/16,900
1997–2002	Quantase phenylalanine kit (Shield Diagnostics)	No second tier from 1998	≥250 μmol/L	–				
2003–2004	Quantase phenylalanine kit (Shield Diagnostics)	Tandem mass spectrometry in house method from 2002	≥180 μmol/L	≥2.0 (2006-)	1,305,706	67	30	1/13,500
2005–2008	Tandem mass spectrometry in house method							
2009–2014	Tandem mass spectrometry non-derivatized kit PerkinElmer							
					4,969,207	245	69	1/15,800

Also included were 33 immigrant/adoptive patients (14 females and 19 males) born between 1966 and 2011 and diagnosed either in their countries of birth or through the Swedish NBS program which is offered to immigrant and adoptive children under the age of 18 years on arrival in Sweden. From 2014 this was lowered to under the age of 8 years.

Genetic Analysis

Genetic analysis has been a part of the routine confirmatory testing of all PKU patients during the past 20 years. Patients born before genetic analyses became a standard procedure were genotyped retrospectively. Eighty-eight index cases born 1966–1985 were included in a previous study (Svensson et al. 1993). The mutation detection rate in that study was 73% and patients not fully genotyped were reanalysed when new methods became available.

Genetic analyses have not been performed in 9 (3.2%) out of the 279 index cases diagnosed by NBS and for another 4 (1.4%) only one mutation has been identified. Mutation analysis has not been requested for seven of the 33 immigrant/adoptive patients.

Genomic DNA was isolated from whole blood following standard procedures which have varied. Since routine genotyping has been in use, screening for mutations has been performed by denaturing gradient gel electrophoresis) or polymerase chain reaction amplification, the latter method most frequently used, followed by direct sequencing. Multiplex ligation-dependent probe amplification was performed to detect large genomic deletions in patients without identified mutations on one or both alleles. Confirmation by carrier analysis of parents was performed in more than 80% of the families.

Mutation nomenclature follows the guidelines and recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen) and novel mutations were validated using the program Mutalyzer (http://mutalyzer.nl/2.0/). Complementary DNA (cDNA) numbering commences from the ATG start codon, where +1 is the A of the ATG translation initiation codon (NM_000271.1).

Classification of Patients with MHP, Mild PKU and Classical PKU

Classification of patients with hyperphenylalaninemia (HPA) according to Phe levels is subjective and several classifications are used, from Phe values ≤360 to ≤1000 μmol/L (Hanley 2011).

We have chosen to define patients with at least one mutation known to be associated with non-PKU mild hyperphenylalaninemia (MHP) or having two mild mutations resulting in repeat Phe levels <500 μmol/L on a normal diet to be classified as MHP patients. PKU patients are classified as classical PKU when they have two null mutations or Phe levels ≥1200 μmol/L in the NBS sample. Patients in between are classified as having mild PKU.

Results and Discussion

Incidence of PKU/MHP in Sweden

During the period 1965 to December 2014, close to five million newborns were screened for PKU in Sweden. PKU was confirmed in 245 newborns and MHP in 69.

The merged incidence of MHP and PKU was 1/15 800, which is similar to what has been reported for many other European countries (Loeber 2007). The ratio of MHP to PKU was 1–3.5. Comparing the period 1965–1989 with 1990–2014, the incidence of PKU/MHP patients has increased from 1/18 300 to 1/14 200 equal to an increase from 5.5 to 7.1 per 100.000 born. This is almost solely due to an increase of patients diagnosed with MHP (Table 2).

Screening Results and Recall Levels

The change in incidence shows the same pattern with an increase in the number of patients diagnosed with MHP when the PKU population is divided according to the different cut offs used for recalls in the neonatal screening (Table 1).

The infants recalled with Phe values <250 μmol/L are of special interest. Of the 15 infants in this cohort only one was sampled after 3 days of age, all the others were sampled at 2–3 days of age (Supplementary 1).

Six patients had Phe values <250 μmol/L also in the repeat samples taken at recall. Three additional infants had a Phe to Tyr ratio less than 2.0 in the sample taken at recall. All but two have at least one allele associated with MHP. Two of the patients were homozygous for p.Leu48Ser (c.143T>C) and p.Gly46Ser (c.136G>A), respectively. The former is known to give an inconsistent phenotype, whilst p.Gly46Ser is associated with a severe phenotype (Djordjevic et al. 2013; Eiken et al. 1996).

Table 2 Incidence of PKU and MHP during the two screening periods

Year	Screened newborns	PKU	MHP	Incidence PKU/MHP
1965–1989	2,322,536	111	16	1/18,300
1990–2014	2,646,671	134	53	1/14,200
1990–2014[a]	*2,646,671*	*134*	*38*	*1/15,400*

[a] Incidences re-calculated excluding 15 MHP patients recalled after lowering the cut off for Phe to ≥180 μmol/L

Taking into account the Phe values and Phe to Tyr ratios seen in the repeat tests of these 15 children, eight of them would not have been recalled as presumptive positive PKU/MHP cases. Their Phe values and Phe to Tyr ratios in the repeat dried blood samples (DBS) did not fulfil the criteria for recall (Phe ≥180 μmol/L and ratio Phe to Tyr at or above 2.0). All eight infants are compound heterozygous for known MHP mutations; p.Ser87Arg (c.261C>A), p.Asp145Val (c.434A>T), p.Arg176Leu (c.527G>T), p.Ala300Ser (c.898G>T), p.Ala322Gly (c.965C>G) and p.Ala403Val (c.1208C>T). When the incidence is re-calculated for the period 1990–2014 excluding the 15 MHP patients recalled with Phe values <250 μmol/L the incidence of MHP patients is still higher than for the period 1965–1989 (Table 2). Lowering the cut off thus cannot explain the increase of detected MHP-patients after 1989.

The increase of MHP patients has raised the question whether we now pick up and treat patients unnecessarily. In the minireview: "Non-PKU mild hyperphenylalaninemia (MHP) – the dilemma", the author did not find any evidence for need of treatment to gain a normal outcome in this group of patients (Hanley 2011).

In Sweden we have seen an increase in the number of patients with a true positive screening test for PKU/MHP defined as carrying two mutations giving persistently elevated Phe values. Comparing the incidence according to the cut offs for Phe, no changes of the total incidence of true positive cases could be seen when the cut off was lowered from ≥360 to ≥250 μmol/L in 1976 (Table 1). From 2003 when the cut off was lowered to ≥180 μmol/L an increase similar to the one seen when the two periods 1965–1989 and 1990–2014 were compared was noted. It is important to have in mind that in 2006 the ratio of Phe to Tyr was added as an additional marker to better distinguish between infants with increased Phe levels due to PKU/MHP and infants with liver disease or having treatment with intravenous amino acid solutions.

Lowering the cut off to levels lower than 180 μmol/L would probably not improve the PKU screening. This is supported in an article from Italy (Trunzo et al. 2013), where they use a Phe cut off ≥120 μmol/L. Of the 30 infants included, 26 were recalled with Phe values 120–180 μmol/L. Eight of these patients were compound heterozygous or homozygous for MHP mutations, in another nine infants only one mutation was detected and in 6 infants no mutations could be found.

Phe Values in First and Second Sampling in Patients with MHP, Mild PKU and Classical PKU

Figure 1a shows the Phe values in true positive infants whose screening samples were collected at age 2–3 days in comparison with those whose samples were collected at age 4–5 days. Included are also the Phe values of the second DBS samples taken when the infants were recalled. The infants were recalled at ages ranging from 5 to 10 days.

Phe values for the MHP group did not change with sampling at an older age. In the group with mild PKU there is an increase between sampling at age 2–3 days and age 4–5 days and later. This is also true in the group with classical PKU.

The ratio between Phe and Tyr was essentially unchanged between the ages at sampling in the MHP group and interestingly it did not change much between the ages at sampling in the group of mild PKU although it was higher in this group. In the group with classical PKU patients there was a clear influence of age at sampling (Fig. 1b).

Impact of Earlier Sampling

In 2008 the age at sampling was brought forward to as soon as possible after 48 h of age. Data from our laboratory shows that the mean age of sampling has decreased from 4.0 (year 2007) to 2.7 (year 2014) days. Earlier sampling may have an effect on the outcome of NBS for PKU since the Phe values may not have had time to reach the highest pre-treatment levels. In the MHP group no differences could be seen when comparing the Phe levels and Phe to Tyr ratios in the screening sample of the three age groups (Fig. 1a, b). In the mild PKU group an increase in the Phe values could be seen when the samples were taken at age 4–5 days compared with 2–3 days. This group seems to reach the highest pre-treatment values at age 4–5 days since there is no difference between samples taken at age ≥6 days and samples taken at age 4–5 days. The Phe to Tyr ratio did not change over time in this group.

Earlier sampling leads to lower Phe values in the first screening sample but does not necessarily lead to any disadvantages for infants with MHP since some who do not need treatment may go undetected. Another issue of earlier sampling is the risk of missing a woman who as an adult may have Phe levels detrimental for the foetus during pregnancy (Smith et al. 1990). A third factor to consider is the detection of infants with cofactor deficiencies. Some of these patients tend to have Phe levels in the MHP region when the neonatal screening sample is taken (Opladen et al. 2012).

Positive Predictive Value

The positive predictive value of the screening for PKU was 0.34 before the ratio of Phe to Tyr was added as a second marker. The majority of the false positives were premature or sick newborns on treatment with intravenous amino acid solutions. In 2006 when the ratio Phe to Tyr ≥2.0 was included, the positive predictive value increased to 0.92 much due to the fact that newborns treated with intravenous amino acid solutions could be sorted out (Table 3). The exclusion of

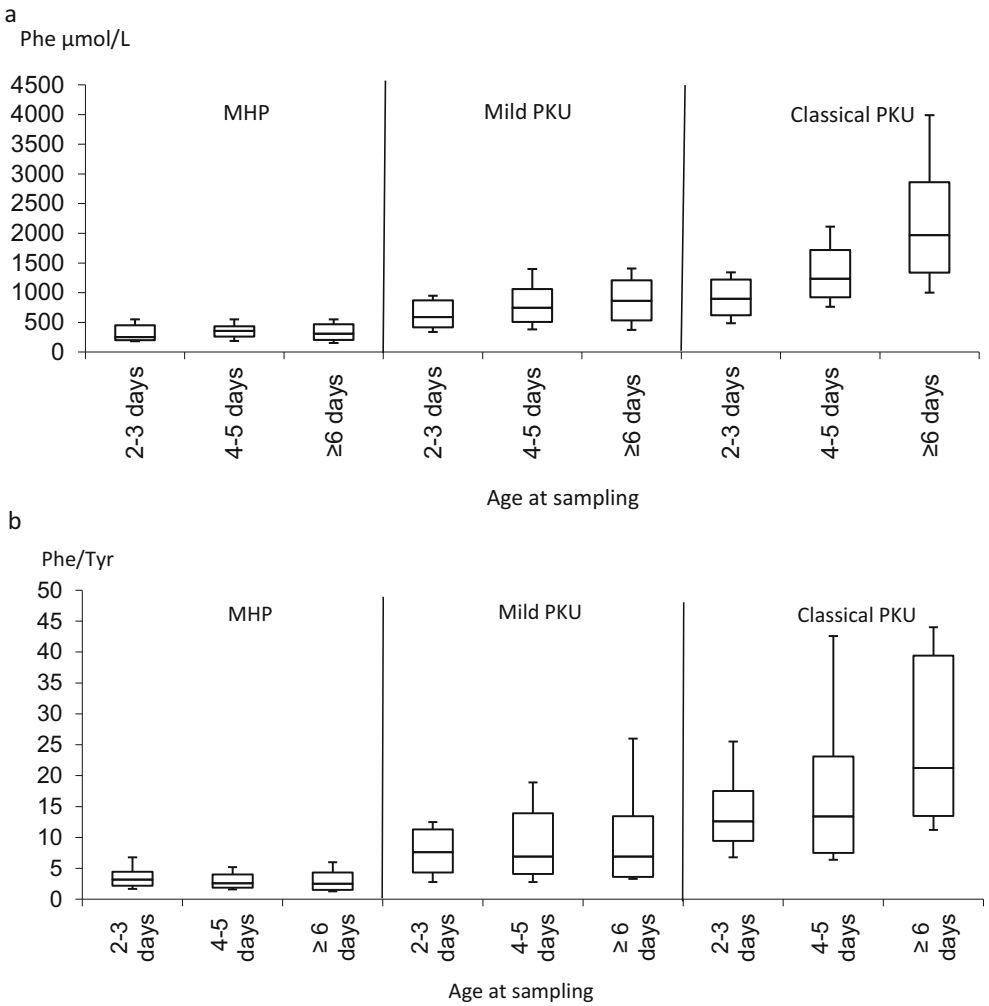

Fig. 1 Phe values and Phe to Tyr ratios divided into different groups according to age at sampling. **a** Phe values in screening samples taken at ages 2–3, 4–5 and ≥6 days, divided into groups with MHP, mild PKU and classical PKU. **b** Phe to Tyr ratio in screening samples taken at ages 2–3, 4–5 and ≥6 days, divided into groups with MHP, mild PKU and classical PKU. The *horizontal lines* in the boxes and the lines outside the boxes indicate median and the minimum and maximum values, respectively. The *top* and *bottom* of each box represent the 90th and 10th percentiles, respectively

Table 3 Positive predictive value in the screening for PKU

Years	Screened newborns	True positive	False positive	Positive predicted value	Ratio Phe/Tyr
1965–2005	3,965,693	245	471	0.34	No
2006–2014	1,003,514	71	6	0.92	Yes

these patients was further improved when the expanded MS/MS screening was implemented in 2010 making it possible to evaluate a total of 11 amino acids. Newborns on treatment with intravenous amino acids and a positive screening for PKU are no longer recalled as presumptive PKU cases; instead a new sample, taken 24 h after completed treatment with amino acids is requested by the laboratory.

Mutation Analysis

The second major change that has had an impact on the increase in the number of MHP patients is the change of mutation spectrum. The mutations detected in patients born from 1990 show an increasing frequency of less severe mutations where MHP mutations represent 14.1% of all mutations compared to 6.9% before 1990. There has been simultaneously a decrease of the frequency of the two most common classical PKU mutations, p.Arg408Trp (c.1222C>T) and c.1315+1G>A, and the milder mutation, p.Tyr414Cys (c.1241A>G), previously associated with MHP but now considered a mild PKU mutation. The prevalence of the mutation p.Arg261Gln (c.782G>A), which is associated with less severe PKU as well as classical PKU, has increased (Fig. 2).

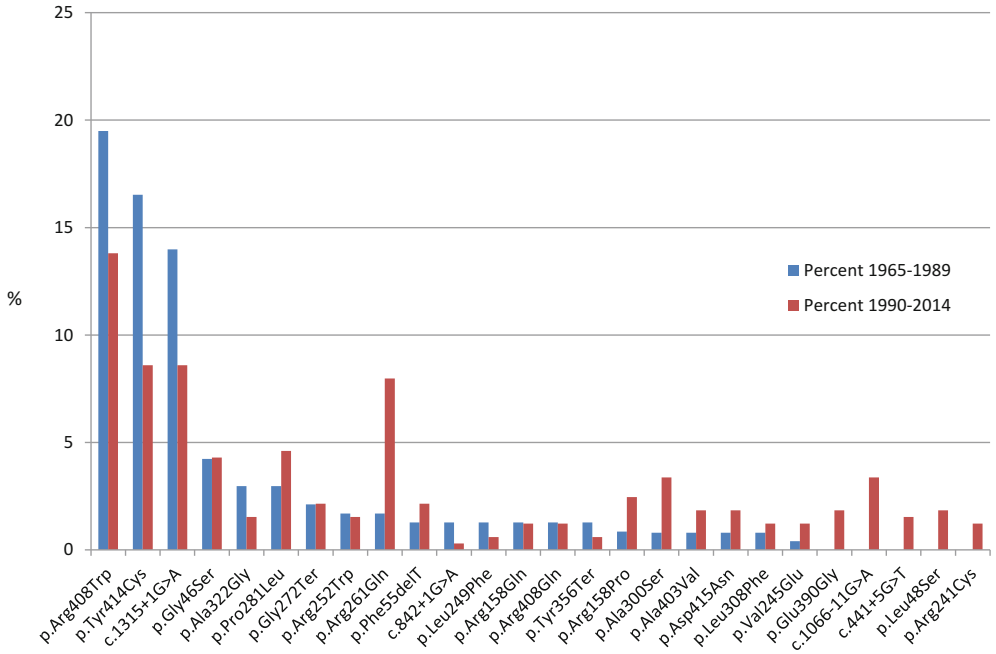

Fig. 2 Mutations with a relative frequency >1.0% of all alleles in the NBS group for the periods 1965–1989 and 1990–2014

Mutations were detected in 536 out of 540 independent alleles (270 index patients) giving a mutation detection rate of 99.3%. For nine additional index patients no DNA was available and 26 of 33 immigrant/adoptive index cases were fully genotyped (52 alleles).

Ninety-four disease causing mutations were detected in the Swedish PKU/MHP-population: 62 missense mutations (66.0%), 16 intronic variations (17.0%), 13 deletions (13.8%) and 3 nonsense mutations (3.2%). A total of 17 of the 61 missense mutations were associated with MHP (Supplementary 2).

Five variants not described earlier in a peer-reviewed journal were detected. The missense variants p.Tyr77His (c.229T>C) and p.Asp143Val (c.428A>T) were run in the functional prediction programs PolyPhen-2, (http://genetics.bwh.harvard.edu/pph2), PROVEAN (http://provean.jcvi.org) and Mutation Taster (http://www.mutationtaster.org/) and both variants were predicted to be disease causing in all three programs. The intron variants c.843-13_843-10delTTCT, c.970-1G>T and c.1315+5G>A were run in Mutation Taster and were all three predicted disease causing. In Fruitfly (http://www.fruitfly.org/seq_tools/splice.html) one was predicted to cause an abolished splice site (c.970-1G>T) whilst the other two resulted in weakened splice sites (c.843-13_843-10delTTCT and c.1315+5G>A). Clinically the five variants are predicted to be associated with classical PKU; c.843-13_843-10delTTCT, c.970-1G>T and c.1315+5G>A, with mild PKU; p.Tyr77His (c.229T>C) and with MHP; p.Asp143Val (c.428A>T), respectively.

Among the 94 different PAH mutations found in the study group, 69 were found in the cohort identified during 1990–2014

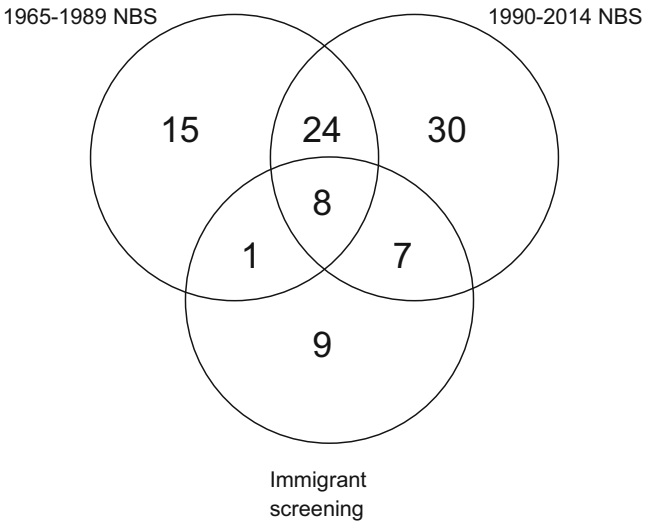

Fig. 3 The Venn diagram describes the heterogeneity of mutations detected in the *PAH*-gene with only 32 mutations being common for the two NBS periods

as compared to 48 during 1965–1989 (Fig. 3). Only 32 of the 94 different mutations were found in both birth cohorts. Out of the 32 mutations, eight were also found in the immigrant/adoptive group. This further more confirms the increase of the heterogeneity of mutations detected in the *PAH* gene.

Shown in Fig. 2 are mutations in the NBS group (index cases) with a relative frequency of >1.0% of all alleles for the periods 1965–1989 and 1990–2014, respectively. The three most frequent mutations, p.Arg408Trp, p.Tyr414Cys and c.1315+1A>G, are the same for both periods, although

the mutations are occurring less frequently in the second period. Four well-known mutations associated with MHP; p.Ala300Ser, p.Ala403Val, p.Asp415Asn (c.1243G>A) and p.Glu390Gly (c.1169A>G) have added together been detected on 8.8% of all alleles during 1990–2014. Five mutations, p.Leu48Ser, c.441+5G>T, p.Arg241Cys, c.1066-11G>A and p.Glu390Gly which were not seen before 1990, are now detected at an increasing frequency. The two mutations showing the highest increase for the two periods are p.Arg261Gln (+6.2%) and c.1066-11G>A (+3.8%), both of which are common in the southern Europe (Zschocke 2003).

The three mutations with the highest frequency among the 26 immigrant/adoption cases were c.168+5G>C (9.6%), p.Arg408Trp (9.6%) and c.1066-11G>A (11.5%). Three of the novel variants were detected in this group, p.Tyr77His, c.970G-1G>T and c.843-13_843-10delTTCT.

In the total Swedish PKU population (NBS and immigration) more than 30 countries are represented. The major immigration influence on the mutation spectrum comes from Turkey, Iraq, Syria and Lebanon. The most frequently occurring mutations in these patients are p.Arg261Gln, p.Arg408Trp, c.1066-11G>A and p.Ala300Ser. All but p.Arg408Trp show an increased frequency also in patients detected by NBS after 1990.

The Change of Homozygosity

During 1965–1989 the two most prevalently occurring homozygous mutations in patients were the same as the mutations with the highest frequencies, p.Arg408Trp and p.Tyr414Cys. These two mutations occurred at the highest frequencies also in the most recent period but were only found in a homozygous state in one patient each. Instead the two most prevalent homozygous genotypes in this period were homozygosity for p.Arg261Gln and c.1066-11G>A. The percentage of patients being homozygous for a mutation was the same for the periods, 18.1% (21 patients) and 16.8% (27 patients), respectively. In the first period one homozygous patient had parents born abroad whilst in the second period 14 patients had parents born abroad. This change could probably be explained by the fact that the native Swedish population has become more movable during the past decades, reducing geographic isolation, whilst immigration from countries with a high rate of consanguineous marriages has become more common.

Conclusions

The Swedish spectrum of *PAH* mutations has during the past 25 years undergone a major change towards becoming more heterogeneous, reflecting the present structure of the Swedish population. Milder mutations common in southern Europe and in the Middle East together with lowering of the cut off values for Phe have led to a shift towards milder genotypes and phenotypes among the PKU patients detected by NBS. The inclusion of the Phe to Tyr ratio as an additional marker has led to an increase in positive predictive value and it also provides a tool to differentiate between mild and classical PKU. Lowering the cut off to Phe below 180 μmol/L most probably will increase the number of false positives and increase the number of newborns with MHP not needing treatment. The lower Phe value in the first screening sample due to earlier sampling age does not necessarily lead to any disadvantages. It does not lead to missed cases in need of treatment and enables children with very mild MHP to remain undiscovered.

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Concise Sentence

Since the start of NBS for PKU in Sweden due to the use of lower cut offs a major shift towards detecting patients with milder mutations and less severe disease are becoming more common in the Swedish population.

Contribution of Individual Authors

Experimental design and execution: AO and HB
Manuscript preparation: AO and UvD
Data analysis: AO, AW and UvD
Clinical investigation: AN and RZ
All authors have read and revised the final manuscript.
Ulrika von Döbeln serves as guarantor for the article, accepts full responsibility for the work, has access to the data and controls the decision to publish.

Conflict of Interest

The authors have nothing to declare.

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Compliance with Ethics Guidelines

Genetic investigation of inborn errors of metabolism, approved by Regional Ethical Committee of Stockholm, 2008/351-31. Informed consent was not required since individual patients cannot be identified in the manuscript.

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CASE REPORT

DMP1-CDG (CDG1e) with Significant Gastrointestinal Manifestations; Phenotype and Genotype Expansion

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Abstract The literature describes eight cases of mutations in the *DPM1* gene generating DMP1-CDG, causing similar phenotype of early onset seizures, microcephaly and developmental delay. Investigations of these patients revealed associated abnormal findings on brain imaging, elevated CK, abnormal clotting factors and mildly deranged serum transaminases. We describe the ninth case of DMP1-CDG, whose clinical presentation includes severe gastrointestinal involvement, i.e. food protein induced enterocolitis syndrome (FPIES). Gastrointestinal manifestations (GIT) of the congenital glycosylation disorders have included deranged liver function, hepatomegaly, liver fibrosis, steatosis and protein-losing enteropathy. This is the first report of a congenital glycosylation disorder being associated with FPIES.

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Introduction

Glycosylation is the post-translational modification of proteins and lipids which occurs in the membrane compartment of endoplasmic reticulum (ER), Golgi complex and lysosomes of cells (Scott et al. 2014; Freitas and Cayuela 2000; Cumming 1992; Meynial-Salles and Combes 1996; Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004; Ashida et al. 2006; Schenk et al. 2001; Maeda et al. 2000), and involves the addition of a glycan oligosaccharide to a protein and lipid backbone (Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004; Ashida et al. 2006; Schenk et al. 2001; Maeda et al. 2000). These glycans affect the conformation and activity of these 'background' proteins, and the resulting glycoproteins are involved in many cellular and physiological processes, such as signal transduction and immune response (Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004; Ashida et al. 2006; Schenk et al. 2001; Maeda et al. 2000). Congenital disorders of glycosylation (CDGs) are rare genetic disorders which affect this post-translational modification of proteins, and various subtypes exist with protean clinical presentations (Maeda et al. 2000). To date, eight cases (Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004) of DMP1-CDG (OMIM 608799) have been described in the literature.

The CDGs exhibit protean clinical manifestations. Previously reported gastrointestinal manifestations (GIT) of the congenital glycosylation disorders include deranged liver function with elevated transaminases, hepatomegaly, liver fibrosis, steatosis and protein-losing enteropathy, as well as non-specific feeding problems, vomiting, diarrhoea and failure-to-thrive (Scott et al. 2014). Protein-losing enteropathy is common in children with MPI-CDG (OMIM 602579, CDG-1b) and has also been reported in types PMM2-CDG (OMIM601785, CDG-1a), ALG6-CDG (OMIM 603147, CDG-1c), ALG12-CDG (OMIM 607143, CDG-1g), ALG8-CDG (OMIM 608104, CDG-1h), as well as in several cases of unclassified CDG (CDG-1x) (Höck et al. 2015; Damen et al. 2004; Agarwal et al. 2007; McKenzie et al. 2007; Kranz et al. 2007; Krasnewich 2014).

Food protein induced enterocolitis syndrome (FPIES) has not been reported in a CDG patient to the best of our knowledge. FPIES is a non-IgE-mediated reaction to food proteins, most commonly cows' milk, soy or rice, which results in profuse vomiting and diarrhoea commencing 1–3 h after ingestion. Onset is in the first year of life, and reactions can be severe, inducing dehydration, hypotension and acidosis. Herein we present a 9th case of DMP1-CDG, who expands the clinical phenotype to involve severe GIT involvement in the form of FPIES.

Clinical Report

Our case is a 10-year-old girl from a non-consanguineous Caucasian family. She was delivered via a spontaneous vaginal delivery at 40 weeks gestation after an uncomplicated pregnancy. Apgar scores were 9 at 1 min and 10 at 5 min. Her birth weight was 3.8 kg and head circumference was 35 cm and length 50 cm.

Her early life was complicated by severe and refractory seizures, global developmental delay, and postnatal microcephaly with her head circumference tracking below the 3rd percentile. Seizures occurred from infancy and were semiologically diverse, including myoclonic, atonic, tonic and absence events. Electroencephalograms demonstrated bilateral epileptogenicity with emphasis on the bilateral central and left posterior regions and a suggestion of focal onset for some of the events, particularly during sleep. Multiple anticonvulsants failed to achieve satisfactory control, and a trial of the ketogenic diet was also unsuccessful. In recent years, however, her seizures have become quiescent, with tonic convulsions occurring once or twice a year and occasional absence seizures which do not affect her activities of daily living or quality of life. She has been weaned off anticonvulsants. She is ambulatory over short distances, has lower limb spasticity, has no verbal expressive language but has receptive and nonverbal language. A combination of oral aversion and vomiting generated a requirement for gastrostomy feeding from an early age. Dysmorphic features include a depressed nasal bridge, prominent glabella, down slanting palpebral fissures, hypertelorism, smooth philtrum, carp shaped mouth, bilateral knee contractures and long great toes with a wide sandal gap.

An MRI scan of her brain at 3 years of age was normal. Consistently elevated creatine kinase (CK) levels, combined with the dysmorphic features prompted consideration of a CDG. APOC-III mass spectrometry revealed a normal pattern. Transferrin isoforms revealed increased levels of disialotransferrin and no asialotransferrin, which is an abnormal pattern typical of *N*-glycosylation defects. DMP1-CDG was suspected and subsequently confirmed at the molecular level.

Utilising next generation sequencing (Otogenetics) we identified two sequence variations in the DPMI gene, i.e. NM_003859 c.1A>C p.(Met1Leu) which was inherited from her father, and an NM_003859 c.274C>G p.(Arg92-Gly) which was inherited from her mother. These mutations were confirmed in the proband and the parents via sanger sequencing (myGenomics). The NM_003859 c.1A>C p.(Met1Leu) mutation is reported in dbSNP with an allele frequency of 0.0002 and a BLOSUM score of 2, and is predicted to interfere with translation initiation. There are however no reports of this variant associated with DMP1-CDG. The NM_003859 c.274C>G p.(Arg92Gly) variant has been previously reported to be associated with DMP1-CDG (Kim et al. 2000).

The patient had a history of profuse vomiting and irritability in the first year of life, which raised suspicion of a cows' milk protein allergy. Extensive skin prick and patch allergy testing (including to pea, soy, rice and target formulas) have all proven negative to date. The first documented episode of FPIES occurred at age 7, when Nutrini Energy formula was switched to Nutrison. After resolution, Nutrini was tolerated for 2 feeds, and the reaction recurred almost immediately when Nutrison was subsequently reintroduced. In retrospect, a similar attack had occurred at almost 4 years of age, when she was inadvertently given a ketogenic formula, possibly Ketocal, instead of her usual formula PediaSure. A ketogenic diet with Ross Carbohydrate Free formula had been used in the past without incident. FPIES was characterised by hypersalivation during the feed, followed by severe vomiting with haematemesis and profuse mucousy diarrhoea with frank PR bleeding. These acute episodes occurred within 90 min of changing formula, and often left the patient very lethargic and required hospitalisation for intravenous fluid rehydration on three occasions.

Aside from her inborn error of metabolism, this child had multiple risk factors for food protein allergies and intolerances. This included her personal history of severe irritability

and vomiting in infancy, suggesting cows' milk allergy. She had a tendency to decompensate rapidly during gastroenteritis infections, suggesting underlying subtle chronic food protein enteropathy. Her half-siblings had similar symptoms, which in one child resolved on a dairy free formula.

Discussion

Dolichol phosphate mannose (DPM) is the sole donor substrate in various glycosylation processes, and has recently shown to be involved in four glycosylation processes: *N*-glycosylation, *O*-mannosylation, *C*-mannosylation and glycophosphatidyl inositol (GPI) anchor assembly (Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004; Ashida et al. 2006; Schenk et al. 2001; Maeda et al. 2000; Lefeber et al. 2009). DPM is synthesised from GDP-mannose and dolichol phosphate, which is catalysed by the enzyme DPM-synthase (Freitas and Cayuela 2000; Cumming 1992; Meynial-Salles and Combes 1996; Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Ashida et al. 2006; Schenk et al. 2001; Maeda et al. 2000). This membrane-bound catalyst is composed of 3 protein subunits (DPM1, DPM2 and DPM3); of which DMP1 (a soluble cytoplasmic catalytic subunit) is anchored to the ER membrane and stabilised by DPM2 and DPM3 (Freitas and Cayuela 2000; Cumming 1992; Meynial-Salles and Combes 1996; Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Ashida et al. 2006). In DMP1-CDG, the biosynthesis of DPM is disrupted through a defect in the DPM-synthase (specifically the gene for the DMP1 subunit) (Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004; Ashida et al. 2006). To date, eight cases (Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004) of DMP1-CDG have been described in the literature, with varying *DMP1* mutations. The clinical features of these patients are summarised in Table 1. A clue to the diagnosis of DMP1-CDG is the elevated CK in keeping with an alpha-dystroglycanopathy (Meynial-Salles and Combes 1996; Yang et al. 2013). Previous links have been suggested between homozygous DPM3 mutations and DPM2-CDG and alpha-dystroglycan-type muscular dystrophy (Lefeber et al. 2009; Barone et al. 2012).

Our patient experienced episodes resembling severe FPIES, which is a non-IgE-mediated allergy to certain foods triggers (McDonald et al. 1984; Sicherer 2000; Mane and Bahna 2014; Fiocchi et al. 2014; Caubet and Nowak-Wegrzyn 2007; Katz et al. 2011). The pathogenesis of FPIES in infants and young children is not completely understood (Sicherer 2000; Mane and Bahna 2014). Several hypotheses have been proposed, and some research has suggested the involvement of antigen-specific T-cells and their pro-inflammatory cytokines which may act to alter the permeability of the intestinal barrier (Mane and Bahna 2014; Fiocchi et al. 2014; Caubet and Nowak-Wegrzyn 2007). The age of onset of FPIES in our patient outside of the infant period is very unusual, and at least 90% of FPIES-affected children experience resolution by 3 years which is not the case with our patient (Katz et al. 2011).

The most recently proposed diagnostic criteria for FPIES are as follows (Miceli Sopo et al 2013) (Miceli Sopo et al. 2013):

1. Less than 2 years of age at first presentation (frequent feature but not mandatory).
2. Exposure to the incriminated food elicits repetitive and important vomiting, pallor, hyporeactivity and lethargy within 2–4 h. Diarrhoea may be present, much less frequently and later. The symptoms last a few hours, usually less than 6 h.
3. Absence of symptoms that may suggest an IgE-mediated reaction.
4. Avoidance of the offending protein in the diet results in resolution of symptoms.
5. Re-exposure or oral food challenge elicits typical symptoms within 2–4 h. Two typical episodes are needed to deliver the definitive diagnosis.

The diagnosis rests entirely on the satisfaction of these clinical criteria, although there are frequently observed but non-specific laboratory findings such as neutrophilia and thrombophilia (Miceli Sopo et al. 2013). Our patient does meet these new criteria which have dispensed with the previously inflexible requirement for presentation in infancy. A neutrophilia was noted during episodes, but no thrombocytosis.

The possibility that our patient displays an incidental association between DPM1-CDG and FPIES cannot be excluded. The cumulative incidence of FPIES is significant, with a 0.34% cumulative incidence in one large cohort study (Katz et al. 2011). The likelihood of a chance association between a very rare disorder and an uncommon one is low; however, such associations do occur and in monogenic disorders their detection is increasing with new diagnostic technology. However, the unusual presentation of FPIES in our patient with a late age at onset and persistence at age 7 poses questions about whether the development of FPIES, its longevity, or both are related to her glycosylation disorder.

In PMM2-CDG, where GIT are common, inflammatory cells and intestinal villous blunting have been observed on microscopic examination of the small intestine. The changes are similar to that seen in food allergy, but no response was observed to elimination diets (Kristianson et al. 1998). The mechanism for protein-losing enteropathy

Table 1 Review of reported cases of DPMI-CDG

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Current Case
DMP1 mutations	c.274C>G (R92G) homozygous	c.274C>G/13bp deletion (c331-343)	c.274C>G/c.628delC	Homozygous C742T>C (S248P)	Homozygous g.IVS4-5T>A	Homozygous g.IVS4-5T>A	c.455G>T pGly152Val /302bp deletion	c.1A>C p.(Met1Leu) c.274C>G p.(Arg92Gly)
CNS	Seizures, hypotonia, developmental delay	Seizures, hypotonia, developmental delay	Seizures, hypotonia, developmental delay	Seizures, hypotonia, developmental delay, neonatal encephalopathy, ataxia	Febrile seizures, hypotonia, developmental delay, ataxia	Hypotonia, developmental delay	Seizures, hypotonia, developmental delay	Seizures, developmental delay, Spasticity
Microcephaly	+ (postnatal)	+	+ (postnatal)	+ (postnatal)	+ (by 6 months)	+ (postnatal)	borderline	+ (postnatal)
MRI brain	Delayed myelination?	Delayed myelination, hypoplastic L inferior frontal lobe, cerebellar atrophy	Widening of fronto-temporal lobe and ventricles	Transient basal ganglia hypodensities, delayed myelination	*N* at 4.5 years, Later mild pontocerebellar atrophy, dentate nucleus hypersignal	Cerebral atrophy, dentate nucleus hypersignal	Mild cerebral volume loss Abnormal/delayed myelination Multiple abnormal T2 hyperintensities	Normal at 3 years
Dysmorphic features	Flat occiput, flat nasal bridge, hyperteloric, high narrow palate, inverted V mouth, short limbs	Inverted nipples	Gothic palate, micro-genitalia hyperteloric dysplastic nails contractures	Trigonocephaly hypertelorism gothic palate micrognathia smooth philtrum prominent fatty cheeks, long 1st toe, sandal gap	Nil	Nil	Camptodactyly	Low nasal bridge Hypertelorism Smooth philtrum Prominent glabella Carp shaped mouth Downslanting palpebral fissures Contractures (knees) Long great toes, wide sandal gap
Muscle	CK+++	CK+	CK +++	CK +	Normal	Normal	CK+++	CK+
Eyes/vision	Cortical blindness	Cortical blindness, strabismus	Absent visual fixation	Optic atrophy Alternating endotropia, nystagmus	Retinopathy	Strabismus, nystagmus	Partial optic nerve atrophy	Normal
GIT	Low antithrombin III	Low antithrombin III, protein C and S, elevated transaminases	Elevated transaminases, hepatosplenomegaly	Low antithrombin, protein C and S, factor XI	Decreased protein C and S	Slight hepatomegaly	Elevated transaminases, low protein C and S, antithrombin III, elevated D dimer	FPIES
Other		FTT			Peripheral neuropathy FTT	FTT		
Reference	Kim et al. (2000)	Kim et al. (2000)	Imbach et al. (2000)	García-Silva et al. (2004)	Dancourt et al. (2006)	Dancourt et al. (2006)	Yang et al. (2013)	Current

in CDGs and other disorders is thought to involve loss of heparan sulphate proteoglycans from the basolateral surfaces of intestinal epithelial cells which directly leads to protein leakage as well as increasing the effects of other factors such as inflammatory cytokines (Bode and Freeze 2006). It is possible that a perturbed intestinal *N*-glycan microenvironment may have predisposed our patient to develop sensitisation to food allergens due to increased gut permeability and/or an altered cytokine environment. This may have contributed to her more severe presentation with FPIES, compared to family members with more subtle food reactions. Gastrointestinal involvement has not been described in any of the DPM1-CDG patients recorded in the current literature (Yang et al. 2013; Dancourt et al. 2006; Imbach et al. 2000; Kim et al. 2000; García-Silva et al. 2004), and as such our patient differs phenotypically from the other patients in the spectrum of this rare disease.

Compliance with Ethics Guidelines

Conflict of Interest

David Coman, Carolyn Bursle, Dominique Brown, Frances Connor, John Cardinal and Sophie Calvert declare that they have no conflicts of interest

Informed Consent

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

Author Contributions

Dr Carolyn Bursle has driven the manuscript development, and been involved in patient care.

Dr Dominique Brown has driven the manuscript development, and been involved in patient care.

Professor David Coman is a metabolic physician coordinating care of patient, and has coordinated the manuscript development and design.

Dr Sophie Calvert is a Paediatric Neurologist, providing neurology care for the patient, and has been involved in the manuscript development.

Dr John Cardinal is a Medical Scientist who has been involved in the manuscript development.

Dr Frances Connor is a Paediatric Gastroenterologist involved in the patient's care and has been involved in the manuscript development.

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RESEARCH REPORT

Classical Galactosaemia and CDG, the N-Glycosylation Interface. A Review

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Abstract Classical galactosaemia is a rare disorder of carbohydrate metabolism caused by galactose-1-phosphate uridyltransferase (GALT) deficiency (EC 2.7.7.12). The disease is life threatening if left untreated in neonates and the only available treatment option is a long-term galactose restricted diet. While this is lifesaving in the neonate, complications persist in treated individuals, and the cause of these, despite early initiation of treatment, and shared *GALT* genotypes remain poorly understood. Systemic abnormal glycosylation has been proposed to contribute substantially to the ongoing pathophysiology. The gross N-glycosylation assembly defects observed in the untreated neonate correct over time with treatment. However, N-glycosylation processing defects persist in treated children and adults.

Congenital disorders of glycosylation (CDG) are a large group of over 100 inherited disorders affecting largely N- and O-glycosylation.

In this review, we compare the clinical features observed in galactosaemia with a number of predominant CDG conditions.

We also summarize the N-glycosylation abnormalities, which we have described in galactosaemia adult and paediatric patients, using an automated high-throughput HILIC-UPLC analysis of galactose incorporation into serum IgG with analysis of the corresponding N-glycan gene expression patterns and the affected pathways.

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Introduction

Inborn errors of metabolism to include the primary Congenital Disorders of Glycosylation (CDG) and galactosaemia can provide vast information regarding disordered metabolic pathways involving glycosylation and potentially modifiable steps (Brinkman et al. 2006; Morava et al. 2015; Sun et al. 2015).

The number of CDG has increased dramatically over the last few years. Over 100 disorders are now described with on-going characterization of new subsets (Rymen and Jaeken 2014; Scott et al. 2014; Cartault et al. 2015; Freeze et al. 2015). While different CDG have well characterized defects in glycosylation, disorders such as galactosaemia, often termed secondary disorders of glycosylation, are less well defined (Morava et al. 2015). An understanding of the shared disturbed metabolic pathways could lead to improved understanding of the pathophysiology of these disorders of glycosylation and possibly improve therapeutic approaches.

Galactosaemia is a group of rare autosomal recessive carbohydrate metabolism disorders caused by deficiency of enzymes involved in the metabolism of the aldose monosaccharide galactose (Fridovich-Keil and Walter

2008). The most severe type of galactosaemia, Classical galactosaemia (OMIM #230400) (subsequently referred to as galactosaemia in this review), is caused by profound deficiency of the galactose-1-phosphate uridyltransferase (GALT) enzyme (EC 2.7.7.12). Following galactose intake in the affected neonate, there is a toxic build-up of intermediates of galactose metabolism. Strict dietary restriction of galactose is lifesaving in the neonate, but mild to severe long-term complications persist, including significant cognitive impairment and infertility in females, regardless of genotype or age at onset of treatment (Schweitzer et al. 1993; Fridovich-Keil and Walter 2008; Krabbi et al. 2011; Coss et al. 2013; Timson 2015). The cause of this pathophysiology is currently under review.

The toxic build-up of galactose intermediates coupled with deficiency of pathway product is proposed to contribute to the development of these complications. These intermediates can result in competitive inhibition of glycosyltransferases (Lai et al. 2003). A shortage of end-product UDP-hexose sugars could also lead to disruption of glycosylation in the posttranslational modification (PTM) of proteins and lipids (Ng et al. 1989; Ornstein et al. 1992).

Dysregulation of a number of genes and pathways has been observed in galactosaemia (Coman et al. 2010; Coss et al. 2014; Maratha et al. 2016), along with abnormal glycosylation profiles of glycoproteins in both treated and untreated patients (Charlwood et al. 1998; Quintana et al. 2009; Coman et al. 2010; Berry 2011).

Early stage perturbations of glycosylation, gene expression and inositol signaling during prenatal galactose intoxication, in combination with long-term galactose restriction and individual endogenous galactose production, likely have a substantial role in determining the long-term complications seen in galactosaemia (Berry et al. 2004; Huidekoper et al. 2005; Coss et al. 2014; Schadewaldt et al. 2014; Maratha et al. 2016). Understanding the role of glycosylation in the development of these complications is essential.

Congenital Disorders of Glycosylation

CDG are a large group of mainly autosomal recessive inherited disorders affecting the glycan synthesis. These can be divided into the following major categories: disorders of protein N-glycosylation or O-glycosylation, disorders of lipid and glycosylphosphatidylinositol (GPI) anchor glycosylation and disorders of multiple glycosylation pathways (Freeze 2006; Hennet 2012), with variable symptomatic severity. Almost all organs are affected with a particular impact on nervous development, immune, hepatic and gastrointestinal systems (Freeze and Aebi 2005; Freeze et al. 2015).

The majority of CDG disorders are caused by defects in the N-glycosylation pathway in which N-glycans are attached to arginine on proteins. N-glycan synthesis starts in the endoplasmic reticulum (ER), where the assembled product is attached. The processing of N-glycans into complex and hybrid structures continues in the Golgi apparatus. CDG-type I (CDG-I) abnormalities result from an abnormality of N-glycan assembly in the ER and CDG-type II (CDG-II) abnormalities result from abnormalities in N-glycan processing after transfer to the protein in the ER or steps occurring in the Golgi apparatus (Freeze 2013; Freeze et al. 2015), as depicted in Fig. 1.

Approximately 1% of the human genome encodes genes involved in glycosylation and over half of all proteins are N-glycosylated (Freeze 1998; Pivac et al. 2011). Glycoproteins are central to many key biological systems such as cell–cell signaling, and are important in coagulation, immunity, fertility, etc. (Zoldos et al. 2010).

Galactosaemia has been reported as a secondary disorder of glycosylation, displaying characteristics of both CDG-I and CDG-II defects with both glycan assembly and processing defects observed (Charlwood et al. 1998; Sturiale et al. 2005; Quintana et al. 2009; Coman et al. 2010; Coss et al. 2012).

CDG and galactosaemia share multiple clinical characteristics. Tables 1 and 2 summarize a number of CDG syndromes (I and II) with symptoms which may also be observed in galactosaemia including neurological involvement, coagulopathies and liver disease. For example, PMM2-CDG, the most common subtype of CDG, is an N-glycan assembly defect caused by lack of phosphomannomutase 2 (PMM2) which converts mannose-6-phosphate to mannose-1-phosphate. The enzymatic deficiency results in reduced GDP-mannose required for the synthesis of the lipid-linked oligosaccharide (LLO) precursor. The symptoms observed are commonly intellectual disability, hypotonia, cerebellar dysfunction, polyneuropathy and stroke-like episodes (Dinopoulos et al. 2007).

The key clinical adverse outcomes seen in galactosaemia include intellectual disabilities, speech abnormalities and primary ovarian insufficiency (POI) in females. Intellectual disability occurs in at least 50% of affected individuals (Waggoner et al. 1990; Schweitzer et al. 1993; Shield et al. 2000; Doyle et al. 2010; Waisbren et al. 2012; Coss et al. 2013; Rubio-Agusti et al. 2013). Abnormal myelination was first documented in galactosaemia in 1971 (Haberland et al. 1971; Lebea and Pretorius 2005). There is considerable variability in IQs documented between galactosaemia patients with scores ranging from very low to well above average.

The presence of speech and language abnormalities (commonly verbal dyspraxia) is well documented in galactosaemia. One well-described entity is Childhood Apraxia of Speech (CAS). Overall, speech and language

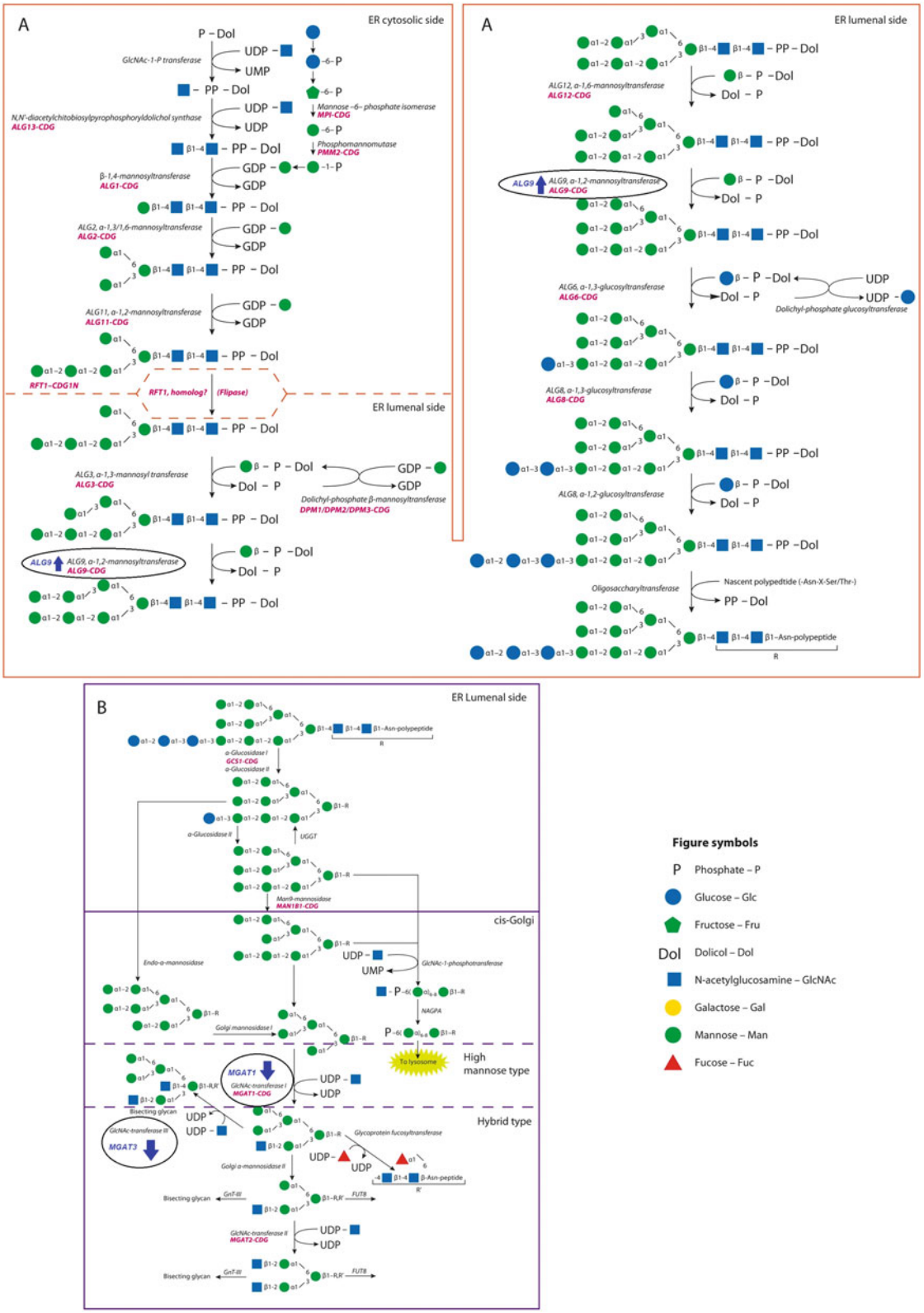

Fig. 1 Schematic representation of: (**a**) N-glycan assembly in rER (Congenital Disorders of Glycosylation-type I, CDG-I) and (**b**) N-glycan processing in Golgi apparatus (Congenital Disorders of Glycosylation-type II, CDG-II). Blue arrows for *ALG9* (Alpha-1, 2-mannosyltransferase), *MGAT1* and *MGAT3* represent respective gene expression pattern observed in our recent study (Maratha et al. 2016)

Table 1 CDG-I conditions and common symptoms

CDG name	Affected protein/gene	Common symptoms
PMM2-CDG	Phosphomannomutase 2	Cognitive/motor dysfunction (de Lonlay et al. 2001), failure to thrive, liver disease, developmental delay (Drouin-Garraud et al. 2001), coagulopathy (Van Geet and Jaeken 1993) and infection (Matthijs et al. 1997)
MPI-CDG	Mannose-6-phosphate-isomerase	Coagulopathy (Marquardt and Denecke 2003)
ALG3-CDG	Dolichyl-P-Man: Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase	Cognitive/motor dysfunction (de Lonlay et al. 2001) and failure to thrive (Kranz et al. 2007)
ALG12-CDG	Dolichyl-P-Man: Man(7)GlcNAc(2)-PP-dolichyl-alpha-1, 6-mannosyltransferase	Cognitive/motor dysfunction, (Thiel et al. 2002), failure to thrive and infection (Chantret et al. 2002)
ALG8-CDG	Dolichyl-pyrophosphate Glc Man9GlcNAc2alpha-1,3-glucosyltransferase	Coagulopathy (Chantret et al. 2003)
ALG2-CDG	Alpha-1,3-mannosyltransferase	Cognitive/motor dysfunction, coagulopathy and liver disease (Thiel et al. 2003)
ALG1-CDG	Chitobiosyldiphosphodolichol beta-mannosyltransferase	Cognitive/motor dysfunction (Dupré et al. 2010) and coagulopathy (Kranz et al. 2004)
ALG9-CDG	Alpha-1,2-mannosyltransferase	Cognitive/motor dysfunction, cerebral atrophy, delayed myelination, epilepsy, failure to thrive, liver disease and skeletal dysplasia (Weinstein et al. 2005; Frank et al. 2004; AlSubhi et al. 2016)
PGM1-CDG	Phosphoglucomutase 1	Hypergonadotropic hypogonadism and growth retardation (Tegtmeyer et al. 2014)
MAN1B1-CDG	Alpha-1, 2-mannosidase	Cognitive/motor dysfunction (Rafiq et al. 2011)

Data collated from (Sparks and Krasnewich 1993) with 2013 updates (Freeze 2013)

Table 2 CDG-II conditions and common symptoms

CDG name	Affected protein/gene	Common symptoms
SLC335C1-CDG; Leukocyte adhesion deficiency II	GDP-fucose transporter 1	Cognitive dysfunction (Etzioni et al. 1992) and infections (Lübke et al. 1999)
B4GALT1-CDG	Beta-1,4-galactosyltransferase 1	Coagulopathy and developmental disability (Peters et al. 2002)
COG7-CDG	COG complex subunit 7	Cognitive dysfunction (Zeevaert et al. 2009) and failure to thrive (Morava et al. 2007)
COG4-CDG	COG complex subunit 4	Cognitive dysfunction, failure to thrive and developmental delay (Ng et al. 2011)

Data collated from (Sparks and Krasnewich 1993) with 2013 updates (Freeze 2013)

disorders are estimated to affect at least 25% of individuals with galactosaemia, commonly presenting in childhood (Schweitzer et al. 1993; Potter et al. 2008, 2013; Timmers et al. 2012; Waisbren et al. 2012; Coss et al. 2013), with pathophysiological correlates studied in fMRI brain studies (Timmers et al. 2015).

Over 80% of galactosaemia females suffer from POI (91% in Irish female patients) (Waggoner et al. 1990; Sanders et al. 2009; Coss et al. 2013). The clinical presentation varies from primary amenorrhea to delayed pubertal development followed by irregular menses or secondary amenorrhea. This results in infertility or subfertility as a predominant feature in galactosaemia females of childbearing age (Rubio-Gozalbo et al. 2010).

The pathophysiology for this presentation is still unknown. Mechanisms proposed include prenatal toxicity with galactose and metabolites possibly causing premature follicular apoptosis/atresia, abnormal cell signaling and hormone/receptor glycosylation abnormalities. Hypoglycosylation of follicle stimulating hormone (FSH) could theoretically alter its function and lead to POI in galactosaemia. However biochemical tests have shown inconclusive results (Prestoz et al. 1997; Gubbels et al. 2011).

Male galactosaemia patients seem to be less affected and have successfully fathered offspring, reaching puberty spontaneously although the age of onset can be delayed (Rubio-Gozalbo et al. 2010). PMM2-CDG patients also virilize normally through puberty but with occurrences of

decreased testicular volume and increased serum FSH concentrations (de Zegher and Jaeken 1995).

Altered leptin signaling secondary to glycosylation abnormalities may be contributory (Knerr et al. 2013). Leptin is a key energy and fat storage regulator (Kratzsch et al. 2002) and abnormal leptin signaling due to hypoglycosylation has also been considered in abnormal fat distribution in PMM2-CDG (Wolthuis et al. 2013).

Osteopenia is another long-term complication associated with galactosaemia. Osteopenia and other skeletal abnormalities are common clinical findings in CDG (Coman et al. 2008; Rimella-Le-Huu et al. 2008). It has been suggested that hypoglycosylation of noncollagenous bone proteins may be responsible for decreased bone mass and increased osteocalcin levels in PMM2-CDG patients (Barone et al. 2002).

Decreased bone mineral density has been consistently observed in galactosaemia patients (Rubio-Gozalbo et al. 2002; Panis et al. 2004; Waisbren et al. 2012; Batey et al. 2013; Coss et al. 2013; Doulgeraki et al. 2014). Decreased bone metabolism has been suggested as the mechanism of reduced bone mineral density in galactosaemia as well as abnormal galactosylation of the collagen matrix (Kaufman et al. 1993).

Reduced levels of insulin-like growth factor 1 (IGF-1), carboxylated osteocalcin, N-terminal telopeptide and C-terminal telopeptide have been reported in serum samples from galactosaemia patients (Panis et al. 2004; Fridovich-Keil and Walter 2008).

Serum IgG N-Glycosylation Abnormalities in Galactosaemia

As discussed earlier, substantial N-glycan abnormalities have been demonstrated in serum transferrin IEF patterns in galactosaemia (Charlwood et al. 1998; Sturiale et al. 2005; Quintana et al. 2009). UDP-galactose substrate deficiency is one of the proposed contributing pathophysiological mechanisms (Gibson et al. 1995; Lai et al. 2003; Parkinson et al. 2013; Jumbo-Lucioni et al. 2014).

We have documented hypoglycosylation and gross N-glycan assembly defects in the whole serum of untreated neonates with galactosaemia similar to what has been observed in CDG-I defects (Coman et al. 2010; Coss et al. 2014). While the N-glycan assembly defects resolve within the first 6 months of life with galactose restriction, it is apparent that, after this initial effect, N-glycan processing defects persist, even in young children (Coss et al. 2014).

We also performed a study of N-glycan processing defects in 10 treated galactosaemia adults on a restricted galactose diet in comparison to matched controls using serum IgG analysis (the most abundant circulating N-glycan glycoprotein), analyzed by NP-HPLC to monitor the effects and potential benefits of galactose supplementation with galactosylation of IgG used as a specific biomarker of dietary galactose tolerance. We demonstrated an increase in non-galactosylated (G0) and monogalactosylated (G1) structures with decreased digalactosylated structures (G2) in diet-restricted galactosaemia patients, indicating continued N-glycan processing defects despite treatment (Coss et al. 2012). Five subjects followed a moderate galactose liberalization trial over 16 weeks. Their IgG N-glycan profiles showed consistent individual alterations in response to diet liberalization with improvement of profiles for three of the five subjects at a galactose intake of 1000 mg/day.

We recently also published a study of 13 children with galactosaemia which indicated that a moderate increase in galactose intake may be well tolerated in children and may improve glycosylation (Knerr et al. 2015).

We previously also identified that children with galactosaemia had lower serum leptin levels than normal controls, expressed as SDS for gender and pubertal age (Knerr et al. 2013). In the above diet relaxation study, there was no statistical significant difference noted in serum leptin levels in the patient control group and the diet relaxation groups at the baseline point. However, patients in the galactose supplementation group had, as a trend, slightly higher leptin levels at the end of the study than patient controls ($p < 0.05$), but within the normal range.

We have now established a rapid automated robotic hydrophilic interaction ultra-performance liquid chromatography N-glycan analysis for the measurement of IgG N-glycan galactose incorporation applied to adult galactosaemia patients which has demonstrated significant differences between the G0/G1 and G0/G2 incorporation ratios and controls (Stockmann et al. 2016).

This analysis of IgG glycosylation has also recently been applied to the CDG condition MAN1B1-CDG using this methodology (Saldova et al. 2015).

To further identify the specific N-glycosylation steps that are affected in galactosaemia, we performed further glycan subset analysis in the IgG glycosylation study of 40 galactosaemia treated patients compared to controls. In this work, we identified a significant increase in core fucosylated neutral glycans and a significant decrease in core fucosylated, afucosylated bisected glycans and N-linked mannose-5 glycans in circulating serum IgG N-glycans (Maratha et al. 2016). Figure 1, amended from this study, illustrates the steps in N-glycan synthesis, which may be affected in this pathway.

Abnormal Gene Expression and Cell Signaling in Galactosaemia

In a pilot microarray study of T-lymphocyte RNA expression from four galactosaemia patients (Coman et al. 2010), we identified extensive dysregulation of genes affecting many signaling pathways including MAP kinase, regulation of actin cytoskeleton, ubiquitin mediated proteolysis, inositol signaling, inflammatory pathways and glycan biosynthesis pathways (Coman et al. 2010), and we subsequently validated dysregulation of a number of N-linked glycosylation biosynthesis genes linked to CDG-1 and CDG-II, e.g. *ALG* (*1, 2, 8* and *9*) in a larger study (Coss et al. 2014).

We also confirmed and noted the dysregulation of the genes *ANXA1* and *ALG9* (Alpha-1,2-mannosyltransferase) (which also responded to differing levels of galactose exposure), in cultured galactosaemia patient fibroblast cells (Coss et al. 2014).

We have subsequently studied the expression of a number of these genes and other related relevant N-glycan biosynthesis genes in peripheral blood mononuclear cells from affected galactosaemia adult patients. We noted significant dysregulation of two key N-glycan biosynthesis genes *ALG9,* which was up-regulated ($p < 0.001$), and *MGAT1,* which was down-regulated ($p < 0.01$) with additional dysregulation of the genes *FUT8,* and *MGAT3* (Maratha et al. 2016). The site of action of these genes is illustrated in Fig. 1.

The *ALG9* (Alpha-1,2-mannosyltransferase) gene product is involved in the addition of the seventh and ninth mannose sugar to the growing N-glycan, essential for the formation of the initial oligosaccharide chain. It has been proposed that the interaction of ALG9 with ALG12 is required for the ultimate formation of the disaccharide glycan, which influences further downstream processing of N-glycans, indicating a potential regulatory role for the *ALG9* gene in glycosylation (Coss et al. 2014). The clinical phenotype for ALG9-CDG has recently been expanded (see Table 1) (AlSubhi et al. 2016).

The decreased expression of the *MGAT1* gene also has significant pathological correlates. The *MGAT1* gene encodes GlcNAc transferase I (Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase), which adds GlcNAc to high-mannose sites, an essential early step in producing all branched complex and hybrid N-glycans.

Inactivation of the *MGAT1* gene in mice was shown to impair oogenesis, and mouse *MGAT1* knockouts were unviable (Shi et al. 2004).

Galactosaemia and CDG: Dietary Treatment Approaches

Our studies of IgG N-glycosylation with varying effects of galactose exposure in galactosaemia adults and children have indicated the presence of significant interindividual tolerance of exogenous galactose in galactosaemia patients. There are reports on individuals who have relaxed the diet at an early age with good outcomes (Lee et al. 2003; Panis et al. 2006). It appears that some affected individuals with galactosaemia may have more ability to utilize alternative, accessory pathways to metabolize galactose and its metabolites than others and may tolerate moderate amounts of exogenous dietary galactose (Coss et al. 2014). This may be influenced by epigenetic regulation (Lauc and Zoldos 2009). As an illustrative example, the overexpression of human UDP-glucose pyrophosphorylase (hUGP2), an 'accessory pathway' enzyme, using both galactose-1-phosphate and glucose-1-phosphate as substrates, rescued GALT-deficient yeast cells from galactose toxicity (Lai and Elsas 2000). The UGP2 reaction may not be relevant under normal physiological conditions as high toxic levels of galactose-1-phosphate seem to be required (Lai et al. 2003), as glucose-1-phosphate is the preferred substrate (Leslie et al. 2005). As referred earlier, it is possible that some patients may have the ability to generate more UDP-galactose, using excess galactose-1-phosphate as a substrate (Lai and Elsas 2000; Fridovich-Keil and Walter 2008).

Considering this variability in accessory pathways of galactose metabolism and linked glycosylation, we propose that the clinical outcomes observed in galactosaemia are multifactorial, influenced by prenatal toxicity and postnatal variation in accessory glycosylation pathways (Coss et al. 2012; Knerr et al. 2015).

Also, while the severe restriction of dietary galactose in the affected newborn is life saving and largely reverses the N-glycan assembly defect, our studies suggest that over restriction of galactose in the long-term may contribute to ongoing N-glycan processing defects, evident in all the galactosaemia patients whom we have studied to date (Coman et al. 2010; Coss et al. 2012; Knerr et al. 2015; Stockmann et al. 2016).

The manipulation of exogenous provided sugar substrates in CDG is informative. At least four subtypes of CDG have been treated with dietary modulation of sugars: MPI-CDG, SLC55C1-CDG, PGM1-CDG (Hendriksz et al. 2001; Harms et al. 2002; Penel-Capelle et al. 2003; de Lonlay and Seta 2009) and SLC35A2-CDG (Ng et al. 2013; Dorre et al. 2015).

SLC55C1-CDG is caused by a decreased affinity of the GDP-fucose transporters resulting in decreased fucose, resulting in immunological defects and severe psychomotor delay (Goreta et al. 2012). Supplementation with oral fucose challenges the defective transporters leading to clinical improvements in some patients, with correction of immunological dysfunction and psychomotor improvement (Marquardt et al. 1999; Jaeken 2010).

PGM1-CDG phosphoglucomutase 1 deficiency (E.C 5.4.2.2) is caused by disruption of the glucose metabolism pathway whereby phosphoglucomutase catalyzes the bidirectional transfer of phosphate from position 1 to 6 on glucose. Deficiency of this enzyme, now characterized clinically by hypoglycaemia, liver disease, cardiomyopathy, short stature, cleft palate and normal intelligence, has previously been associated with a primary muscle disease, Glycogen Storage Disease, XIV (Morava 2014). There are a limited number of patients reported in the literature; one of the first reported suffered from exercise-induced rhabdomyolysis and muscular glycogenosis (Stojkovic et al. 2009; Timal et al. 2012; Morava 2014). The disruption of this pathway (caused by reduced PGM1) results in dysregulation of glycolysis and disruption of the galactose metabolism pathway. It has been suggested that the build-up of glucose-1-phosphate competes with galactose-1-phosphate for the UDP-glucose pyrophosphorylase enzyme. This drives the product of the pyrophosphorylase pathway towards UDP-glucose, reducing the level of UDP-galactose (Perez et al. 2013). If this is the dysregulated pathway of PGM1-CDG, then it would indicate there is some biologically relevant level of UDP-galactose produced from the UDP-glucose pyrophosphorylase pathway, which has direct relevance for galactosaemia. A study of PGM1-CDG patients treated with a combination of D-galactose and complex carbohydrate supplementation improved serum transferrin hypoglycosylation and ameliorated clinical symptoms. This study indicated increased levels of activated UDP-galactose in the treated patients which improved glycosylation (Morava 2014).

In addition, Ng et al. in 2013 reported a disorder of the X-linked gene UDP-galactose transporter SLC35A2. This disorder leads to galactose-deficient glycoproteins as measured by N-glycans from whole serum using MALDI-TOF. This showed increased levels of hypogalactosylated glycans, particularly biantennary species (Ng et al. 2013). Interestingly, in 3 affected children, the neonatal profile improved and normalized during the first few years of life. In a recently reported child with this transporter deficiency, dietary galactose supplementation resulted in nearly complete normalization of the abnormal transferrin glycosylation pattern (Dorre et al. 2015).

The beneficial effect of galactose supplementation for PGM1-CDG and SLC35A2 deficiency suggests the physiological need for supplementary exogenous galactose in the presence of UDP galactose limited bioavailability. This is of possible relevance to galactosaemia.

Conclusion

There are many biochemical and clinical similarities between galactosaemia and CDG syndromes. Early (prenatal and perinatal) dysregulation of glycosylation in galactosaemia must be a major determinant of both neurological/cognitive and reproductive deficits, while ongoing abnormalities in glycosylation and associated gene dysregulation and associated cell signaling abnormalities may also have relevant pathophysiological consequences.

The persistence of aberrant glycosylation and disruption of CDG-related genes in long-term treated galactosaemia patients suggest that this is a major area in galactosaemia research. This requires further investigation which may offer new biomarkers to monitor affected individuals and enhance our understanding of this and related conditions.

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Synopsis

An understanding of the link between galactosaemia and CDG, with a focus on abnormal N-glycosylation.

Compliance with Ethics Guidelines

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

Ashwini Maratha and Eileen Treacy have been involved in the planning, conception, writing, drafting and reviewing this review. Hugh-Owen Colhoun assisted in writing this review. Ina Knerr, Karen Coss and Peter Doran have assisted in reviewing this review.

Conflict of Interest

The authors Ashwini Maratha, Hugh-Owen Colhoun, Ina Knerr, Karen Coss, Peter Doran and Eileen Treacy declare that they have no conflict of interest.

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CASE REPORT

Argininosuccinic Acid Lyase Deficiency Missed by Newborn Screen

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Abstract Argininosuccinic acid lyase (ASL) deficiency, caused by mutations in the *ASL* gene (OMIM: 608310) is a urea cycle disorder that has pleiotropic presentations. On the mild end, ASL deficiency can manifest as nonspecific neurocognitive abnormalities without readily identifiable signs to differentiate it from other causes of intellectual disability or learning disabilities. Dietary management and arginine supplementation, if initiated early, may ameliorate symptoms.

Because of the nonspecific nature of the symptoms and the possibility for therapeutic management, ASL deficiency is part of the recommended uniform screening panel for newborn screening in the USA. We report here a case of ASL deficiency that was missed on newborn screening in the USA.

The case reported here has two known pathogenic mutations – one with no residual activity and one with reported 10% residual activity. Review of this newborn screening results showed subtle elevation of citrulline, overlapping the normal range. These findings suggest that newborn screening may be missing other patients with ASL deficiency with at least one hypomorphic allele. This case was diagnosed incidentally, but in retrospect had symptoms best attributed in full or in part to his ASA deficiency, including protein aversion, developmental delay, and seizures. This case highlights the importance of considering ASL deficiency in patients with nonspecific abnormal neurocognitive signs, such as epilepsy and developmental delay, even when newborn screening was normal.

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Introduction

Argininosuccinic acid lyase (ASL) deficiency caused by mutations in the *ASL* gene is a potentially severe, but treatable inborn error of metabolism. There are two forms of ASL deficiency: the newborn form, which can present indistinguishably from proximal urea cycle disorders with neonatal hyperammonemia, and the late-onset form, which has less specific features (Tuchman et al. 2008; Erez et al. 2011; Kölker et al. 2015). The late-onset form of ASL deficiency presents with nonspecific intellectual disability or learning disability (Erez et al. 2011). In some patients, hepatitis and cirrhosis also occur, but these may not be present on initial presentation. Hyperammonemia may be intermittent or completely absent (Erez et al. 2011). This absence of specific features means that a high index of suspicion is required to make the diagnosis. Trichorrhexis nodosa is a pathognomic finding of ASA Lyase deficiency but it requires a priori suspicion to detect and is only present in 50% of patients (Fichtel et al. (2007); Ficicioglu et al. 2009). Prior to the newborn screening era, the diagnosis of late-onset ASL deficiency was delayed by more than a year in some cases (Kölker et al. 2015).

Making the diagnosis of ASL deficiency is critical, because long-term dietary protein restriction and arginine supplementation may improve cognitive outcome (Ficicioglu et al. 2009), although some cases that have been properly identified and treated still have complications, which is thought to be due to depletion of the synthetic pool used for NO and creatine generation. Therefore, because of the benefit in many cases from early initiation of therapy and the absence of specific features, ASL deficiency is on the Recommend Uniform Screening Panel for newborn screening in the USA (American College of Medical Genetics' Newborn Screening Expert Group, 2006).

Here we report a case of ASL deficiency that was missed by properly performed newborn screening in the USA; a pair of siblings with ASL deficiency have been reported to have been missed on newborn screening in Australia (Estrella et al. 2014). ASL deficiency was picked up incidentally on whole exome sequencing (WES) performed for this patient's comorbid dysmorphia. In retrospect, the reported patient had symptoms at least partially explained by his ASL deficiency, including protein aversion and seizures. This case raises the possibility that there may exist several other missed symptomatic cases, highlighting the importance of considering ASL deficiency in patients with nonsyndromic developmental delay, with normal newborn screening.

Clinical Case Report

We present a 26-month-old patient with a reportedly normal New Jersey newborn screen. He first came to attention of clinical genetics at age 26 months, when he was admitted for medically nonresponsive focal seizures. Review of the past medical history was notable for reflux, poor feeding, and lifelong short stature. The patient had an overall disinterest in food, but on open-ended questioning about the dietary history following diagnosis, parents volunteered that he seemed particularly averse to eating meat, and was noted to vomit after being fed meat on several occasions. Developmental history was notable for global developmental delay, with severe expressive language delay out of proportion to his other development. He had hyperactivity. Family history was unremarkable; he was the first child born to nonconsanguineous European and Armenian parents. His mother was pregnant at the time of consultation.

On examination, the patient was small: weight, height, and head circumference were less than the fifth percentile (weight 50th percentile for 10 months; height 50th percentile for 13 months; and head circumference 50th percentile for 11 months). He had dysmorphic features including an upswept frontal hairline, deep-set eyes, large ears, a bulbous nasal tip, and mild retrognathia. These features led the dysmorphologist to suspect Floating Harbor Syndrome (FHS) (Nikkel et al. 2013); however, the seizures were worse than previously reported in FHS. Therefore, to expedite the workup because of ongoing maternal pregnancy, rapid WES was selected as the best diagnostic modality. WES confirmed the clinically suspected diagnosis of FHS with a *de novo* pathogenic nonsense *SRCAP* mutation (c.7330C > T; p.R2444X); however, additionally there were biallelic known pathogenic mutations in *ASL* (c.765dupG; p.M256DfsX79 and c.1135C > T; p.R379C), with confirmed biparental inheritance. The p.M256DfsX79 variant has been previously reported as a pathogenic variant in a patient with infantile-onset ASL deficiency (Balmer et al. 2014); the p.R379C has been previously reported as a pathogenic variant in a patient with late-onset ASL deficiency, and has been shown to have 10% residual enzyme activity (Engel et al. 2012; Hu et al. 2015).

Because of these genetic results, the patient's previous metabolic laboratory results were evaluated. The newborn screen had shown a very subtle elevation of citrulline, below both the presumptive and borderline cutoff labels established by the state. The New Jersey state newborn screening does not use ASA as a primary target. Based on the R4S post-analytical tool calculated using only citrulline (no – ASA tool), the case score (cumulative) was 2. His subsequent diagnostic plasma amino acids at ages 12 months (obtained for failure to thrive) and 26 months (obtained for epilepsy) as well as cerebrospinal fluid amino acids at age 26 months all showed subtle citrullinemia, whose diagnostic significance was not appreciated at the time due to the low level of the elevation and absence of measured ASA (Table 1). Plasma ammonia level and urine organic acids obtained at 26 months (for epilepsy) were normal. His plasma citrulline level increased dramatically following initiation of tube feeding through his gastrostomy tube at age 14 months (Table 1). The absence of detected ASA is explained because in the reference laboratory, co-elution of ethanolamine, and ASA impairs detection of mild elevations of ASA. Plasma amino acids sent following molecular diagnosis, including dedicated testing for ASA, was performed and showed striking elevation of ASA, confirming the diagnosis (Table 1). Following diagnosis, the patient was started on a low protein diet. Arginine supplementation was unable to be started because it was denied by insurance.

The patient continued to have refractory seizures, with hypsarrhythmia on EEG. He was nonresponsive to trials of multiple antiepileptic agents, including levetiracetam, topiramate, and oxcarbazepine. He had good clinical response to clobazam; however, he developed a severe allergic reaction, and it needed to be discontinued. An interdisciplinary team convened to discuss the risks and benefits of

Table 1 Patient's biochemical results

	Pre-diagnostic testing			Routine care			During steroid wean			
	12 months	26 month	26 months[a]	26 months	26 months	27 months	28 months[b]	29 months	29 months	29 months
Gln (303-837)	808.1	607	1029.2	671.9	620.1	706.4	593	843.8	895.9	678.1
Cit (7-47)	55.0	83.7	10	72.9	110.6	75.6	112	132.3	92.7	73.7
ASA (0)	ND[c]	ND[c]	ND[c]	ND[c]	60.5	ND[c]	57	56.3	51.0	45.2
Arg (15-120)	34.6	37.0	24.3	67.4	29.2	18.5	44	35.7	45.9	45.1
Orn (13-151)	36.9	32.3	2.9	39.9	19.6	17.5	19	28.1	39.2	17.6
Ammonia (9-33)		27	<9	<9	<9	18	13	24	11	26

Units in nmol/mL for all amino acids; units in umol/L for ammonia
ND not detected
[a] Sample is from CSF. Norms are Gln 228-1008; cit 0-3
[b] Sample was performed in alternate lab with reference ranges: gln 329-976; cit 11-45; ASA <2; Arg 31-132; Orn 22-97
[c] In this reference lab, ASA coelutes with ethanolamine, so lack of detection is not sensitive

corticosteroid therapy to treat refractory hypsarrhythmia. The perceived risks were that as corticosteroids encourage a catabolic state, proteolysis might be induced, which could result in hyperammonemia; however, because the patient had refractory seizures, limited therapeutic options and had previously clinically responded to corticosteroids, it was determined that the risks outweighed the benefits and high-dose prednisone was initiated. Weekly laboratory studies, including plasma amino acids, were performed during steroid therapy. As expected, corticosteroids provoked increased citrullinemia, hyperglutaminemia, as well as an increase in ASA; however hyperammonemia did not develop (Table 1). Although corticosteroids resulted in fewer clinical seizures and normalization of his EEG, he had a regression in his expressive sign language during steroid therapy. Additionally, clinical seizures and hypsarrhythmia returned following his steroid course. He subsequently has failed to respond to many additional antiepileptic drugs (lacosamide, vigabatrin, and clorazepate). The possibility of ketogenic diet is currently being considered.

The patient did make developmental progress following transition to a low protein diet; however, several medical changes such as changes in his antiepileptic medication were attempted at the same time, so the direct benefits of treatment for ASL deficiency are unclear.

Methods

For all standard runs, amino acids were analyzed using ultra-performance liquid chromatography, as described previously. A standard column temperature of 43° C was used, at which temperature ASA can only be resolved from ethanolamine when present in high quantities. For the dedicated urea cycle run performed at 28 months of age, liquid chromatography and tandem mass spectrometry were used.

Discussion

Here we present a case of a patient with molecularly diagnosed and biochemically confirmed ASL deficiency who was diagnosed incidentally by WES, sent to diagnose his suspected FHS. Although this patient had biochemical testing prior to diagnosis, his amino acid elevations were too subtle to provoke metabolic referral. This case is the first report of ASL deficiency missed on newborn screen in the USA and this is the second reported family to have ASL deficiency missed on newborn screening (Estrella et al. 2014). In this case, it is difficult to determine the specific contribution of ASL deficiency to the phenotype because of his comorbid FHS; however we speculate that his seizures may be related because they are highly unusual for FHS, both in their semiology (infantile spasms) and in their severity (Table 2). His regression while on corticosteroid therapy is also likely attributable to his ASL deficiency, which has important implications for the considerations of corticosteroid therapy for idiopathic infancy. Similarly, his vomiting may be related because it seemed to improve with transition to a low protein diet and the family voluntarily reported protein aversion. It is possible that his seizures and development would further improve if arginine therapy could be initiated; however, abnormal EEG and neurocognitive features such as intellectual disability have been reported even in patients diagnosed and treated appropriately from birth (Ficicioglu et al. 2009; Mercimek-Mahmu-

Table 2 Patient's phenotypic features

Sign	Patient	ASL deficiency[a]	FHS[b]
Seizures	Medically intractable infantile spasms	32%; no infantile spasms; highly responsive to medical therapy	11% (Nikkel et al. 2013)
Intellectual disability	Moderate intellectual disability, with severe expressive language delay	70%; usually mild-mod; verbal IQ more affected	Common mild-mod. Predominant speech and language involvement
Short stature	Height 50th %ile for 13 months at age 26 months	Not reported	95% below the 5th %ile for height
Reflux	Severe, requires gastrostomy tube	Not reported	25%; severe, may require gastrostomy tube
Protein aversion	Vomiting provoked by proteins	Associated, but not quantified	Not reported
Dysmorphic facies	Deep-set eyes, large ears, bulbous nasal tip, and retrognathia	Not reported	Universal deep-set eyes, large, low-set ears, and broad nasal tip

[a] Tuchman et al. (2008), Ficicioglu et al. (2009)
[b] Nikkel et al. (2013)

toglu et al. 2010). This is thought to be due to the duality of arginine pools in the cell; arginine used for ureagenesis seems to be distinct from arginine for NO donation and creatine synthesis. Critically, arginine supplementation does not seem to replete the biosynthetic arginine pool (Erez et al. 2011).

This case may represent an unappreciated population of ASL that is missed on newborn screen, since the clinical presentation of ASL deficiency may be quite mild or late onset (Ficicioglu et al. 2009). Although it is difficult to say conclusively how clinically significant his ASL deficiency is because of his comorbid FHS, we can speculate based on his genotype. Our patient had one mutation, p.R379C, associated with the late-onset form of ASL deficiency and known to have 10% residual enzyme activity, and when homozygous results in a phenotype of tremor and mild intellectual disability (Kleijer et al. 2002); his second mutation was a null mutation, p. M256DfsX79, which in the homozygous state causes "severe" disease (Balmer et al. 2014). Together, along with the aspects of the patient's phenotype not explained by FHS that were explicable by ASL deficiency (Table 2), these findings suggest that this patient is clinically affected by ASL deficiency. It is important to note that even patients with biallelic mutations associated with late-onset disease, such as p.R379C, are at risk for clinical consequences from ASL deficiency and may be even less likely than the reported patient to be detected on newborn screening.

For all newborn screening conditions, the possibility of false negative newborn screen exists, due to the nature of newborn screening as a large-scale screening test. Therefore, consideration must be given to both optimization of the newborn screen and to the clinical consideration of disorders of intermediary metabolism in patients with a negative newborn screen. In previous discussion of false negative newborn screening, it has been proposed that ratios be used to help improve the detection (Hall et al. 2014). In this case, retrospective use of the R4S CLIR system did show an increased index of suspicion that the newborn screening values were abnormal, and the case score was 2 that indicates possible ASA lyase deficiency. This case highlights the importance of clinicians eliciting a dietary history and other clinical features that may suggest an inborn error of metabolism, and having a low threshold to pursue diagnostic laboratory testing, especially for treatable metabolic disease.

In conclusion, newborn screening using citrulline as a primary marker may not be able to detect all cases of ASL deficiency. Including ASA as a primary target for newborn screening may increase the sensitivity of newborn screening for this condition. Although we cannot clearly establish the contribution of ASL deficiency to this patient's phenotype, this case speaks more generally to the importance of considering ASL deficiency in patients presenting with intellectual disability, epilepsy, liver dysfunction, and/or citrullinemia even in the face of normal newborn screening. ASL deficiency is a potentially treatable cause of non-syndromic intellectual disability, epilepsy, and late-onset liver disease, therefore this case highlights how critical it is for clinicians to be aware that a normal newborn screen does not rule out ASL deficiency.

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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

Argininosuccinic acid lyase deficiency is an important consideration in a patient with nonsyndromic intellectual disability and can be missed by newborn screening.

Details of the Contributions of Individual Authors

RDG performed clinical and biochemical evaluation of the patient, conceived, and wrote the manuscript. EB and MAD performed clinical evaluation of the patient and were responsible for genetic testing. CF conceived the manuscript, provided oversight, and served as the guarantor for the article.

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CASE REPORT

Very Long-Chain Acyl-Coenzyme A Dehydrogenase Deficiency and Perioperative Management in Adult Patients

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Abstract Surgery and anesthesia pose a threat to patients with very long-chain acyl-CoA dehydrogenase deficiency (VLCADD), because prolonged fasting, stress, and pain are known risk factors for the induction of metabolic derangement. The optimal perioperative management in these patients is unknown and the use of volatile agents and agents dissolved in fatty acids has been related to postoperative metabolic complications. However, the occurrence of metabolic derangement is multifactorial and depends, amongst others, on the severity of the mutation and residual enzyme activity. Current guidelines suggest avoiding both volatile anesthetics as well as propofol, which seriously limits the options for providing safe anesthesia. Therefore, we reviewed the available literature on the perioperative management of patients with VLCADD. We concluded that the use of some medications, such as volatile anesthetics, in patients with VLCADD might be wrongfully avoided and could in fact prevent metabolic derangement by the adequate suppression of pain and stress during surgery. We will illustrate this with a case report of an adult VLCADD patient undergoing minor surgery. Besides the use of remifentanil, anesthesia was uneventfully maintained with the use of sevoflurane, a volatile agent, and continuous glucose infusion. The patient was monitored with a continuous glucose meter and creatinine kinase measurements.

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Introduction

In patients with a very long-chain acyl-CoA dehydrogenase deficiency (VLCADD; EC # OMIM201475) the enzyme responsible for one of the first steps in the metabolism of fatty acids is deficient (Leslie et al. 1993; Redshaw and Stewart 2014). VLCADD is an autosomal recessive disorder with an estimated prevalence of 1:31,500–1:85,000 (Lindner et al. 2010; Arnold et al. 2009). The disorder is highly variable, and ranges from severe infantile disease to completely asymptomatic elderly individuals. Since the introduction of worldwide newborn screening, it has become clear that a significant number of the identified newborns with VLCADD actually have a very low risk for metabolic decompensation and may even remain fully asymptomatic if left untreated.

The pathophysiology of VLCADD is complex. As in other fatty acid oxidation defects, a combination of hypoglycemia and toxicity of fatty acid intermediates can result in liver, brain, and heart injury. There is evidence of more general mitochondrial dysfunction in these disorders, including generation of reactive oxygen species and

calcium imbalance (Wajner and Amaral 2015). During catabolic circumstances such as prolonged fasting, stress, illness, and surgery, metabolic derangement can occur, resulting in hypoglycemia, myopathies (including cardiomyopathy), metabolic acidosis, and rhabdomyolysis (Leslie et al. 1993; Redshaw and Stewart 2014). Current treatment consists of avoiding catabolism with regular feedings and in some patients a restriction of long chain fatty acids, supplementation of medium chain triglycerides, and frequent carbohydrate intake to prevent activation of fatty acid metabolism (Arnold et al. 2009; Das et al. 2010). The metabolic derangement during surgery depends, amongst others, on disease severity which is related to residual enzyme activity. Nonsense mutations in the encoding gene (ACADVL) may result in a severe and early presentation of the disorder, but the more frequent missense mutations are associated with both severe and attenuated presentations. Perioperative care in patients with VLCADD should thus be individualized, but the evidence is scarce and the literature is conflicting (Table 1). The use of volatile agents and agents dissolved in a fatty solution is controversial, since they have been related to metabolic derangement (Fierobe et al. 1998). This limits the option to provide safe and stress-reducing anesthesia. The available evidence might however be biased by reports of symptomatic patients and consequently concern more severe presentations of VLCADD.

Thus, a critical review of the available perioperative management is needed, as adequate management of VLCADD patients during surgery is crucial to prevent metabolic deterioration. We will highlight this by presenting a case report and critically review the available literature on perioperative management of patients with VLCADD.

Case Report

Our patient is a 24-year-old woman with relatively mild VLCADD, diagnosed at the age of 16 months after severe hypoglycemia caused by metabolic derangement. (ACADVL mutations c. 104delC (p.Pro35LeufsX26) and c.848T>C (p.Val283Ala); lymphocyte enzyme activity <0.15 (1.84–4.8); fibroblast enzyme activity 0.24 (1.8–5.24) lcFAO flux (([9,10-3H(N)]-oleic acid oxidation rate)) 37% of normal.)

Later she was diagnosed with mild psychomotor retardation. She was admitted for a surgical removal of a cyst in the neck. We used the following strategy:

The patient was scheduled as the first patient of the elective program. Glucose 10% was started at a rate of 2,500 ml/24 h (~2 mg/kg/min), in a solution containing 0.45% NaCl and 10 mmol KCl per 500 ml. We inserted a continuous intravenous glucose monitor for glucose measurements (Fig. 1). Premedication consisted of temazepam 10 mg the evening prior to surgery and clonidine 150 mcg, paracetamol 1,000 mg, and esomeprazole 40 mg the morning of the procedure. Anesthesia was induced with thiopental 4.5 mg/kg, remifentanil infusion 0.3 mcg/kg/min, and rocuronium 40 mg. For maintenance of anesthesia we used remifentanil 0.19 mcg/kg/min and sevoflurane 1.6 vol.% end-tidal. At the end of the procedure 3 mg of morphine was administered as a loading dose for postoperative pain relief. Dehydrobenzoperidol 0.625 mg and dexamethasone 4 mg were administered to prevent postoperative nausea and vomiting. The procedure was uneventful and took 60 min. The patient was extubated before leaving the operation room. Creatine kinase (CK) before and after surgery was 61 mcg/L and 36 mcg/L, respectively.

Table 1 Summary of the statements of the articles included

First author (year)	Preoperative care	Peroperative care	Glucose infusion (recommended)	Laboratory measurements (recommended)	Postoperative management (recommended)
Kleemann (1986)	Heptabarbital, morphine, and promethazine were used	– Volatile agents: recommended	–	–	–
Vellekoop (2011)	Adequate premedication recommended, such as benzodiazepines	– Volatile agents: avoid – Propofol: avoid – Etomidate: avoid	Age and weight based (infants: 6–8 mg/kg/min)	Pre- (during) and postoperative monitoring of glucose and CK	–
Redshaw (2014)	Benzodiazepines: safe to use	– Volatile agents, thiopentone and opiates: safe to use – Propofol: caution – Etomidate: not recommended	Age and weight based (adults: 2 mg/kg/h)	If decompensation is considered: extensive laboratory measurements	– Maintaining glucose infusion until normal oral intake – Prophylaxis with anti-emetics – Prevent catabolism by monitoring early signs of infection, pain, and surgical complications

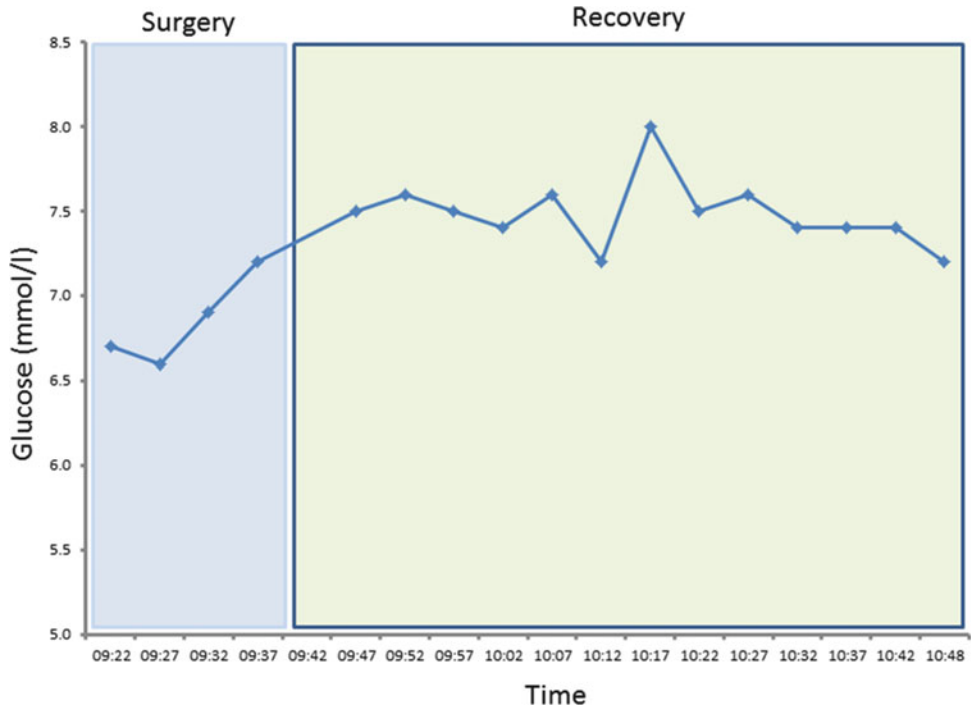

Fig. 1 The results of the continuous intravenous glucose monitor

The patient was released from the recovery room after resuming oral intake. Her stay on the surgical department was also uneventful and the same evening she was discharged home.

Principles of Perioperative Management

Preoperative Care

Stress prior to surgery is a known trigger for metabolic derangement (Vellekoop et al. 2011; Redshaw and Stewart 2014). Preventing stress with the use of premedication could be effective. However, the available literature that addresses premedication to prevent preoperative stress is inconclusive. Kleemann et al., who investigated the levels of free fatty acids as a sign of stress prior, during, and after surgery, claim that premedication did not prevent a stress-induced rise of free fatty acids, using heptabarbital on the preoperative night and morphine with promethazine prior to the transfer to the operating room (Kleemann et al. 1986). However, evidence towards the uneventful use of benzodiazepines such as midazolam is also available (Steiner et al. 2002; Schmidt et al. 2009). Despite the conflicting and scarce literature, premedication is not likely to result in additional harm for the patient and as mentioned above, some data indicate that it is possibly helpful in reducing the perioperative stress response. Patients should be planned as early as possible to minimize the fasting period and thereby reducing the period at risk of deterioration. Finally, measuring the fatty acid oxidation flux predicts the clinical severity of VLCAD deficiency, and could be useful to predict severity of the disease before surgery (Diekman et al. 2015).

Peroperative Care

The principal aim of anesthetizing VLCADD patients is minimizing the surgical stress response, using regional and general anesthesia where considered appropriate. Some anesthetic medication is discussed separately.

Volatile Agents

According to several authors, volatile agents should be avoided, because they are associated with a significant rise of free fatty acid concentrations and this could lead to metabolic derangement (Leslie et al. 1993; Steiner et al. 2002; Vellekoop et al. 2011). However, the assumption is based on the study of Kleeman et al., who investigated plasma concentrations of free fatty acids as the marker of catabolism secondary to stress at multiple points during surgery in a small cohort. They showed that the induction of anesthesia caused a statistically significant rise in free fatty acids, which can be attributed to stress prior to surgery and not to the anesthetic used. Kleeman et al. even showed a decrease of free fatty acids 10 min after the administration of enflurane and concluded that enflurane provides adequate protection against stress during minor surgery.

Another mentioned risk of volatile agents is malignant hyperthermia, which could cause rhabdomyolysis (Fierobe et al. 1998; McKenney and Holman 2002; Vellekoop et al. 2011; Rosenberg et al. 2015). Malignant hyperthermia is a hereditary disorder that is characterized by a rise of body temperature (1°C/5 min) and can be fatal if not timely detected and treated (Vanholder et al. 2000; Rosenberg et al. 2015). The incidence of malignant hyperthermia during anesthesia is between 1:10,000 and 1:250,000 (Rosenberg et al. 2015). It can be triggered by all inhalation anesthetics, except nitrous oxide (Rosenberg et al. 2015). The risk of malignant hyperthermia could be important in VLCADD patients, since the risk of rhabdomyolysis is already increased. However, although both diseases share some symptoms, the etiology is different. And to our knowledge, there are no reports about an association of VLCADD with malignant hyperthermia. Thus, the risk for malignant hyperthermia in patients with VLCADD is presumably comparable to patients without VLCADD.

Propofol

The use of propofol in patients with VLCADD is controversial (Leslie et al. 1993; Steiner et al. 2002; Vellekoop et al. 2011; Redshaw and Stewart 2014). Propofol is dissolved in a solution containing 0.1 g fat/ml. The amount administered depends on the age and weight of a patient. For the induction of anesthesia in adults, 2–3 mg/kg of propofol is used; to maintain anesthesia, a continuous infusion of 4–10 mg/kg/h is administered, depending on the administration of other agents. During a one-hour procedure, the minimum dosage fat within propofol may equate a sixth of the daily fat allowance of an adult. Since VLCADD patients may have a fat restricted diet to prevent organ lipidosis and the accumulation of fat oxidation intermediates, it is recommended to avoid the use of propofol, to improve long-term morbidity and mortality (Redshaw and Stewart 2014).

A case report of a child without VLCADD suggests that propofol can lead to depression of the mitochondrial respiratory chain by disruption of fatty acid oxidation (Wolf et al. 2001). This could mean that patients with VLCADD might be at greater risk for the propofol infusion syndrome when using a continuous infusion of propofol. However, there is no data to support the latter assumption.

Additionally, cases demonstrating the uneventful use of propofol have been described as well (Vellekoop et al. 2011; Martin et al. 2014; Redshaw and Stewart 2014). Most likely, induction with propofol will not lead to immediate problems when sufficient glucose is provided (explained below) and its use in VLCADD is therefore only relatively contraindicated. However, low fat induction agents, e.g., thiopental, are available and perhaps preferable. For maintenance of anesthesia we still would recommend using volatile agents, unless contraindicated, especially for long procedures.

Other Agents

As discussed above, benzodiazepines are safe. In addition, opiates are considered safe in patients with fatty acid metabolic disorders (Steiner et al. 2002; Vellekoop et al. 2011; Redshaw and Stewart 2014). For the lipid formulation of etomidate (e.g., Etomidaat-®Lipuro, B. Braun, Melsungen, Germany), the same considerations apply as with propofol.

Prophylaxis of postoperative nausea and vomiting with anti-emetics is important, as this reduces not only the stress from postoperative nausea and vomiting, but also promotes early restart of oral intake.

Glucose Infusion and Laboratory Measurements

Patients with fatty acid metabolic disorders are unable to address their fatty acids when the available glucose storage becomes depleted. Therefore, patients in need of surgery should receive constant supply of glucose from the moment they fast to prevent cell damage, especially in muscles. The amount of glucose infusion depends on various factors such as residual enzyme activity (the type of VLCADD), the type of procedure and the amount of stress. It is also based on age, because it is presumed that the need for glucose diminishes with aging. According to Vellekoop et al., a glucose infusion of 6 mg/kg/min should prevent catabolism in healthy children undergoing minor surgery, therefore they advise an amount of 8 mg/kg/min for infants with VLCADD. Nishina and coworkers advise 2 mg/kg/min in healthy children who undergo minor surgeries (Nishina et al. 1995). The BIMDG (British Inherited Metabolic Diseases Group) advises 3 mg/kg/min for adults with a fatty acid metabolic disorder, including VLCADD (http://www.bimdg.org.uk/guidelines/guidelines-adult.asp). Redshaw and colleagues however advise to give 2 mg/kg/h (Redshaw and Stewart 2014). Thus, recommendations vary widely and for adults there is no clear consensus, which reflects the paucity of data in this patient group. There is however a clear relation between increasing endogenous glucose production (and thus need) during fasting, ranging from above 8 mg/kg/min in the newborn to 2 mg/kg/min in the adult human (Huidekoper et al. 2014). In our view, the easiest way to prescribe glucose for the adult patient is to use a fixed 10% solution and give 2 mg/kg/min. For children however glucose infusion needs to be increased – depending on age – up to 6–8 mg/kg/min (Vellekoop et al. 2011).

In case of hyperglycemia (plasma glucose >10 mmol/l) the amount of glucose should never be diminished, but must be treated with insulin (http://www.bimdg.org.uk/guidelines/guidelines-adult.asp).

To be able to monitor whether catabolism is sufficiently suppressed, it is important to monitor glucose and serum CK frequently. An increased CK is a sign of lysis of muscle cells, which could indicate rhabdomyolysis (Vanholder et al. 2000). The latter can even occur despite the administration of glucose, because during stress cortisol and catecholamines are released. According to Nishina et al., this could affect the insulin receptor which results in insulin resistance in the peripheral tissue. Rhabdomyolysis can thus occur when glucose levels are within normal range. A relative shortage of insulin causes a delay in glucose uptake within the cells, despite a normal glucose level. Insulin resistance is also associated with the accumulation of lipid intermediates such as long chain acyl-coA, which are presumed to be toxic and responsible for metabolic derangement (Morris and Turnbull 1998; Hoy et al. 2009). Thus, in case of an increase in CK, additional glucose needs to be administered.

Postoperative Management and Discharge

After surgery, the glucose infusion must be maintained until the patient can resume the normal oral intake (http://www.bimdg.org.uk/guidelines/guidelines-adult.asp). As previously mentioned, glucose infusion must be maintained even in case of hyperglycemia. To prevent catabolism, early signs of infection, pain, and surgical complications should be monitored as well and treated promptly (Redshaw and Stewart 2014).

Summary and Recommendations

After reviewing the literature we performed an uneventful surgical procedure in a patient with VLCADD. Although a rare disease, complications are serious and precautions are necessary. The most important cornerstones of the perioperative care are to minimize the fasting period and surgical stress. In addition, one needs to provide adequate glucose infusion (~2 mg/kg/min of glucose 10% in adults) and measure glucose and CK (summary Table 2). Volatile agents can be used safely; propofol and etomidate in a fatty emulsion are relatively contraindicated. Preoperative knowledge on functional fatty acid oxidation despite the VLCADD, e.g., by measuring the fatty acid oxidation flux, could be useful when preparing for surgery.

Take-Home Message

Despite the conflicting literature addressing the perioperative management in patients with VLCADD (very long-chain acyl-CoA dehydrogenase deficiency), volatile agents can be used safely if other important precautions are provided, such as an adequate glucose infusion and the minimization of the fasting period and surgical stress, taking into account the severity of the mutation.

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– Contribution of the individual authors:
– MM Welsink-Karssies: Data collection, reviewing the available literature and writing a first draft of the manuscript, and processing the adjustments of the other authors
– JAW Polderman: data collection, critically reviewing the manuscript
– EJ Nieveen van Dijkum: surgeon performing the procedure, critically reviewing the manuscript
– BP Preckel: critically reviewing the manuscript
– WS Schlack: critically reviewing the manuscript, data collection
– G Visser: reviewing the available literature, critically reviewing the manuscript

Table 2 Our current recommendations according to the available literature

Preoperative care	Peroperative care	Glucose infusion	Laboratory measurements	Postoperative management
Low threshold for benzodiazepines as premedication	– Volatile agents: preferred – Propofol: relatively contraindicated – Etomidate (lipid formulation): relatively contraindicated	Age and weight based (~2 ml/kg/h of glucose 10 % in adults)	Glucose and CK levels before and after surgery. If surgery >3 h, sample CK every 3 h during surgery. Increase glucose infusion if CK increases.	– Maintaining glucose infusion until normal oral intake – Prophylaxis with anti-emetics – Prevent catabolism by monitoring early signs of infection, pain, and surgical complications

- CE Hollak: reviewing the available literature, critically reviewing the manuscript, data collection, and treating metabolic specialist of the patient
- J Hermanides: anesthesiologist during the procedure, reviewing the available literature, critically reviewing the manuscript, and data collection

- J. Hermanides serves as guarantor for the article, accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.
- The conflict of interest form of the authors is included in the online submission.
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- The authors hereby state that an ethics approval was not required.
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RESEARCH REPORT

Paracentric Inversion of Chromosome 21 Leading to Disruption of the *HLCS* Gene in a Family with Holocarboxylase Synthetase Deficiency

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Abstract Holocarboxylase synthetase (HLCS) deficiency is a rare autosomal recessive disorder that presents with multiple life-threatening metabolic derangements including metabolic acidosis, ketosis, and hyperammonemia. A majority of HLCS deficiency patients respond to biotin therapy; however, some patients show only a partial or no response to biotin therapy. Here, we report a neonatal presentation of HLCS deficiency with partial response to biotin therapy. Sequencing of *HLCS* showed a novel heterozygous mutation in exon 5, c.996G>C (p.Gln332His), which likely abolishes the normal intron 6 splice donor site. Cytogenetic analysis revealed that the defect of the other allele is a paracentric inversion on chromosome 21 that disrupts *HLCS*. This case illustrates that in addition to facilitating necessary family testing, a molecular diagnosis can optimize management by providing a better explanation of the enzyme's underlying defect. It also emphasizes the potential benefit of a karyotype in cases in which molecular genetic testing fails to provide an explanation.

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Introduction

Biotin is a water-soluble vitamin that acts as the coenzyme of four carboxylases involved in gluconeogenesis, fatty acid synthesis, and the catabolism of several amino acids: acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase. Biotin activates the four inactive apocarboxylases through a covalent bond that is catalyzed by holocarboxylase synthetase (HLCS) in a two-step process. In the first step, HLCS activates the carboxyl group of biotin with the addition of an adenylate group to form the reaction intermediate biotinyl-5′-AMP. In the second step, the biotin moiety of biotinyl-5′-AMP is transferred to one of the apocarboxylases (Chapman-Smith and Cronan 1999).

HLCS deficiency (OMIM #253270) is an autosomal recessive disorder that presents with multiple life-threatening metabolic derangements including metabolic acidosis, ketosis, and hyperammonemia. Characteristic findings are similar to those in other organic acidurias and include tachypnea, hypotonia, lethargy, vomiting, and seizures (Sweetman et al. 1982). In addition, cutaneous symptoms that range from periorificial and erythrodermic dermatitis to alopecia are frequently observed (Esparza et al. 2011).

The majority of HLCS patients respond to biotin therapy. However, a few patients show only a partial or very limited response to biotin therapy depending on the genotype (Peters et al. 2000; Santer et al. 2003; Wilson et al. 2005; Van Hove et al. 2008). Thus, obtaining a molecular diagnosis can result in optimized management by providing a better understanding of the underlying enzyme defect and the extent to which a response to biotin therapy is anticipated.

Here, we describe a patient with a neonatal presentation of HLCS deficiency with partial response to biotin therapy and two novel *HLCS* aberrations. Sequencing of *HLCS* revealed a novel heterozygous variant in exon 5, c.996G>C (p.Gln332His), which may abolish the normal intron 6 splice donor site. Cytogenetic analysis revealed a paracentric inversion on chromosome 21 that was shown by FISH analysis to disrupt the other *HLCS* allele. Familial testing for these variants unexpectedly showed an additional pathogenic variant in the patient's mother, which was also passed onto the patient's sibling. Further familial analysis allowed for a number of interesting conclusions regarding the genotype–phenotype correlation of the family's *HLCS* aberrations.

Case Report

The infant boy was born by vaginal delivery at term to a 29-year-old gravida 5 para 2 mother after an uncomplicated pregnancy. Apgar scores and cord blood gases were normal. Birth parameters included a weight of 2.63 kg (<3rd centile), a length of 49.5 cm (43rd centile), and a head circumference of 33 cm (9th centile). The family history (Fig. 1) includes a mother of Korean ancestry who was adopted without further details known. She is healthy except for a history of palmar hyperkeratosis. The paternal ancestry is Italian, Irish, and French Canadian with no consanguinity present between the patient's parents. There is a known chromosome 21 inversion present in the paternal grandmother that was identified as part of workup for advanced maternal age. The patient also has a healthy 1-year-old sister whose newborn screen was normal. She has a history of hypopigmented hyperkeratotic skin lesions of uncertain etiology.

The patient was initially alert and fed well but within hours of delivery he developed poor feeding, tachypnea, and grunting. Physical exam was otherwise normal. An echocardiogram was only notable for a small patent foramen ovale and a patent ductus arteriosus. A cranial ultrasound showed multiple cystic lesions at the frontal horn and caudal thalamic notches of variable sizes that suggested multiple choroid plexus cysts or an intrauterine hematoma with resolving cystic change. The serum glucose level and oxygen saturation were normal. A capillary blood gas analysis revealed metabolic acidosis with a pH of 7.12 and an anion gap of 32 meq/L. Plasma lactate was 13.2 mmol/L (normal 0.5–2.2 mmol/L) with an elevated lactate:pyruvate ratio of 65 (normal < 25). Initial ammonia level was 136 μmol/L and quickly decreased with resuscitation. Plasma amino acids were within normal limits. Semiquantitative urine organic acids (compound peaks compared to an internal standard) showed massive amounts of lactic acid, very large amounts of 3-hydroxyisovaleric acid, large amounts of 3-methylcrotonylglycine, and a small amount of methylcitric acid. Newborn screening performed in the state of Michigan and drawn at 25 h of life showed a 3-hydroxyisovalerylcarnitine/2-methyl-3-hydroxybutyrylcarnitine (C5-OH) level of 3.45 μmol/L (<1.0 μmol/L) and a propionylcarnitine (C3) level of 7.1 μmol/L (<4.0 μmol/L), consistent with multiple carboxylase deficiency. A serum biotinidase enzyme assay was normal, suggesting a diagnosis of HLCS deficiency.

Empiric treatment with biotin 5 mg daily was started on the first day of life due to lactic acidosis and increased to 10 mg daily divided into two doses on day 3 when biochemical data supported the diagnosis of HLCS deficiency. Dietary protein was restricted to 1.5 g/kg/day. Tachypnea resolved and lactate slowly decreased to 5.5 mmol/L upon discharge on day 9.

At 2 years of age, he has had two hospitalizations for lactic acidosis, though not accompanied by clinical decompensation. During these hospitalizations, the biotin dose was increased to control lactic acidosis with noted decreases in lactate concentrations. Developmentally, he displays only mild expressive language delay. Current growth parameters include a weight of 12.7 kg (76th centile), a length of 81.4 cm (6th centile), and a head circumference of 51.7 cm (>95th centile). His physical exam is otherwise normal. He is maintained on carnitine 100 mg/kg/day and sodium bicarbonate titrated between 1 and 3 meq/kg/day. Biotin has been continued at a dose of 30 mg twice daily, which has maintained lactic acid levels in a range from 2.6 to 12.1 mmol/L during well periods and episodes of biochemical decompensation, respectively. The patient's biochemical labs were repeated during hospitalizations and during times of clinical stability and continued to show the presence of multiple abnormal metabolites at approximately the same relative concentrations (data not included).

Confirmation of the biochemical diagnosis by lymphocyte carboxylase enzyme activity assay, obtained during a period of biochemical stability, demonstrated significantly decreased activity of propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, and pyruvate carboxylase

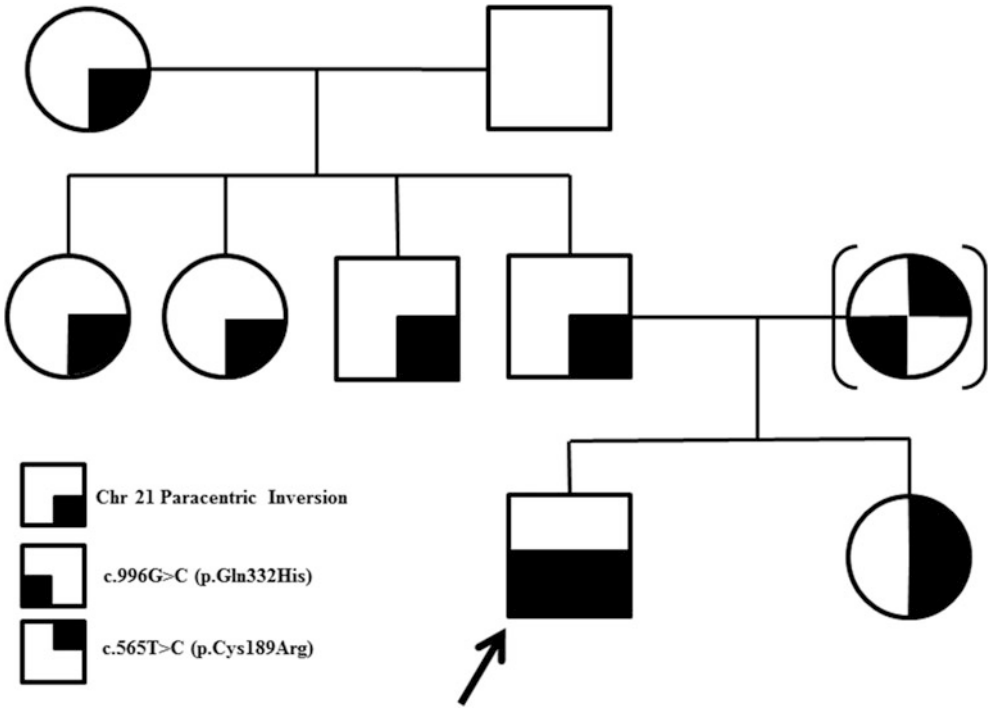

Fig. 1 Pedigree of the proband

(Table 1). Sequencing of the *HLCS* gene showed a heterozygous missense variant, c.996G>C (p.Gln332His). This residue is highly conserved and the last amino acid encoded in exon 5. The variant alters the terminal guanine and likely abolishes the normal intron 6 splice donor site. Three bioinformatics tools, SIFT, PolyPhen-2, and MutationTaster, predict the p.Gln332His change to be "not tolerated," "probably damaging," and "disease causing," respectively. A deletion and amplification analysis of *HLCS* via array comparative genomic hybridization was normal.

A karyotype was also obtained and revealed the same chromosome 21 paracentric inversion previously identified in the paternal grandmother (Fig. 2a). Subsequent fluorescent in situ hybridization (FISH) studies showed that the breakpoint was between the RP11-166F15 and the 24H16 loci, which are directly flanking and somewhat overlapping the *HLCS* gene proximally and distally (Fig. 2c). The FISH signal with probe RP11-383L18, which hybridizes entirely within the HLCS locus, was split (Fig. 2d), indicating that *HLCS* is disrupted by the inversion. The *HLCS* missense variant and the paternally inherited chromosome 21 inversion were therefore determined to be the molecular cause of the patient's HLCS deficiency.

Targeted molecular testing confirmed that the patient's mother carried the c.996G>C (p.Gln332His) variant. In addition, she was identified to have two additional variants: c.834T>C, a homozygous known synonymous benign variant, and c.565T>C (p.Cys189Arg), a heterozygous variant of unknown significance in *trans* with the c.996G>C (p.Gln332His) variant as it was not identified in her son. The p.Cys189 residue is fairly conserved across HLCS proteins; while a cysteine can be found at this position in many mammalian species, a leucine can be found at this position in nematode and fruit fly, and a valine can be found at this position in yeast. The amino acid change is nonconservative with the cysteine residue changed to a positively charged arginine. The variant is extremely rare, as it is not present in the Exome Aggregation Consortium (ExAC) database, dbSNP, or Exome Variant Server (EVS). The bioinformatics tools SIFT, PolyPhen-2, and Mutation Taster all predict the p.Cys189Arg substitution to be "deleterious." The mother is entirely asymptomatic except for palmar punctate keratitis, with no history to suggest a previous metabolic decompensation. Biochemical labs including lactate, plasma amino acids, semi-quantitative urine organic acids, plasma acylcarnitine profile, and serum biotinidase activity were all normal except for a mild elevation of propionylcarnitine at 0.97 μmol/L (normal < 0.88 μmol/L) and urine organic acids that showed a small amount of 3-hydroxyisovaleric acid and a trace amount of 3-methylcrotonylglycine. These results interpreted together were consistent with multiple carboxylase deficiency. A lymphocyte carboxylase enzyme activity assay confirmed mild HLCS deficiency with propionyl-CoA carboxylase activity of 9.1 pmol/min/mg protein (normal ≥ 70), 3-methylcrotonyl-CoA carboxylase

Table 1 Patient characteristics

Patient	Disease severity	Lactic acid (mmol/L; $N < 2.2$)	Ammonia (μmol/L; $N < 60$)	UOA abnormalities	ACP abnormalities (μmol/L)	PCC activity ($N > 70$)	3-MCC activity ($N > 31$)	PC activity ($N > 6$)	Genotype
Proband	Severe	13.2	136	Lactate 3-Hydroxybutyrate Acetoacetate 3-Hydroxyisovalerate 3-Methylcrotonylglycine Methylcitrate	C3: 160.39 ($N < 0.870$) C5-OH: 2.346 ($N < 0.110$)	8.67	2.84	0	p.Gln332His 46,XY,inv(21)(q21.1q22.13)
Proband's mother	Mild	1.7	–	3-Hydroxyisovalerate 3-Methylcrotonylglycine	C3: 0.970 ($N < 0.880$)	9.1	2.1	5.5	p.Cys189Arg p.Gln332His
Proband's sister	Mild	1.3	–	None	None	40	2	7	p.Cys189Arg 46,XY,inv(21)(q21.1q22.13)

UOA Urine organic acid, *ACP* Acylcarnitine profile, *PCC* Propionyl-CoA Carboxylase (pmol/min/mg protein), *3-MCC* 3-Methylcrotonyl-CoA Carboxylase (pmol/min/mg protein), *PC* Pyruvate Carboxylase (pmol/min/mg protein)

activity of 2.1 pmol/min/mg protein (normal ≥ 31), and pyruvate carboxylase activity of 5.5 pmol/min/mg protein (normal ≥ 6) (Table 1). Full *HLCS* gene sequencing was performed and no additional variants were identified. Based on the combination of biochemical abnormalities, dermatologic findings, and the analysis of the mother's *HLCS* variants, which were felt to be pathogenic, the patient's mother was diagnosed with mild HLCS deficiency, started on 5 mg of biotin, provided with an emergency room protocol and will be followed regularly in the biochemical genetics clinic.

The patient's sister was also evaluated and found to carry the chromosome 21 inversion. *HLCS* molecular testing showed that she also carries the c.565T>C (p.Cys189Arg) variant identified in her mother but as expected not the c.996 G>C (p.Gln332His) variant. Lymphocyte carboxylase enzyme activity was performed and was consistent with mild multiple carboxylase deficiency with propionyl-CoA carboxylase activity of 40 pmol/min/mg protein (normal ≥ 70), a 3-methylcrotonyl-CoA carboxylase activity of 2 pmol/min/mg protein (normal ≥ 31), and a pyruvate carboxylase activity of 7 pmol/min/mg protein (normal ≥ 9) (Table 1). Biochemical labs including lactate, urine organic acids, and plasma amino acids were all within normal limits. Given the molecular results and enzymatic testing she was placed on biotin 5 mg daily and provided with an emergency room protocol.

Methods

Patient

Exempt-status was granted by The University of Michigan's Institutional Review Board. Informed consent was obtained from all patients for which potentially identifying information is included in this article.

Molecular

Sequencing and deletion and amplification analysis of *HLCS* was performed by Prevention Genetics (Marshfield, WI).

Cytogenetic

Chromosome analysis was performed at the 550-band level of resolution using the GTG banding method. FISH was performed using BAC probes RP11-166F15 (chr21:38,044,629-38,215,919; hg19) (BlueGnome, UK), RP11-24H16 (chr21:38,291,517-38,442,545; hg19) (BlueGnome, UK), RP11-383L18 (chr21:38,187,159-

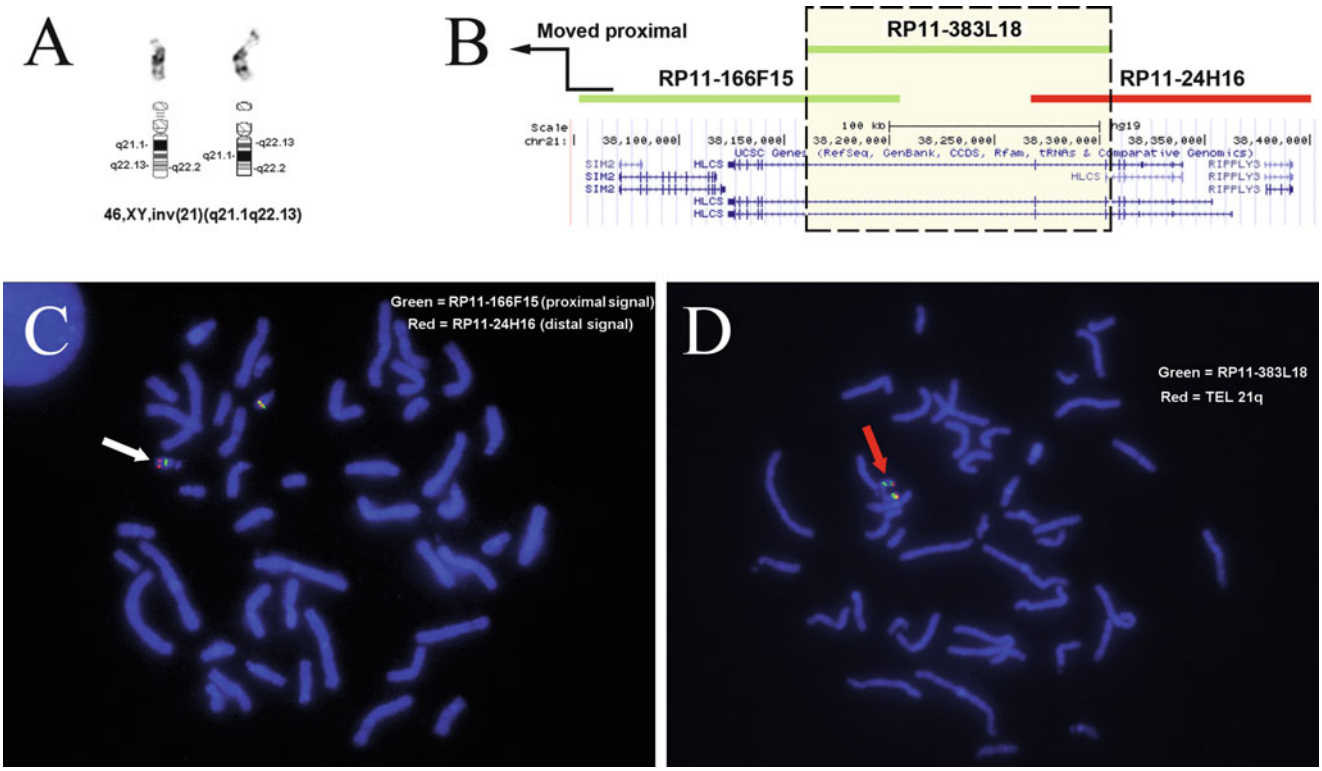

Fig. 2 Cytogenetic analysis of the proband which identified the paracentric inversion of chromosome 21 that disrupts *HLCS*. (**a**) G-banded chromosomes 21 and ideogram showing the paracentric inversion of chromosome 21. (**b**) Location of BAC probes used in FISH analysis. The *yellow box* outlines the map position of RP11-383L18 within the HLCS locus where the breakpoint occurred. (**c**) FISH analysis showing the breakpoint of the inversion is proximal to the RP11-24H16 locus (*red*) and distal to the RP11-166F15 locus (*green*) as it hybridized more proximal to its normal location (*white arrow*). (**d**) FISH analysis showing that the breakpoint occurred within the RP11-383L18 locus (*green*) as it hybridized to both its normal location and a more proximal location (*red arrow*)

38,390,773; hg19) (BlueGnome, UK), and 21qter (Abbott Molecular).

Enzymatic

Lymphocyte carboxylase enzyme activity was performed by the University of California San Diego Biochemical Genetics Laboratory. Briefly, lymphocytes recovered from blood by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich) were incubated with $Na \cdot H^{14}CO_3$ and (a) propionyl-CoA, (b) 3-methylcrotonyl-CoA, and (c) pyruvate plus acetyl-CoA, to produce the nonvolatile products ^{14}C-methylmalonyl-CoA, ^{14}C-methylglutaconyl-CoA, and ^{14}C-citrate through the propionyl-CoA carboxylase, methylcrotonyl-CoA carboxylase, and pyruvate carboxylase (plus citrate synthase) reactions, respectively. Unreacted $Na \cdot H^{14}CO_3$ is removed as $^{14}CO_2$ by acidification with formic acid and drying under heat, and quantification of retained ^{14}C is used to calculate the activity of each enzyme (Van Hove et al. 2008). As part of the laboratory quality assurance, continuing assay performance validation includes adjustment of normal ranges at semiannual intervals, based on frequency distribution of control results obtained over each interval.

Discussion

Our patient demonstrated the classic biochemical and clinical findings associated with HLCS deficiency and harbored two novel aberrations of the *HLCS* gene. His clinical presentation included tachypnea and poor feeding but no significant skin involvement. Cranial ultrasound also demonstrated subependymal cysts, a finding observed in most reported cases of holocarboxylase deficiency due to the p.L216R pathogenic variant (Wilson et al. 2005; Slavin et al. 2014). His partial response to high dose biotin (60 mg daily) is evident by the age-appropriate developmental skills and normal physical examination but with persistently elevated lactic acid concentration and abnormal metabolites on urine organic acids.

Currently, there are 41 pathogenic variants reported in *HLCS* (http://www.hgmd.cf.ac.uk, accessed June 2016; Aoki et al. 1999; Yang et al. 2001). Biotin responsive

patients often have pathogenic variants identified within the biotin-binding domain (between amino acids 448 and 701), causing an increased K_m for biotin (Suzuki et al. 1994). Biotin supplementation in these patients overcomes this increased K_m restoring enzymatic activity. These patients are clinically asymptomatic on the suggested dose of biotin 10–20 mg daily and require no additional therapies, such as protein restriction or carnitine supplementation with good clinical outcomes documented with biotin doses as low as 1.2 mg daily (Dupuis et al. 1999; Bailey et al. 2008; Tammachote et al. 2010). Conversely, individuals with pathogenic variants outside of the biotin-binding domain are considered biotin-unresponsive and are the most severe cases (Mayende et al. 2012). These patients display normal biotin affinity but a decreased HLCS V_{max} (Suzuki et al. 1994). Though these variants are labeled as biotin-unresponsive, all patients to date with HLCS deficiency display some degree of clinical responsiveness, though this response may only be partial as evident by persistent excretion of abnormal metabolites (Suzuki et al. 1994). This clinical improvement may be due to biotin's ability to increase transcription of the *HLCS* gene and/or the observation that the maximum activity of HLCS occurs at supraphysiologic biotin concentrations (Aoki et al. 1997; Solórzano-Vargas et al. 2002). In line with this observation, very large doses of biotin (i.e. 1.2 g daily) have been found to ameliorate symptoms in individuals homozygous for the p.L216R variant, a non-K_m-variant which causes one of the most severe forms of HLCS deficiency (Slavin et al. 2014).

The patient described here was found to have two novel pathogenic variants in the *HLCS* gene making it difficult to predict how these alterations respond to biotin therapy. The paracentric inversion disrupts transcription of the *HLCS* allele and is predicted to result in a complete absence of functional HLCS protein. Importantly, the presence of two null-variants has been suggested to result in perinatal lethality, as no patients harboring two null variants have been identified to date (Suzuki et al. 1994). This suggests that the p.Gln332His variant, though predicted to disrupt splicing, likely results in the production of at least some functional protein (Suzuki et al. 1994). This residual protein, though harboring a non-K_m-variant, has shown partial responsiveness to biotin supplementation clinically.

In our case, the molecular diagnosis allowed for familial testing which surprisingly identified the patient's mother and sister to also be affected with mild HLCS deficiency. Interestingly, the patient's mother was found to have a novel, nonconservative missense variant, p.Cys189Arg, in addition to the likely pathogenic variant, p.Gln332His. Enzymatic analysis confirmed the mild HLCS deficiency with the only disease manifestation being palmar hyperkeratosis and urine organic acids that showed the presence of a few abnormal metabolites. The patient's sister was found to carry the chromosome 21 inversion, which also likely results in a null allele, as well as the p.Cys189Arg variant identified in her mother. The sister's history was normal except for hyperkeratotic skin lesions. Subsequently, enzymatic analysis confirmed mild HLCS deficiency but with no abnormal urine metabolites or lactic acid elevation.

Comparison of the enzymatic activities of all three patients reveals a number of interesting conclusions. The proband's severe clinical presentation is consistent with the significantly decreased activity in all three enzyme complexes. The absence of any appreciable pyruvate carboxylase (PC) activity combined with the "biotin-unresponsive" pathogenic variant likely explains the proband's persistent lactic acid elevations on high dose biotin. The significantly decreased propionyl CoA carboxylase (PCC) activity in the proband's mother compared to the only mildly decreased activity in the proband's sister provides a plausible explanation for the mother's acylcarnitine abnormalities. Interestingly, the patient's sister was found to have the lowest activity of 3-methylcrotonyl-CoA carboxylase when compared to her brother and mother, but with an absence of 3-methylcrotonylglycine in the urine or C5-OH abnormalities on acylcarnitine analysis. The exact reasons for this are uncertain at this point but it has previously been shown that there is known biochemical heterogeneity between patients (Burri et al. 1985).

This case supports the necessity of determining an HLCS patient's molecular diagnosis. If a non-K_m-variant is identified, treating physicians should expect that a patient will only experience therapeutic benefit with a very high biotin dose. In line with this, our patient's case also argues for a revision of how we classify *HLCS* variants and patients. Labeling an HLCS patient and/or an *HLCS* variant as "biotin-unresponsive" may inadvertently cause a patient to miss out on a potentially beneficial medication. As the exact mechanisms for biotin responsiveness have yet to be fully elucidated, an alternative classification is not readily apparent. Our case, in combination with the recent report by Slavin et al. (2014), provides evidence that large doses of biotin can ameliorate some symptoms even when the detected mutations suggest severe disruption in the production of a functional protein. These individuals, however, will require closer observation, especially during periods of stress and illness. Furthermore, supplementary therapies such as carnitine, sodium bicarbonate, and protein restriction may be necessary. Additionally, when standard molecular testing does not reveal an abnormality, an underlying chromosomal rearrangement should be considered.

Take-Home Message

The molecular characterization of patients with holocarboxylase synthetase deficiency is an important aspect of their workup, as it facilitates necessary family testing and provides useful insight into their potential response to biotin therapy.

Details of Contributions of Individual Authors

Shane C. Quinonez: manuscript preparation, Table 1 creation.

Andrea H. Seeley: manuscript preparation, Fig. 1 design, and Table 1 creation.

Thomas W. Glover: cytogenetic laboratory interpretation, Fig. 2 design, and manuscript review.

Cindy Lam: cytogenetic laboratory interpretation and Fig. 2 design.

Bruce A. Barshop: biochemical laboratory interpretation and manuscript review.

Catherine E. Keegan: manuscript review and planning/organization of manuscript.

Manuscript Guarantor

Catherine E. Keegan MD, PhD.

Compliance with Ethics Guidelines

Shane C. Quinonez declares that he has no conflict of interest.

Andrea H. Seeley declares that she has no conflict of interest.

Thomas W. Glover declares that he has no conflict of interest.

Cindy Lam declares that she has no conflict of interest.

Bruce A. Barshop declares that he has no conflict of interest.

Catherine E. Keegan declares that she has no conflict of interest.

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Details of Ethics Approval

Not required by our institution. Informed consent was obtained from all patients for which potentially identifying information is included in this article.

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RESEARCH REPORT

Delayed Infusion Reactions to Enzyme Replacement Therapies

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Abstract *Background*: There are currently ten intravenous enzyme replacement therapy (ERT) products available for the treatment of eight different lysosomal diseases (LD) in the USA. Additional ERT products are in clinical trials. The most common ERT adverse events are infusion reactions (IR). While IR are often defined as hypersensitivity or anaphylactoid reactions occurring concurrently with (i.e., during) infusion administration (CIR), there exists the potential for delayed infusion reactions (DIR), which present after completion of infusion administration.

Hypothesis: Concurrent infusion reactions (CIR) are not the only infusion reactions associated with enzyme therapy.

Methods: This study evaluated the occurrence of infusion reactions in 46 patients with LD who had received ERT for a minimum of 2 years. Infusion reactions were evaluated according to symptoms, time of onset, and duration of reactions. The frequency of infusion reactions with each ERT product was compared to that reported in the FDA-approved product package insert.

Results and Conclusions: In this study, DIR were observed and occurred as often as CIR in the study population, despite not being characterized or reported in most ERT product package inserts. Effective methods for managing DIR and CIR differed, thus emphasizing the importance of monitoring for both types of infusion reactions in order to optimize outcomes for patients using ERT.

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Introduction

Intravenous (IV) enzyme replacement therapy (ERT) is FDA approved for the treatment of eight different lysosomal diseases (LD): Gaucher disease (OMIM catalogue number 230800), Fabry disease (OMIM catalogue number 301500), Pompe disease (OMIM catalogue number 232300), mucopolysaccharidosis type I (MPS I) (OMIM catalogue number 607014), mucopolysaccharidosis type II (MPS II) (OMIM catalogue number 309900), mucopolysaccharidosis type IV A (MPS IVA) (OMIM catalogue number 253000), mucopolysaccharidosis type VI (MPS VI) (OMIM catalogue number 253200), and lysosomal acid lipase deficiency (LAL-D) (OMIM catalogue number 278000). There are currently ten FDA-approved ERT products (Table 1) and additional ERT products are in development or in clinical trials OMIM 2016.

The most common adverse reactions associated with ERT are infusion reactions (IR). IR symptoms are most frequently defined as those of an allergic or type I hypersensitivity reaction, occurring during or shortly after the infusion administration. Commonly reported IR symptoms include flushing, hives, fever, chills, hypertension/

Table 1 FDA-approved enzyme replacement therapies currently available for lysosomal diseases

	Enzyme Replacement Therapy (ERT)			
Lysosomal disease	Generic (trade) names	Manufacturer (IBD)[a]	FDA-approved dosing	Dosing frequency
Fabry disease	Agalsidase beta (Fabrazyme® (2003)) EC 3.2.1.22	Genzyme (1994)	1 mg/kg	Biweekly
Gaucher disease type I	Imiglucerase (Cerezyme® (2014)) EC 3.2.1.45	Genzyme (1994)	2.5–60 units	Three times weekly–biweekly
	Velaglucerase alfa (VPRIV® (2010)) EC 3.2.1.45	Shire (2010)	60 units/kg	Biweekly
	Taliglucerase (Elelyso® (2012)) EC 3.2.1.45	Pfizer (2012)	60 units/kg	Biweekly
Pompe disease – infantile and late onset	Alglucosidase alfa (Lumizyme®) EC 3.2.1.3	Genzyme (2010)	20 mg/kg	Biweekly
MPS I	Laronidase (Aldurazyme® (2002)) EC 3.2.1.76	BioMarin (2003)	0.58 mg/kg	Weekly
MPS II	Idursulfase (Elaprase®) EC 3.1.6.13	BioMarin (2006)	0.5 mg/kg	Weekly
MPS IV type A	Elosulfase alfa (Vimizim® (2013)) EC 3.1.6.4	BioMarin (2014)	2 mg/kg	Weekly
MPS VI	Galsulfase (Naglazyme® (2013)) EC 3.1.6.12	BioMarin (2005)	1 mg/kg	Weekly
Lysosomal acid lipase deficiency (LAL-D)	Sebelipase alpha (Kanuma™ (2015)) EC 3.1.1.13	Alexion (2015)	1 mg/kg	Weekly

[a] IBD: International Birth Date
Note: Biweekly denotes ERT infusions once every 2 weeks (14 days)

hypotension, arrhythmias, rigors, dyspnea, bronchospasm, and wheezing. These symptoms are usually readily ameliorated by reducing the rate of the infusion or temporarily discontinuing the infusion, administration of antipyretics (e.g., acetaminophen), and/or anti-inflammatory agents (e.g., ibuprofen), and/or a steroid (e.g., hydrocortisone), and/or antihistamines (e.g., diphenhydramine).

Delayed infusion reactions (DIR) to ERT were first described in 2008 (Utz et al. 2008). An 8-week study of 23 patients receiving ERT noted two distinct types of IR: (1) concurrent infusion reactions (CIR), occurring during or within 4 h after completion of ERT infusion with symptoms corresponding to those described in the product package inserts (PI), resembling a type I hypersensitivity reaction, (2) DIR, with a late onset and symptoms that begin within 4–24 h after completion of the infusion and continuing for a duration of 24–72 h. DIR often presented as a combination of flu-like symptoms that included fatigue/lethargy, body aches (e.g., leg pain), headache, abdominal pain, nausea, vomiting, and diarrhea.

A number of case reports and articles have described infusion reactions to ERT and its management (Miebach 2009; Nicholls et al. 2012; Kim et al. 2008). Some studies have focused on the mechanism and physiology of CIR (Haller et al. 2016). Studies to identify the incidence, associated symptoms, and management of DIR have not been conducted. Of the ten ERT products that are FDA approved for use, only the PI for alglucosidase alfa (Lumizyme® 2014) and idursulfase (Elaprase® 2014) mention the possibility of an infusion reaction occurring after completion of the infusion.

Hypothesis

This study hypothesized that CIR are not the only drug reactions associated with ERT, and that DIR may be associated with all ERT drugs.

Methods

This study was conducted under University of Minnesota Institutional Review Board approval.

Retrospective and prospective chart reviews were performed at the Advanced Therapies Clinic of the University of Minnesota Medical Center (UMMC 2016) to identify patients with LD treated with ERT between 2008 and 2015. All sexes and age groups were included in this study. Patients must have received ERT infusions for at least 2 consecutive years. This allowed reasonable comparison of IR incidence in the study population to that reported in

Table 2 Demographics of subjects and their lysosomal diseases

Total patients ($N = 46$)	Fabry disease ($n = 17$)	Gaucher disease ($n = 7$)	Pompe disease ($n = 9$)	MPS I ($n = 4$)	MPS II ($n = 4$)	MPS IV ($n = 1$)	MPS VI ($n = 3$)	LAL-D ($n = 1$)
Sex	11 (64.7%)	3 (43%)	5 (56%)	1 (25%)	4 (100%)	0 (0%)	2 (67%)	1 (100%)
Male ($n = 27$)	6 (35.3%)	4 (57%)	4 (44%)	3 (75%)	0 (0%)	1 (100%)	1 (33%)	0 (0%)
Female ($n = 19$)								
Age	14 (82.4%)	7 (100%)	8 (89%)	0 (0%)	1 (25%)	1 (100%)	1 (33%)	1 (100%)
Adult ($n = 33$) ($\geq$18 years old)	3 (17.6%)	0 (0%)	1 (11%)	4 (100%)	3 (75%)	0 (0%)	2 (67%)	0 (0%)
Pediatric ($n = 13$) ($<$18 years old)								

product package inserts, as PI data was from clinical trials taking place over 1–2 years.

Patients were counseled about CIR and DIR prior to starting treatment with ERT. Any CIR was documented by the infusion nurse during ERT administration, and DIR were reported by the patient or caregiver voluntarily, or were discovered when they were questioned about DIR during pharmacotherapy consultation, followed by documentation in the patient electronic medical record (EMR).

IR to ERT were evaluated according to symptom presentation, time of onset, and duration. A subanalysis was performed to evaluate the association of sex, age, and the presence or absence of antidrug antibodies (ADA) with IR. ADA were measured through processes provided by individual ERT manufacturers for determining and interpreting presence or absence of ADA. The percentage of patients who had experienced any type of IR to a given ERT were compared to that reported in the corresponding PI. Preventative and treatment interventions employed for the management of IR in the study population were also noted. When EMR did not include specific information required for this study (i.e., history of presence or absence of IR symptoms), the patients and/or their representatives were contacted for that information. Subject characteristics were summarized with mean and standard deviation for continuous variables, and frequencies and percentages for categorical variables. Differences between groups in proportions and corresponding confidence intervals and *P*-values were based on a two-sided chi-squared test. Confidence intervals and two-sided *P*-values for individual proportions were based on the exact binomial distribution. All analyses were performed using R v3.1.1 (R Core Team 2014).

Results

Patients

Between 2008 and 2015, 46 patients with an LD were identified who had received ERT for a minimum of 2 years: 27 (59%) male and 33 (73%) adult patients. The mean age was 40.2 ± 20.5 years (range: 9–85 years). The patient demographics based on sex, age, and LD are presented in Table 2.

Infusion Reaction Analyses

Of the 46 patients evaluated, 25 patients (54.3%) reported at least one IR to ERT. Of these 25 patients, 16 patients (34.8%) experienced a DIR at least once, 16 patients (34.8%) experienced a CIR at least once, and 7 patients (15.2%) experienced a CIR and DIR at least once during their history of ERT. Three patients (6.5%) reported having, at one or more times, CIR during the infusion administration followed by a DIR occurring after completion of the very same infusion.

The percentage of patients experiencing specific symptoms of CIR and DIR are shown in Table 3. The most common CIR symptom was dyspnea/shortness of breath (69%), while the most common DIR symptom was fatigue/lethargy (88%). DIR symptoms such as fatigue/lethargy were distinguished from the sedative side effects of antihistamine and antiemetic premedications since only 3 (22%) out of 14 patients who experienced fatigue/lethargy had received sedative antihistamines, and no patients were premedicated with sedating antiemetics prior to ERT infusions.

Subanalysis of the percentage of patients experiencing CIR and DIR for sexes and age groups showed that in patients with Fabry disease, DIR was higher in females compared to males (67% versus 36%, $P = 0.492$). Statistical subanalyses of other groups were not feasible due to small sample sizes.

When data were available, the presence (positive) or absence (negative) of ADA was documented for patients in each LD group. The ADA measured by the manufacturers were IgG. No IgE levels were reported. It was found that ADA values were not consistently available for patients at times that corresponded with infusion reactions. Furthermore, some ERT (i.e., taliglucerase and elosulfase alfa) do

Table 3 Frequency of infusion-associated symptoms in CIR (during infusion) and DIR (post-infusion)

Infusion reaction symptoms	Subjects with symptoms *during infusion* ($n = 16$)	Subjects with symptoms *post-infusion* ($n = 16$)
Chills	1 (6%)	0
Fever/Pyrexia	0	0
Rigors	0	0
Dyspnea/Shortness of breath	11 (69%)	0
Chest pain/Tightness	4 (25%)	0
Throat tightness/ Bronchospasm/ Wheezing	1 (6%)	0
Dizziness/ Lightheadedness	3 (19%)	0
Blood pressure changes (hypertension or hypotension)	1 (6%)	0
Arrhythmias (tachycardia or bradycardia)	0	0
Flushing	0	0
Rash	1 (6%)	0
Urticaria/Hives	0	0
Pruritus/Itching	0	0
Nasal congestion	0	0
Edema (peripheral, facial, etc.)	0	0
Somnolence/Drowsiness/ Sleepiness	0	0
Headache	0	2 (13%)
Gastrointestinal side effects (nausea, vomiting, diarrhea, abdominal pain)	0	4 (25%)
Paresthesia	0	0
Pain in extremities (leg pain)	0	1 (6%)
Back pain	0	0
Myalgia	0	0
Fatigue/Lethargy	0	14 (88%)
Flu-like body ache	0	2 (13%)

CIR concurrent infusion reaction
DIR delayed infusion reaction

not have ADA measurement services provided by the manufacturers. Due to these limitations and the small sample sizes, calculation of odds ratios and confidence intervals for ADA was not possible.

The percentage of patients who experienced IR was compared to that reported by the PI for the ERT they were receiving (Table 4). The incidence of any type of IR was found to be highest among patients with Fabry disease (71%, $P = 0.152$), followed by Pompe disease (44%, $P = 0.001$) and Gaucher disease (33%, $P = 0.623$ and 29%, $P = 0.098$ for velaglucerase alfa and imiglucerase, respectively). The small number of patients with MPS disorders (I, II, IV A, and VI) and LAL-D prevented reporting a meaningful IR incidence, precluding formal comparisons to their respective PI.

Discussion

This is the first study to characterize DIR to ERT and compare infusion reaction occurrence to that reported in the FDA-approved package inserts. The symptom profile of CIR was clearly distinguished from DIR in this study population. Dyspnea, chest tightness, and dizziness were the most common symptoms noted in patients with CIR, while fatigue, gastrointestinal side effects (nausea, vomiting, and diarrhea), flu-like body aches, and pain in the extremities were the most common symptoms reported by patients with DIR.

Alglucosidase alfa (2010) is the only ERT product whose PI mentions the possibility of a "delayed-onset reaction"; however, the PI does not clarify when the symptoms of "delayed-onset reaction" occur in relation to infusion administration (Lumizyme® 2014). Moreover, the reported incidence of IR in the PI is stated as "≥3%," leaving questions about the actual overall IR risk for patients. The percentage of patients with Pompe disease at the University of Minnesota who experienced any IR to alglucosidase alfa was higher than that reported by the PI, and the difference was statistically significant (44% versus 3%, $P = 0.001$). The PI for idursulfase described a "late-emergent or biphasic anaphylactoid/anaphylactic reaction" (Elaprase® 2014). It is important to note that the biphasic reaction is distinguished from the DIR observed in the University of Minnesota study population. Specifically, the "biphasic reaction" to idursulfase had CIR symptoms occurring during the infusion, followed by an identical set of CIR symptoms recurring 24 h later.

The overall percentage of Fabry disease patients in this study who had ever experienced any type of IR to agalsidase beta was greater than that reported in the PI (71% versus 50–55%, $P = 0.152$). Although the study population was small, a similar trend was seen among patients with Gaucher disease using imiglucerase (29% versus 6.6%, $P = 0.098$), as well as those who switched to velaglucerase alfa (33% versus 22.5%, $P = 0.623$). Even though these differences were not statistically significant, one might question if the discrepancies were due to underreporting of DIR in the clinical trials.

The subanalysis demonstrated higher incidence of DIR among females compared to males in patients with Fabry disease (67% versus 36%, $P = 0.492$). This difference is *not statistically significant*, but the trend is *notable* (DIR was observed almost twice as frequently in female patients compared to males).

Table 4 Comparison of the incidence of infusion reactions observed in the study population and FDA-approved package inserts

Lysosomal disease	ERT generic (trade) names	Infusion reactions in FDA-approved PI	CIR (95% CI)	DIR (95% CI)	IR (95% CI)	*P*-value
Fabry disease	Agalsidase beta (Fabrazyme® (2003))	50–55%	41% (18%, 67%)	47% (23%, 72%)	71% (44%, 90%)	0.152
Gaucher disease type I	Imiglucerase (Cerezyme® (2014))	6.6–9%	29% (4%, 71%)	29% (4%, 71%)	29% (4%, 71%)	0.098
	Velaglucerase alfa (VPRIV® (2010))	22.5% C	33% (4%, 78%)	33% (4%, 78%)	33% (4%, 78%) C	0.623
		51.9% N	–	–	–	–
	Taliglucerase (Elelyso® (2012))	44–46%	0	0	0	–
Pompe disease – infantile and late onset	Alglucosidase alfa (Lumizyme® (2014)))	≥3%	22% (3%, 60%)	33% (7%, 70%)	44% (14%, 79%)	0.001
MPS I	Laronidase (Aldurazyme® (2002))	32–49%	50%	25%	75%	–
MPS II	Idursulfase (Elaprase®)	68.8%	25%	25%	50%	–
MPS IV type A	Elosulfase alfa (Vimizim® (2013))	71.2%	0%	0%	0%	–
MPS VI	Galsulfase (Naglazyme® (2013))	56–70%	67%	33%	67%	–
Lysosomal acid lipase deficiency (LAL-D)	Sebelipase alpha (Kanuma™ (2015))	20%	0%	0%	0%	–

CIR concurrent infusion reactions in study population
DIR delayed infusion reactions in study population
IR infusion reactions (CIR or DIR) in study population
PI package insert
C patients who switched from Cerezyme to VPRIV, *N* Naïve patients who initially received VPRIV as ERT
A adults, *P* pediatrics
Dashes (–) insufficient data available

A clear association was not observed between the presence of ADA and the occurrence of IR in this study, consistent with information reported by most PI. Unfortunately, the sample size and limited available laboratory data prevented a more comprehensive analysis of ADA. Additionally, the incidence of ADA positivity may be influenced by a number of factors related to assay methodology, sample handling, timing of sample collection relative to dosing, concomitant medications, and underlying disease, and due to this, direct comparisons between different ERT are not recommended (Kishnani et al. 2016).

Measures taken to prevent and treat IR to ERT at the University of Minnesota are presented in Fig. 1. Strategies to prevent CIR included slowing the infusion rate, and administration of premedications that include oral acetaminophen, intravenous (IV) diphenhydramine antihistamine and IV corticosteroid. If a DIR was persistent and causing a significant impact on quality of life (e.g., patient missing days at work or school for 2–3 days after each ERT infusion due to DIR), the use of a longer-acting steroid premedication (e.g., IV methylprednisolone) was often effective in preventing DIR. Whenever possible, premedications were gradually tapered off or reduced to the minimum effective dose with which IR could be prevented.

Of the 25 patients who experienced any type of IR in this study, 17 patients received prophylactic therapy to prevent IR with subsequent infusions: eight with history of CIR, three with history of DIR, and five with history of both CIR and DIR. Prophylactic measures were successful in preventing all CIR from reoccurring. In contrast, successful prevention of DIR was achieved in only 50% of the patients. Patients who did not receive prophylactic therapy included six patients with DIR symptoms consisting of mild-moderate fatigue for 24 h after completion of infusion, who chose not to receive prophylactic therapy. One patient was lost to follow-up due to relocation to a different state.

It is notable that two patients chose to discontinue ERT due to infusion reactions. One of these patients experienced

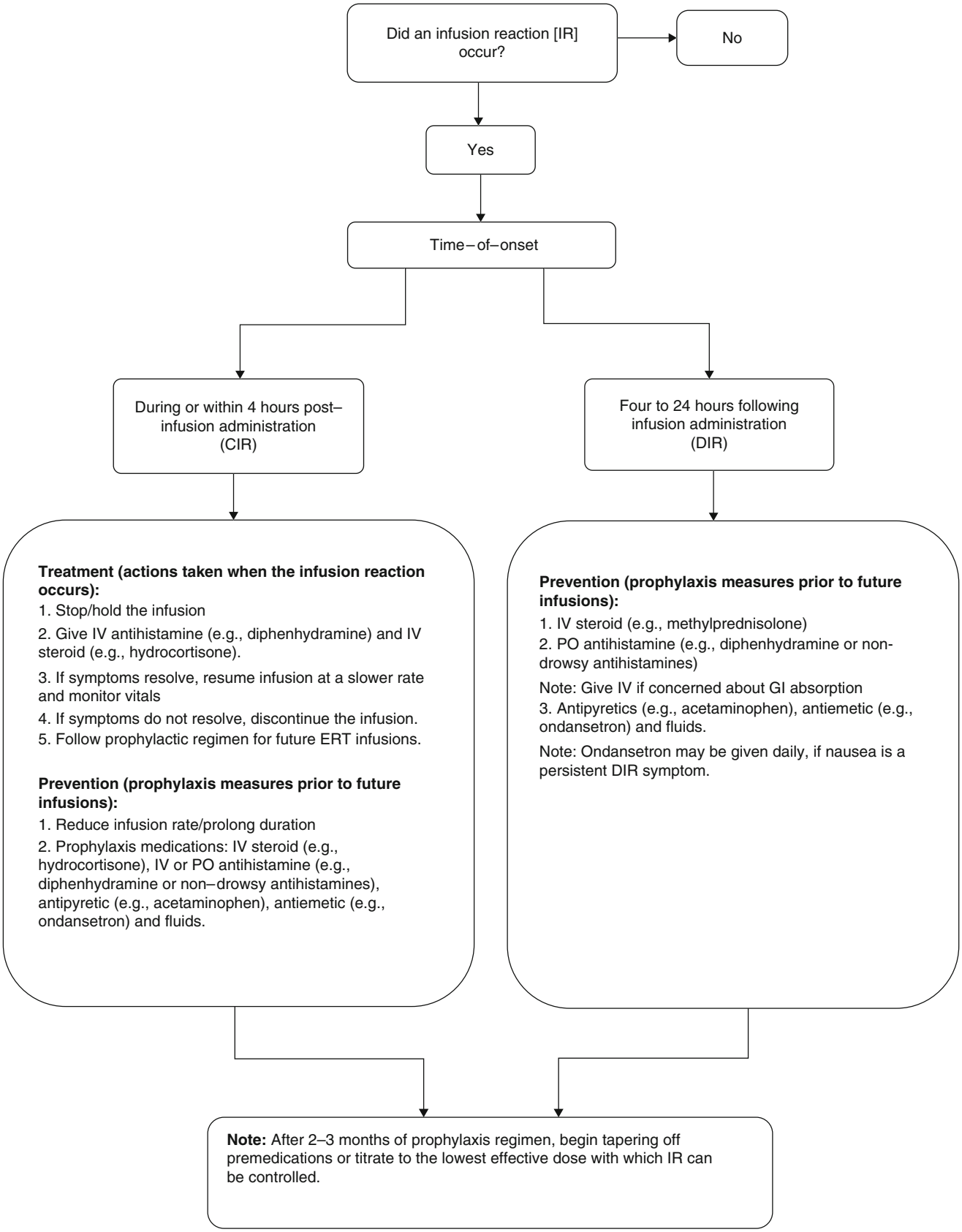

Fig. 1 Prevention and treatment strategies for CIR and DIR (guidelines from University of Minnesota Medical Center (UMMC 2016) Advanced Therapies Program). *CIR* Concurrent infusion reactions, *DIR* Delayed infusion reactions

both CIR and DIR, and had partial improvement in the severity and duration of symptoms following prophylactic management. Despite some improvement, the patient chose to discontinue ERT due to the impact of IR on their quality of life. The other patient experienced a CIR and opted for discontinuing ERT without trying premedications. These two cases, in particular, underscore the importance of proper identification and management of infusion reactions to improve patient adherence to therapy.

Limitations of This Study

An important limitation of this study is the sample size of certain LD groups, which prevented statistical analyses of IR incidence by sex, age, ADA response, as well as the comparison of IR incidence to that of their respective PI. Future studies that include larger patient sample sizes and ADA values (e.g., IgG, IgE, and neutralizing ADA) ordered consistently and in relation to infusion reactions would improve analysis and understanding of infusion reactions (CIR and DIR) to ERT.

Conclusions

The recognition and management of infusion reactions to ERT are critical to ensure patient safety during and after ERT administration. In this study, a distinct form of IR to ERT was observed, which can be distinguished by symptoms, time of onset, and duration. Pharmacovigilance and adverse event reporting systems for ERT have previously characterized IR as occurring during the infusion administration, but have rarely captured information on reactions occurring after completion of the infusion. Inadequate management of IR has resulted in patients discontinuing ERT, and this has occurred in a setting in which ERT has been the only treatment option for debilitating, and often catastrophic diseases. Recognizing DIR will lead to safer and more effective administration of ERT, while enhancing the quality of life for patients who are receiving potentially lifelong therapy with ERT.

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One Sentence Synopsis of Article

Although not reported in most product package inserts, delayed infusion reactions to enzyme replacement therapy must be recognized, and their successful prevention and treatment require distinction from concurrent infusion reactions.

Contributions of Individual Authors

Dr. Jeanine R. Utz is the Principal Investigator and Director of the Pharmacotherapy of Inherited Metabolic Diseases (PIMD) fellowship program. She provides ongoing clinical care for the patients in the study. Dr. Utz conceived, designed, and conducted the study, orchestrated funding, coordinated efforts of collaborators, and participated in the interpretation of data. She is the guarantor for this article, accepts full responsibility for the work and the conduct of this study, and provided final approval of the version to publish.

Dr. Chester B. Whitley is the Co-Investigator for this study, Director of the Advanced Therapies Program and Gene Therapy Center. He provides continuous medical care for the patients in the study, has made significant contributions to formulating the hypothesis and study design, and offered critical revisions of the article.

Dr. Kyle D. Rudser is a senior biostatistician at the Clinical and Translational Science Institute. He offered statistical expertise in the study design, data monitoring, and analyses for this project, conducted the statistical analysis and interpretation of the results, and wrote relevant portions of the paper.

Dr. Zahra Karimian is a postdoctoral research associate and fellow in the PIMD program. Contributed substantially to the project through acquisition, reporting, analysis, and interpretation of the data, and wrote major portions of the paper.

All authors are responsible for the validity of the entire work and manuscript submitted for publication.

Funding and Conflict of Interest

Dr. Jeanine Utz is the recipient of an unrestricted educational grant from Genzyme-Sanofi which helps fund the PIMD fellowship in addition to the NIH LDN grant (U54NS065768). She provides consultation for the scientific

content of the annual WORLD Symposium meeting and is on the Speakers bureau for Genzyme, Shire, and Pfizer.

Dr. Chester Whitley is the Principal Investigator for the NIH Lysosomal Disease Network (LDN) grant (U54NS065768). He provides consultation for gene therapies being developed by Sangamo and the scientific content of the annual WORLD Symposium meeting.

Dr. Kyle D. Rudser is a recipient of the NCATS award (UL1TR000114) and the NIH LDN grant (U54NS065768).

Dr. Zahra Karimian is a postdoctoral research fellow in the Pharmacotherapy of Inherited Metabolic Diseases (PIMD) program, which is supported by an unrestricted educational grant from Genzyme-Sanofi in addition to the NIH LDN grant (U54NS065768).

The contents of this article have not been influenced by the financial resources disclosed above and the authors declare that they have no conflict of interest.

Compliance with Ethics Guidelines

This study was conducted under University of Minnesota Institutional Review Board (IRB) 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 (5). Informed consent was obtained from all patients for being included in this study. Patients were made aware that participation in the study was voluntary, that they had the right to withdraw from the study at any time, and would continue to have access to unrestricted health care despite withdrawal.

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RESEARCH REPORT

Novel *PEX3* Gene Mutations Resulting in a Moderate Zellweger Spectrum Disorder

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Abstract *Background*: Peroxisome biogenesis disorders (PBDs) may have a variable clinical expression, ranging from severe, lethal to mild phenotypes with progressive evolution. PBDs are autosomal recessive disorders caused by mutations in *PEX* genes, which encode proteins called peroxins, involved in the assembly of the peroxisome.

Patient Description: We herein report a patient who is currently 9 years old and who is compound heterozygous for two novel mutations in the *PEX3* gene.

Results: Mild biochemical abnormalities of the peroxisomal parameters suggested a Zellweger spectrum defect in the patient. Sequence analysis of the *PEX3* gene identified two novel heterozygous, pathogenic mutations.

Conclusion: Mutations in *PEX3* usually result in a severe, early lethal phenotype. We report a patient compound heterozygous for two novel mutations in the *PEX3* gene, who is less affected than previously reported patients with a defect in the *PEX3* gene. Our findings indicate that *PEX3* defects may cause a disease spectrum similar as previously observed for other *PEX* gene defects.

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Introduction

Peroxisomal disorders can be subdivided into peroxisome biogenesis disorders (PBDs; MIM 601539) and the single peroxisomal enzyme deficiencies (Wanders 1999; Wanders and Waterham 2004). PBDs include the Zellweger spectrum disorders (ZSDs), which are Zellweger Syndrome (ZS), Neonatal Adrenoleukodystrophy (NALD) and Infantile Refsum Disease (IRD), and Rhizomelic Chondrodysplasia Punctata (RCDP) type 1 (Wanders 1999; Wanders and Waterham 2004; Muntau et al. 2000a, b; Shimozawa et al. 2005). Patients with a ZSD may present with a continuum of symptoms of varying severity; the ZS presentation represents the most severe clinical phenotype. ZSDs can be caused by mutations in any of at least 13 different *PEX* genes, which encode proteins called peroxins that are involved in peroxisomal protein import and peroxisome assembly (Ebberink et al. 2012).

The *PEX1*, *PEX6* and *PEX12* genes are the most affected genes in patients with a ZSD (Ebberink et al. 2012). In contrast, *PEX3* defects are very rare. Patients with a defect in *PEX3* are usually severely affected, thus resulting in early lethality.

We herein report a patient, who is currently 9 years old and who is compound heterozygous for two novel mutations in the *PEX3* gene. The patient is less affected than previously reported patients with a defect in the *PEX3* gene.

Patient Description

This 9-year-old boy is the first child of non-consanguineous healthy parents. The pregnancy was uneventful and he was born at term by caesarean delivery due to podalic version.

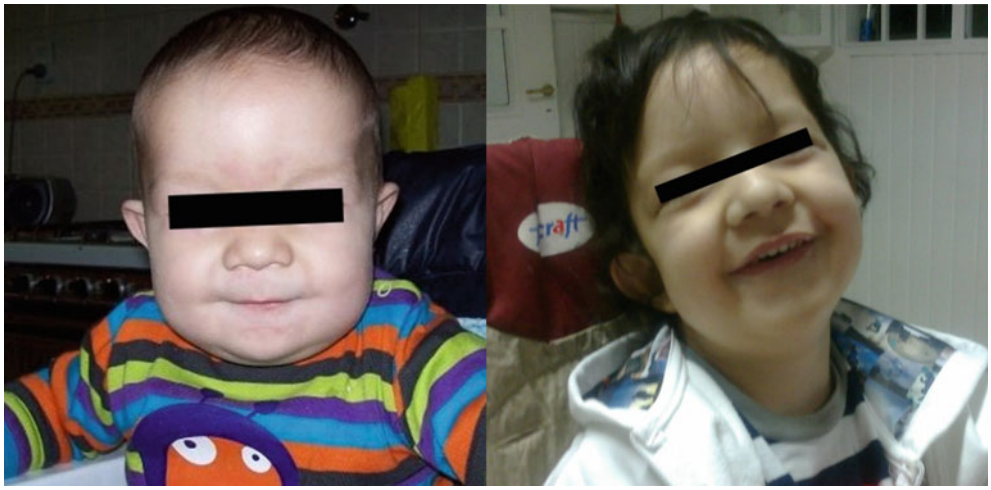

Fig. 1 Patient at 8 months and at 4 years of age

He presented with neonatal jaundice that resolved spontaneously. He had a high forehead, posteriorly rotated, low set ears and inverted nipples. At the age of 8 months, he presented with psychomotor retardation, axial and peripheral muscular hypotonia and nephrocalcinosis. Evaluation at the age of 18 months revealed progressive spastic paraparesis, neurogenic bladder and nystagmus. At the evaluation at 4 years of age, he showed severe spastic paraparesis, brisk reflexes and axial hypotonia. He had neither cutaneous abdominal reflex nor tickling. He managed to remain seated but needed motor assistance using a wheelchair for ambulation. He also needed constant care and assistance. The ophthalmologic exam showed bilateral cataracts at the age of 4 years, which were surgically corrected at the age of 5. At 5 years of age, he had a non-febrile focal clonic seizure on left arm and left hemiface lasting less than 5 min. He repeated a similar episode with 24 h associated to an abnormal EEG so he was treated with clobazam. He had an isolated fever record 24 h later the event. He was on clobazam until 9 years of age and did not repeat any other seizure (Fig. 1).

Central nervous system (CNS) magnetic resonance imaging (MRI), CNS spectroscopy and CNS MRI angiography performed at 26 months and at 3 and 4 years of age were normal. However, increased gadolinium reinforcement at the medullary cone on spine MRI was described on the studies performed at 3 and 4 years of age. Somatosensory evoked potential showed spinal cord compromise at 19 months and at 3 years of age. Elbow and knee radiographies performed at 2 years of age were normal. Visual evoked potentials, electroretinography and auditory evoked potentials performed at 4 years of age were also normal.

He had a normal 46 XY karyotype. Screening for HTLV1 by PCR on CSF was negative. Laboratory and basic metabolic investigations performed (creatine kinase, aldolase, lactic acid, ammonia levels, uric acid, pristanic and phytanic acids, urinary organic and bile acids, and transferrin isoelectric focusing) were all normal. However, erythrocyte plasmalogen levels were C16 DMA/C16:0 0.025 (reference values 0.045–0.082) and C18DMA/C18:0 0.065 (reference values 0.125–0.265); and the ratios for the very long chain fatty acids (VLCFAs) were elevated in plasma at the age of 3 years: C24:0/C22:0 1.30 (reference values 0.68–0.98) and C26:0/C22:0 0.0383 (reference values 0.0064–0.0216).

Peroxisomal parameters were subsequently studied in cultured skin fibroblasts. VLCFA levels were also elevated in fibroblasts: C22:0 4.63 (μmol/g) (reference values 3.84–10.20), C24:0 8.91 (μmol/g) (reference values 7.76–17.66) and C26:0 0.47 (μmol/g) (reference values 0.18–0.38). Ratio C24:0/C22:0 1.93 (reference values 1.55–2.30) and ratio C26:0/C22:0 0.10 (reference values 0.03–0.07).

Immunofluorescence microscopy analysis with antibodies against catalase, a peroxisomal matrix protein, revealed an abnormal peroxisomal mosaic pattern with cells with import-competent peroxisomes and cells without import-competent peroxisomes (Fig. 2). Moreover, the patient's cells with import-competent peroxisomes showed lower numbers and increased size of peroxisomes. Immunoblot analysis of the peroxisomal proteins acyl-CoA oxidase 1 (ACOX1) and 3-ketoacyl-CoA thiolase revealed normal processing of these proteins within the peroxisome. The activity of the peroxisomal enzyme dihydroxyacetone phosphate acyltransferase (DHAPAT), the first enzyme of the plasmalogen biosynthesis pathway, was low normal compared to the reference values. Despite the relative mild biochemical abnormalities of the peroxisomal parameters, these results strongly suggested a Zellweger spectrum

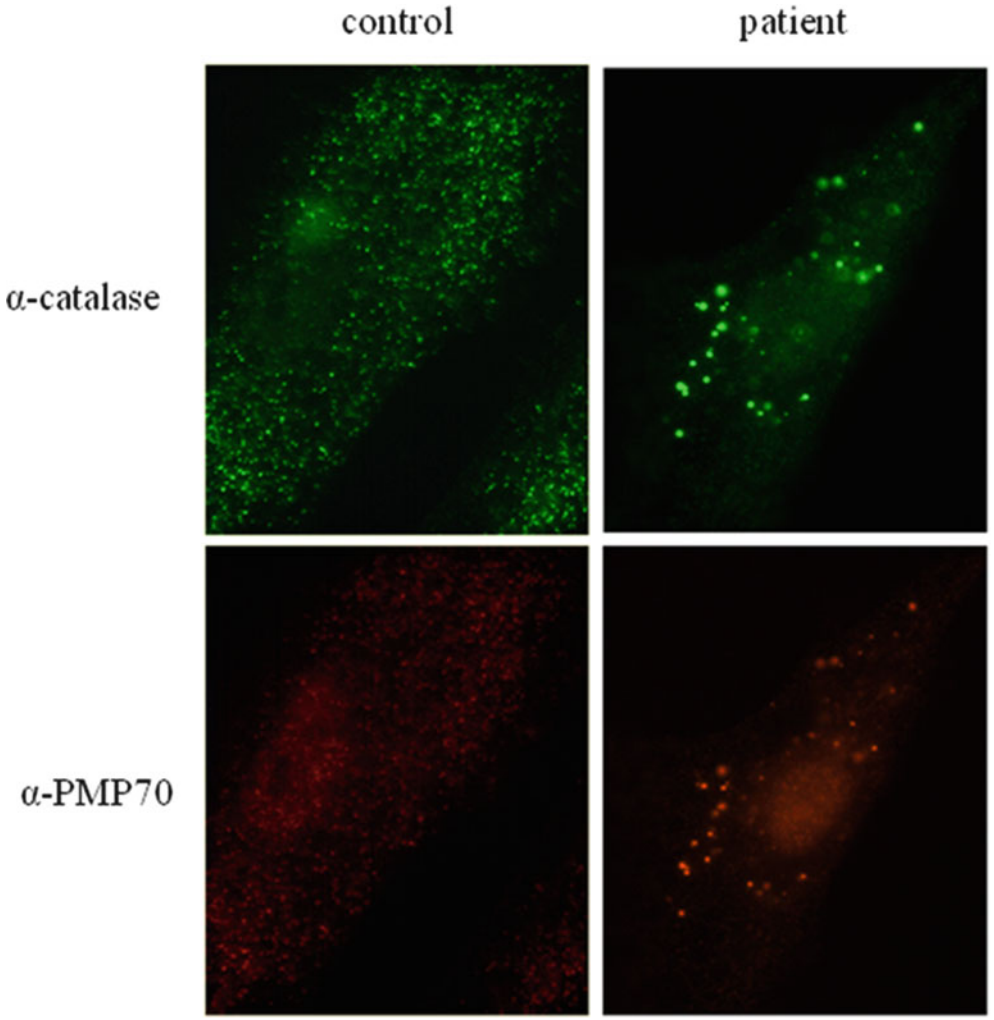

Fig. 2 Immunofluorescence microscopy analysis for catalase, a peroxisomal matrix protein, and PMP70, a peroxisomal membrane protein (PMP) in cultured fibroblasts from the patient

defect in the patient. To determine which *PEX* gene would be defective in the patient, we sequenced all exons and flanking intron sequences of the *PEX1*, *PEX2*, *PEX5*, *PEX10*, *PEX12*, *PEX13*, *PEX14*, *PEX16*, *PEX19* and *PEX26* genes, but did not identify potential pathogenic mutations. However, sequence analysis of the *PEX3* gene (reference sequence MN_003630.2) identified two heterozygous, pathogenic mutations in the *PEX3* gene: c.898C>T (p.Arg300*) inherited from the mother and c.991G>A (p.Gly331Arg) inherited from the father. Both mutations have not been reported previously. The c.898C>T mutation interrupts the reading frame by a premature STOP codon, which theoretically leads to a truncated PEX3 protein. Alternatively, the *PEX3* mRNA might be targeted for nonsense mediated decay. The c.991G>A mutation changes the glycine at position 331 into an arginine. This glycine is highly conserved among PEX3 proteins from different species ranging from fruit fly to man, and the amino acid change is predicted as deleterious by SIFT, disease causing by MutationTaster and probably damaging by PolyPhen-2 prediction software (Fig. 3).

Discussion

We herein report the clinical, neuroradiological, biochemical and molecular characterization of a male patient affected with a ZSD due to two novel heterozygous mutations in the *PEX3* gene. The relatively mild clinical and biochemical phenotype of our patient expands the clinical spectrum for *PEX3* patients and indicates that dependent on the severity of the disease-causing mutations *PEX3* defects result in a disease spectrum similar as previously observed for other *PEX* gene defects.

Patients with PBDs can be classified into 13 genetic complementation groups reflecting defects in 13 different *PEX* genes. These complementation groups can be subdivided into two groups according to the dysfunction. Defects in *PEX3*, *PEX16* and *PEX19* result in a complete defect in

Fig. 3 Sequence analysis of *PEX3*. Pathogenic mutations

peroxisomal assembly and cell lines often do not show peroxisomal membrane structures (ghosts) (Ebberink et al. 2011). In cell lines with defects in the other *PEX* genes, peroxisomal membrane structures are always present and sometimes partial matrix protein import can be observed (Wanders 1999; Shimozawa et al. 2005; Muntau et al. 2000a, b; Ghaedi et al. 2000; Matsui et al. 2013).

PEX3 is an integral membrane protein. It is supposed to play a role in the insertion of membrane proteins into the peroxisomal membrane. Since PEX3 interacts with PEX16 and PEX19 in yeast and human cells, it has been proposed that a defective PEX3 affects this interaction and leads to degradation or mislocalization of the peroxisomal membrane proteins (PMPs) (Muntau et al. 2000a, b; Matsui et al. 2013).

The commonly observed clinical presentation of patients with mutations in *PEX3* includes the severe ZS phenotype (Muntau et al. 2000a, b; Ghaedi et al. 2000). Clinical findings in patients presenting with ZS include facial dysmorphism, neonatal jaundice, hepatomegaly, liver dysfunctions, calcification of joints, renal cortical cysts, cataracts, hearing impairment, retinal degeneration, seizures, polymicrogyria and developmental delay (Wanders 1999). ZSD atypical phenotypes presenting with a relatively mild clinical phenotype with slow progression and mild elevation of biochemical markers have recently been described (Regal et al. 2010; Sevin et al. 2011; Thoms and Gärtner 2012; Zeharia et al. 2007).

In addition, Matsui et al. recently reported another patient with a milder phenotype due to a *PEX3* mutation (Matsui et al. 2013). Similarly, the patient described here presented a mosaic pattern of catalase positive particles and peroxisomal membrane structures. It has been suggested that the severity of the phenotype could be related to the number of residual peroxisomes in tissues and the preservation of peroxisomal function. The mosaic pattern found in our patient's fibroblasts and the relative mild biochemical aberrations are in agreement with this hypothesis.

Pathological findings on brain MRI in ZSD include abnormalities in neuronal migration and differentiation, such as cortical dysplasia and neuronal heterotopias, particularly perisylvian polymicrogyria or incomplete opercularization. Germinolytic cysts along the frontal horns of the lateral ventricles and cerebellar cortical dysplasia are also described. White matter abnormalities consist of dysmyelination rather than demyelination, involving cerebral and cerebellar hemispheres (Wanders 1999). Cerebellar atrophy without any other changes has also been described in phenotypes presenting as autosomic recessive cerebellar ataxias. Late-onset white matter disease is a distinct phenotype in ZSD, and the physiopathological explanation for this phenotype is still unresolved. Patients can present with abrupt clinical deterioration preceded by normal development or moderate delay in the first months of life. Clinical findings include hypotonia, retinopathy, sensory deafness, seizures and cerebral demyelination in centrum semiovale, sparing U fibres and even central cerebellar white matter. When conventional MRI techniques fail to detect white matter abnormalities, then diffusion-weighted and diffusion tensor imaging may be useful. Although our patient presented a normal CNS on MRI follow-up, progressive changes cannot be definitely ruled out.

Conclusions

Several patients with ZSD presenting with atypical phenotypes have been recently reported. These patients showed milder phenotypes, slow progression of symptoms, spastic paraparesis, ataxia, normal or mild elevation of biochemical markers and normal images on CNS MRI. Mutations in *PEX3*, *PEX16* and *PEX19* genes usually result in a severe, early lethal phenotype and in cells entirely lacking remnant peroxisomal membranes. However, our patient still has peroxisomal remnants and displays a much milder phenotype than that previously

observed in patients with *PEX3* mutations. Our findings indicate that *PEX3* defects may cause a disease spectrum similar as previously observed for other *PEX* gene defects.

Declaration of Conflicting Interests

Delfina Marchione works for Genzyme Sanofi Company.

The authors Clarisa Maxit, Inés Denzler, Guillermo Agosta, Janet Koster, Ronald Wanders, Sacha Ferdinandusse and Hans Waterham declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Author Contributions

CM, ID, MD and AG: conception, manuscript, acquisition of clinical data, preparation and revision of intellectual content, and final approval of the manuscript. KJ, WRJA, FS and WHR: acquisition of biochemical and genetic data, review and critique, and final approval of the manuscript.

Ethical Approval

This work was performed in agreement with the ethical rules of Hospital Italiano de Buenos Aires. No ethical approval was necessary for the writing of this case report.

The authors received an informed consent form from the patient's parents.

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

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RESEARCH REPORT

Improved Measurement of Brain Phenylalanine and Tyrosine Related to Neuropsychological Functioning in Phenylketonuria

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Abstract *Introduction*: Researchers hypothesized that in phenylketonuria (PKU) high brain phenylalanine (Phe) levels and low brain tyrosine (Tyr) levels affect neuropsychological functioning. However, traditional magnetic resonance spectroscopy (MRS) yielded uncertain results of brain Phe and could not adequately measure brain Tyr. This pilot study examined the potential of correlated spectroscopy (COSY) to quantify these biomarkers and explain variability in neuropsychological functioning.

Methods: Nine adults with early treated classic PKU received magnetic resonance imaging (MRI) with COSY and a battery of neuropsychological tests. Brain Phe and Tyr in parietal white matter (PWM) were compared to results in gray matter of the posterior cingulate gyrus (PCG).

Results: Brain Phe ranged from 101 to 182 (mean = 136.76 ± 23.77) μmol/L in PCG and 76 to 185 (mean = 130.11 ± 37.88) μmol/L in PWM. Brain Tyr ranged from 4.0 to 7.4 (mean = 5.44 ± 1.01) μmol/L in PCG and 4.1 to 8.4 (mean = 5.90 ± 1.48) μmol/L in PWM. Correlation coefficients were largest for brain Phe PWM and measures of auditory memory (rho = −0.79), anxiety (rho = 0.79), and executive functioning (rho = 0.69). Associations were in the expected direction, with higher brain Phe and lower brain Tyr related to poorer functioning. The two participants with severe structural MRI abnormalities had low brain Tyr levels in PCG and 3/5 of the participants with moderate to severe MRI abnormalities had higher than average brain Phe levels.

Conclusion: COSY has the potential to quantify brain Phe and Tyr at low concentrations and in specific brain regions. In this pilot study, these biomarkers were associated with indices of neuropsychological functioning. Additional studies are needed to validate the COSY results.

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Introduction

Phenylketonuria (PKU, OMIM 261600), an autosomal recessive disorder, affects approximately 1:11,000 individuals in the USA. In PKU, mutations in the gene responsible for the liver enzyme phenylalanine hydroxylase (PAH) result in reduced or absent conversion of phenylalanine (Phe) to tyrosine (Tyr) and subsequently to elevated plasma concentrations of Phe and reduced concentrations of Tyr (Scriver and Kaufman 2001). Untreated PKU results in progressive, neurological decline by 6–12 months of age (Koch et al. 1971). Even with early detection and treatment with a Phe-restricted diet, patients experience neurocognitive deficits (Waisbren et al. 1994) and psychiatric disturbances as they get older (Bilder et al. 2013; Weglage et al. 2013) as well as white matter abnormalities detected through magnetic resonance imaging (MRI) (Mastrangelo et al. 2015).

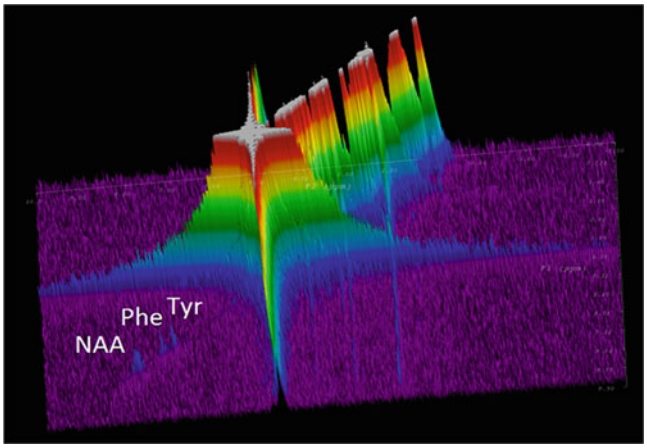

Fig. 1 Three-dimensional rendering of the correlated spectroscopy (COSY) spectrum. Phenylalanine (Phe), tyrosine (Tyr), and *N*-acetyl-aspartate (NAA) can be readily visualized and quantified using this method

Hypotheses attribute neuropsychological deficits in PKU to: (1) reduced myelin as observed in MRI and (2) reduced dopamine (a metabolite of Tyr) in the brain (Surtees and Blau 2000). Evidence for the dopamine hypothesis derives indirectly from neuropsychological tests showing impaired functioning on tasks associated with the prefrontal cortex, such as executive functions (Diamond et al. 1997) as well as abnormal MRI findings in brain regions dependent on dopamine (Bodner et al. 2012). However, direct measurement of Phe concentrations in the brain via conventional magnetic resonance spectroscopy (MRS) is challenging due to overlapping spectra of Phe and Tyr, as well as relatively low concentrations of brain Phe (Kreis et al. 2009). Research to improve quantification of brain Phe and Tyr and to understand the impact of these biomarkers is important not only in understanding the pathology in PKU, but also in understanding the biological mechanisms leading to phenotypic variability (Ramus et al. 1999). In addition, quantification of brain Phe and Tyr can lead to individualizing treatment and biomarkers for clinical trials.

A potential method to overcome limitations of conventional spectroscopy is two-dimensional shift Correlated Spectroscopy (COSY). This method has been used for the unambiguous identification of cerebral metabolites that could not be detected using conventional MRS methods due to spectral overlap (Thomas et al. 2001; Lin et al. 2012). By obtaining multiple acquisitions at different echo times, a second chemical shift domain allows for metabolites to be identified by two chemical shifts instead of just one based on scalar coupling of different proton groups. The concentration of the metabolite is therefore shown in the third dimension. By visualizing COSY data in three dimensions, smaller resonances that would have been obscured by larger resonances can be measured (Fig. 1). Different brain regions separating white and gray matter tissue can be assessed with this method as a smaller voxel can be used. In addition, the COSY method can measure Tyr (Ramadan et al. 2011), and other amino acids (Lin et al. 2015).

The aims of this pilot study were to: (1) demonstrate the potential of the COSY method in quantitative measurement of Phe and Tyr in distinct regions of the brain and (2) determine if there is a relationship between these biomarkers and measures of neuropsychological functioning.

Methods

Overview

Nine adults with early treated classic PKU detected by newborn screening (four men, five women; mean age 29 ± 4 years) comprised the sample. Eligibility required pretreatment/off-diet blood Phe concentration above 1,200 μmol/L, Phe tolerance of less than 300 mg/day, or genotype associated with classic PKU. Study participants were identified and recruited by health care providers (psychologists, dieticians, or metabolic physicians) who followed the patients at Boston Children's Hospital. One sibling pair with identical genotypes for the PAH gene was included in the sample.

Blood draws, neurological examinations, and neuropsychological testing were conducted at the Boston Children's Hospital Clinical Translational Studies Unit (CTSU). The Committee on Clinical Investigations at Boston Children's Hospital approved the study and all participants provided written informed consent.

Magnetic Resonance Imaging and Spectroscopy

MRI and MRS were performed on a 3T Siemens scanner with a 32-channel coil at Boston Children's Hospital. Participants were given ample time to become familiar with the procedures and offered earplugs and/or headphones to block out the sound. The structural MRI sequences included a volumetric multiecho T1-weighted three-dimensional (3D) magnetization-prepared rapid gradient-echo (MP-RAGE) sequence (slice thickness 1 mm, 20–25 cm FOV), axial T2-weighted sequence (slice thickness 2.5 mm skip 0, 20–22 cm FOV), axial T2-weighted FLAIR (slice thickness 4 mm skip 0, 20 cm FOV), and diffusion-weighted images (35 directions, B values: 0 and 1,000 s/mm^2).

Structural MRI sequences including the diffusion-weighted images were reviewed by a fellowship trained board certified pediatric neuroradiologist (SPP), who was blinded to the MR spectroscopy data, clinical examination, and blood levels of Phe and Tyr. The MRI scans were assessed for the degree of T2 prolongation and diffusion restriction and each finding was classified into mild, moderate, and severe

categories. The MRI appearance was classified into these categories by combining the degree of T2 prolongation, diffusion restriction, and degree of parenchymal volume loss indicated by sulcal and extraaxial space prominence. The "mild" category included scans of subjects with hazy T2 prolongation in the periventricular and deep white matter without diffusion restriction and no significant prominence of the extraaxial spaces. The "moderate" category included scans with more pronounced T2 prolongation and scattered areas of mildly restricted diffusion in some of these areas of T2 prolongation and mild sulcal and extraaxial space prominence. The "severe" category included scans with confluent areas of T2 prolongation and decreased diffusion and generalized parenchymal volume loss in the supratentorial and infratentorial brain indicated by moderate sulcal and extraaxial space prominence.

COSY was used to quantify brain levels of Phe and Tyr. Spectroscopy was performed using both 1D MRS (PRESS; TR 2 s, TE 30 ms, 64 avgs) and COSY (TR 1.5 s, initial TE 30 ms, 64 increments of 0.8 ms, 8 avgs). MRS in the gray matter region, posterior cingulate gyrus (PCG) ($3 \times 3 \times 3$ cm^3), and parietal white matter (PWM) ($4 \times 3 \times 2$ cm^3) was acquired with unsuppressed water reference (Thomas et al. 2001; Ramadan et al. 2011; Lin et al. 2012).

Post-processing of the COSY data quantified the levels of Phe as the primary measure using Felix NMR. Secondary measures included Tyr. Values for Phe and Tyr reported here were expressed in unadjusted terms in addition to ratios to illustrate the capability of measuring low elevations. For the 2D COSY spectra, metabolite concentrations were calculated from the volume of the peak and calibrated to the LCModel estimation. Felix NMR was used to measure the volume of Phe and Creatine (Cr) resonances from the 2D COSY spectra. The Felix NMR was used at the D1 settings: data size to 512, spectrometer frequency of 123.23, and a sweep of 2,000.0. Next, the D2 settings were set to 64, spectrometer frequency 123.23, and a sweep width of 1,250.0. The acquisition mode was set to magnitude, as well as the acquisition in the D2. Next, the window function was set to "skewed sinebell^2," along with CNV based solvent suppression at a value of 30. The phase shift and the skew parameter were set to 0.0 and 0.3, respectively. Consequentially, the D2 window function was set to sinebell^2 with a phase shift of 0.0. Using the chemical shift of creatine 3.02 (Govindaraju et al. 2000), the resulting COSY data were referenced. Using the chemical shifts of Phe 7.32 and Tyr 7.18, a dba was created to measure the peaks of Phe and Tyr.

Neuropsychological Evaluation

All participants received a brief neuropsychological evaluation in order to further describe the sample and identify functional outcomes sensitive to variations in brain Phe and Tyr. The Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler 1999) was administered for IQ determination. The Beery Visual Motor Integration Test, Sixth Edition (VMI) (Beery et al. 2010) provided a measure of visual motor skills. The California Verbal Learning Test – Adult Version (CVLT-A) (Delis et al. 2000) measured verbal learning and auditory memory. The Delis–Kaufman Executive Functioning Verbal Fluency subtest measured aspects of processing speed (Delis et al. 2001). The Behavior Rating Inventory of Executive Function – Adult Version (BRIEF-A) (Gioia et al. 2000) provided a self-reported index of executive functioning and the Beck Anxiety Inventory (Beck and Steer 1993) and Beck Depression Inventory, Second Edition (Beck et al. 1996), provided self-reported indices of mood. Participants also completed a self-report measure of day-to-day functioning, the Adaptive Behavior Assessment System, Second Edition (ABAS-II) (Harrison and Oakland 2003).

Serum Measurements

For determination of the plasma amino acids, Phe and Tyr, venipuncture was performed by nurses at the Clinical Translational Studies Unit (CTSU) and sent to a CLIA approved laboratory.

Neurological Examinations

All participants were evaluated with a standardized neurological examination performed by the two adult neurologists on the research team.

Statistical Analysis

Results from COSY were derived from direct measurement of the crosspeak volume and are presented as means with standard deviations. Due to the small sample size, exploratory analyses were conducted using nonparametric statistical methods. The Mann–Whitney test was used to compare COSY results derived from the ratios of Phe/creatine and Tyr/creatine in individuals with PKU and a group of nine healthy adults who comprised an historical control group. Spearman's correlation coefficients were used to identify associations between COSY and laboratory results and Phe and Tyr concentrations in gray and white matter regions. Preliminary analyses, also using Spearman's correlation coefficients, examined associations of blood and brain Phe and Tyr concentrations with neuropsychological test results.

Results

To validate the COSY method, several studies were conducted prior to scanning research participants. Solutions of the Phe and Tyr, or phantoms, were made up in 250 ml

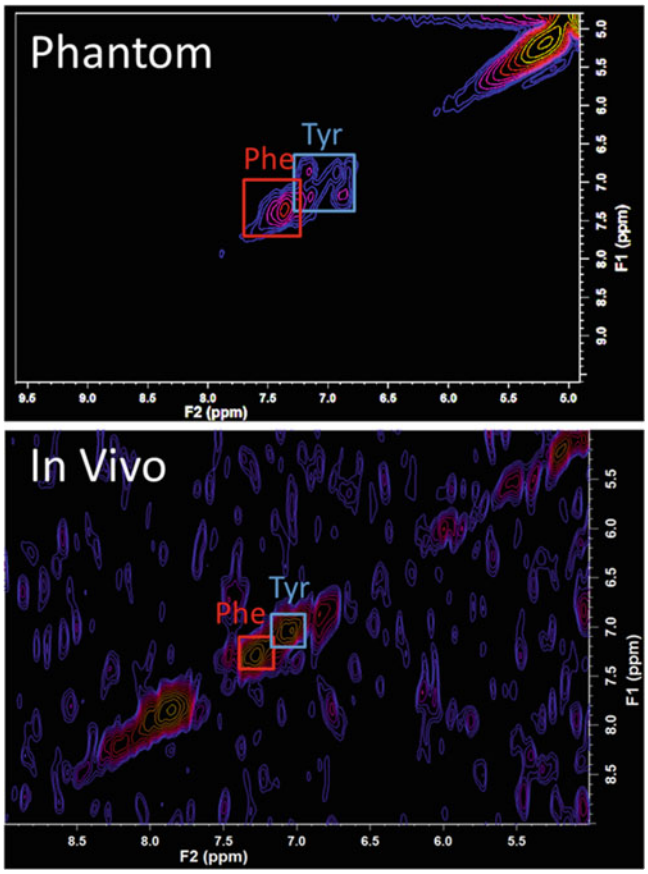

Fig. 2 COSY measures of Phe and Tyr in phantoms (*top*) and human subjects (*bottom*)

MR compatible spheres at physiological concentrations with creatine at 10 mM to serve as a chemical shift reference. Along with these amino acids, creatine was measured for normalization purposes. The resulting COSY volumes of Phe and Tyr were normalized with their respective creatine volumes. Characterization of downfield resonances including Phe and Tyr was validated using phantom solutions of different concentrations to ensure the specificity of the COSY measurements (Fig. 2). Scans were conducted with phantoms with concentrations consisting of 0.01, 0.1, 1, and 1 mM at the chemical shifts of F2 = 7.35 + 0.016 and F1 = 7.50 + 0.27 ppm. For Tyr, the chemical shifts of F2 = 7.17 + 0.008 and F1 = 6.87 + 0.0019 ppm were measured. The same MRS protocol was used for phantom and human studies. Results of the phantom studies showed good correlations between cross-peak volumes and the concentrations of Phe ($r = 0.98$) and Tyr ($r = 0.84$).

To further validate COSY, average measurements of Phe and Tyr from de-identified human brain MRI scans with COSY were used. Nine healthy adults (ages 28 ± 3 years and matched for sex and age within 3 years of the enrolled study participants with PKU) had previously participated in a similar study that obtained measurements of brain Phe and Tyr in the PCG. For the previous study, brain Phe and Tyr results were expressed as ratios, with creatine as the denominator to account for variability in brain volumes. One of the subjects was scanned repeatedly five times with the same protocol to ensure reproducibility of the methods. Reproducibility of these measures in the human subject scanned repeatedly showed a variation of 9% for Phe and 22% for Tyr. The increased variability of Tyr is likely due to its lower concentration.

Comparisons with our results in individuals with PKU were in the expected direction for brain Phe. The mean brain Phe/creatine ratio in individuals with PKU was 0.039 ± 0.008, significantly higher than the brain Phe/creatine ratio in controls which was 0.025 ± 0.004 (Mann–Whitney test, $p = 0.02$). Mean brain Tyr/creatine ratios did not differ significantly between the PKU group and controls (0.0085 ± 0.0027 and 0.0076 ± 0.0018, respectively). However, given the low concentration of Tyr and its variability, it is likely that there are not a sufficient number of subjects to show a difference, if it exists.

All participants with PKU were identified by newborn screening and treated with a Phe-restricted diet since infancy. None had comorbid physical health conditions and all were living independently. Four participants discontinued treatment in middle childhood, but eight were currently on a Phe-restricted diet, albeit with varying levels of metabolic control. Four received Tyr supplementation and one young woman (Participant #9) reported that she was taking supplemental large neutral amino acids (LNAAs), including Tyr, but did not restrict her protein intake.

Table 1 summarizes results from the participants with PKU. Blood and brain Phe and Tyr levels varied among these treated individuals. Blood Phe ranged from 277 to 1,512 μmol/L. Brain Phe ranged from 101 to 182 μmol/L in PCG (gray matter region) and from 76 to 185 μmol/L in PWM (white matter region). Blood Tyr ranged from 17 to 131 μmol/L. Brain Tyr ranged from 4.0 to 7.4 μmol/L in gray matter and from 4.1 to 8.4 μmol/L in white matter. Spearman rank correlations indicated that blood Phe correlated with blood Tyr (rho = −0.94, $p < 0.01$) with higher blood Phe levels associated with lower blood Tyr. Blood levels of Phe and Tyr did not correlate significantly with brain levels of these biomarkers. Blood Phe levels were on average 6.7 times higher than brain Phe PCG levels and 7.0 times higher than brain Phe PWM levels. Blood Tyr levels were on average 12 times higher than brain Tyr levels in the PCG and 11 times higher than brain Tyr levels in the PWM. Brain Phe did not differ in white and gray matter regions, while Tyr levels tended to be higher in white matter than in gray matter.

Table 1 Description of the sample, biomarkers, neurological exam, and neuropsychological testing results

Participant #	1	2	3	4	5	6	7	8	9	Mean or %	SD
Sex	F	F	F	M	M	F	M	M	F	55% females	–
Age in years	32	25	38	32	31	27	22	27	31	29.44	4.72
Diet discontinued at some time in past?	Yes	No	Yes	No	Yes	No	Yes	No	Yes	55% Yes	
On diet?	Some	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	78% Yes	–
Supplements	No	No	No	Tyr	Tyr	No	No	Tyr	LNAA	44% Yes	–
Blood Phe μmol/L	1,189	771	862	277	708	1,336	865	743	1,512	918.08	373.27
Brain Phe (PCG) μmol/L	138	157	125	101	135	113	143	182	137	136.76	23.77
Brain Phe (PWM) μmol/L	185	118	138	76	161	109	149	78	159	130.11	37.88
Blood Tyr μmol/L	17	74	28	131	115	55	18	90	54	64.68	41.49
Brain Tyr (PCG) μmol/L	5.6	5.6	4.0	6.3	4.5	5.1	4.9	7.4	5.0	5.44	1.01
Brain Tyr (PWM) μmol/L	4.6	8.4	5.3	4.1	5.8	7.3	4.4	7.2	6.0	5.90	1.48
Neurological exam	Normal	Normal	Normal	Normal	Brisk reflexes	Normal	Postural tremor	Postural tremor	Normal	67% Normal	–
MRI changes	Moderate	Moderate	Mild	None	Moderate	Severe	Mild	Mild	Severe	89% Yes	–
Periventricular abnormality	Moderate	Moderate	Mild	Mild	Moderate	Severe	Mild	Mild	Severe	100% Yes	–
Restricted diffusion in white matter	Mild	Mild	None	None	Mild	Severe	Mild	None	Severe	67% Yes	–
Neuropsych. test											
WASI FS IQ	64	98	98	101	103	106	112	116	129	103.00	17.71
Verbal Fluency	12	12	–	12	12	12	12	9	15	12.00	1.60
Visual Motor Integration	77	103	92	103	103	–	103	92	107	97.50	9.94
Visual Perception	92	81	–	45	97	–	97	97	–	84.83	20.48
CVLT-1	−2.50	−1.00	–	−1.50	−1.00	−1.5	−1.00	0.00	0.05	−1.06	0.83
CVLT-5	−2.00	1.00	–	0.00	−1.00	0.00	−0.05	0.50	−1.50	−0.38	1.02
BRIEF-A, GEC	60	49	59	46	71	37	38	46	62	52.00	11.60
ABAS-II, GAC	94	116	96	110	118	108	115	113	96	107.33	9.50
Beck Anxiety	9	1	2	0	11	–	–	2	21	6.57	7.63
Beck Depression	12	1	14	0	7	1	0	2	2	4.33	5.36

Abbreviations: *SD* standard deviation, *F* female, *M* male, *Tyr* tyrosine, *LNAA* large neutral amino acid, *Phe* phenylalanine, *PCG* posterior cingulate gyrus (gray matter region of the brain), *PWM* periventricular white matter (white matter region of the brain), *IQ* intelligence quotient, *Neuropsych* neuropsychological

Neuropsychological tests:

WASI FS IQ: Wechsler Abbreviated Scale of Intelligence, Full-Scale IQ

Verbal Fluency: Delis–Kaplan Executive Function System, Verbal Fluency Subtest (normative mean = 10 ± 3)

Visual Motor Integration: Beery Test of Visual Motor Integration, Sixth Edition (normative mean = 100 ± 15)

Visual Perception: Beery Test of Visual Perception, Sixth Edition (normative mean = 100 ± 15)

CVLT-1: California Verbal Learning Test – Adult Version, Trial 1 (*z*-scores)

CVLT-2: California Verbal Learning Test – Adult Version Trial 5 (*z*-scores)

BRIEF-A, GEC: Behavior Rating Inventory of Executive Function – Adults, Global Executive Composite (scores greater than 65 indicate difficulties in executive functions)

ABAS-II, GAC: Adaptive Behavior Assessment System, Second Edition, General Adaptive Composite (normative mean = 100 ± 15)

Beck Anxiety: Beck Anxiety Inventory (Scores 0 – no anxiety; 1–7 minimal anxiety; 8–15 mild anxiety; 6–25 moderate anxiety; and 26–63 severe anxiety)

Beck Depression: Beck Depression Inventory, Second Edition (0 – no depression; 1–13 minimal depression; 14–19 mild depression; 20–28 moderate depression; and 29–63 severe depression)

Table 2 Spearman rank order correlation (rho) between Phe biomarkers and neuropsychological tests

	Blood Phe	Brain Phe PWM	Brain Phe PCG	Blood Tyr	Brain Tyr PWM	Brain Tyr PCG
WASI Full-Scale IQ	0.23	−0.18	0.18	0.18	0.19	0.02
Beck Depression[a]	0.14	0.55	0.03	−0.31	0.09	−0.37
Beck Anxiety[a]	0.58	0.79	0.13	−0.38	0.13	−0.49
CVLT Trial 1	0.10	−0.05	0.45	0.12	0.32	−0.16
CVLT Trial 5[a]	−0.41	−0.79	0.30	0.53	0.50	0.51
Visual Motor Integration	0.01	−0.08	−0.17	0.37	0.11	−0.15
ABAS-II (GAC)	−0.60	−0.18	0.28	0.58	0.22	−0.02
BRIEF-A (GAC)	−0.03	0.69	0.03	−0.02	−0.03	−0.34

Abbreviations: *Phe* phenylalanine, *Tyr* tyrosine, *PCG* posterior cingulate gyrus (gray matter region of the brain), *PWM* parietal white matter (white matter region of the brain), *IQ* intelligence quotient, *WASI* Wechsler Abbreviated Scale of Intelligence, *Beck Depression* Beck Depression Inventory, Second Edition (higher scores indicate greater depression), *Beck Anxiety* Beck Anxiety Inventory (higher scores indicate greater anxiety), *CVLT-1* California Verbal Learning Test – Adult Version, Trial 1, *CVLT-2* California Verbal Learning Test – Adult Version Trial 5, *ABAS-II, GAC* Adaptive Behavior Assessment System, Second Edition, General Adaptive Composite (higher scores indicate better functioning), *BRIEF-A, GEC* Behavior Rating Inventory of Executive Function – Adults, Global Executive Composite (higher scores indicate greater difficulties in executive functions)

[a] Only eight subjects completed these measures

In the one sibling pair (Participants #3 and #5), blood Tyr levels were 27.9 μmol/L (without Tyr supplementation) and 115.2 μmol/L (with Tyr supplementation), but brain Tyr levels were similar (4.0 and 4.5 μmol/L in gray matter; 5.3 and 5.8 μmol/L in white matter, respectively). Both had similar scores on IQ tests and reported emotional disturbance (depression in one, anxiety in the other).

All study participants exhibited MRI changes in at least one domain, with five individuals rated as having moderate or severe changes overall, five with moderate or severe changes in periventricular regions, and two with severely diminished diffusion in white matter. Neurological examinations were rated as normal, although two individuals exhibited mild postural tremor and one young man had slightly brisk reflexes. Three of the five participants with moderate to severe MRI abnormalities had higher than average brain Phe levels in both white and gray matter. The two participants with severe structural MRI abnormalities (participants #6 and #9) had lower than average brain Tyr levels in gray matter, as well. Participant #9 who reported LNAA supplementation and an unrestricted diet exhibited severely abnormal MRI changes, periventricular abnormalities, and restricted diffusion, despite having a normal neurological exam and the highest IQ in the sample. She suffered clinically significant anxiety and had the highest blood Phe level in the sample, as well as high brain Phe levels in both PCG and PWM brain regions.

IQ ranged from 64 to 129, with all but one participant performing within the average range for the general population (IQ above 85). All but two research participants experienced anxiety or depression, as determined by scores above the cutoff on the self-reported Beck scales or reports of being treated for these conditions. Verbal processing skills appeared intact, as indicated by scores generally higher than the population norm on the Delis–Kaplan Verbal Fluency subtest (where scores 7–13 represent the average range). Visual motor skills were also within the average range (85–115) for all but the young woman with IQ in the range of intellectual disabilities. Six of the eight individuals receiving the California Verbal Learning Test performed 1 standard deviation or more below the normative mean on the first trial (CVLT-1), indicating poor auditory memory/processing skills. Three of these individuals performed within a standard deviation of the normative mean after hearing the list of words 5 times (CVLT-5), suggesting that the issue may be more related to processing than to memory skills. One research participant received a score above the cutoff indicating executive functioning deficits (scores >65) on the BRIEF-A, but four participants rated themselves higher than the population norm of 50. None of the respondents indicated difficulties in day-to-day functioning as indicated by scores within the average range (85–115) or above on the ABAS-II.

Table 2 presents associations between Phe and Tyr biomarkers and neuropsychological testing results. Spearman rank order correlation coefficients (rho) were largest for associations between brain Phe PWM and measures of auditory memory (CVLT, rho = −0.79) and the self-reported measures of anxiety (rho = 0.79) and executive functioning (rho = 0.69). In general, associations were in the expected direction, with higher brain Phe and lower brain Tyr related to poorer functioning. The correlations tended to be stronger in white matter than in gray matter. Brain Phe and Tyr were not sensitive indicators of full-scale

IQ and measures of verbal fluency, visual motor integration, and overall adaptive behavior.

Discussion

This pilot study demonstrated the potential of the COSY method to measure even small elevations in brain Phe in white and gray matter. This study also demonstrated that it is possible to differentiate brain Tyr from brain Phe levels.

As measured by COSY, brain Phe and Tyr within different brain regions varied among individuals. If validated in future studies, this finding may provide answers to some of the more vexing questions regarding the specific neuropsychological deficits associated with PKU, despite lifelong treatment. The range of Phe was greater than the range of Tyr in both gray and white matter. This may suggest a capacity for the brain to establish equilibrium in terms of Tyr accumulation. This latter explanation is consistent with our finding that LNAA supplementation in one subject did not appear to result in elevated brain Tyr. Moreover, in our sibling pair, Tyr supplementation in one sibling compared to the other resulted in higher blood Tyr but no difference in brain Tyr. Alternatively, the prefrontal cortex and not the PCG may be the relevant site for investigating variations in Tyr levels in the brain, as has been suggested by previous investigators (Diamond et al. 1997; Bodner et al. 2012). Blood Tyr levels, as well as blood Phe levels, are known to fluctuate depending on recent food intake (Cleary et al. 2013). This potentially could also affect results of COSY measurements of brain Tyr.

Phe in white matter as compared to gray matter tended to be associated with neuropsychological testing results (specifically, depression, anxiety, auditory memory, and executive functioning). The opposite tended to be true for brain Tyr, which was more closely associated with executive functioning in gray matter as compared to white matter, consistent with the hypotheses proposed by Diamond et al. (1997).

Our study differed in some respects from previous studies. Pietz et al. (1999) assessed brain Phe in patients with PKU by quantitative 1H MRS during an oral Phe challenge with and without additional supplementation with all other LNAAs. Baseline plasma Phe was ~1,000 μmol/L and brain Phe was ~250 μmol/L in both series, with blood Phe 4 times greater than brain Phe. Significant correlations were found between plasma Phe concentrations and brain Phe only in the group without LNAA supplementation. We did not find close associations between blood and brain Phe levels. Moreover, participants in our study taking supplements, including the one on LNAAs, did not show decreased brain Phe. In the Pietz study, correlations between plasma and brain Phe were significant when specimens were obtained 12–24 h after a Phe loading. Our values for blood and brain Phe were obtained within a few hours on the same day and without loading doses of Phe.

Möller et al. (2003) used proton MRS and noted that white matter alterations correlated with higher brain Phe and several untreated patients with normal IQ had lower concentrations of brain Phe despite high blood Phe concentrations. All but two of the participants in our study had white matter changes, despite variable brain Phe. The definition of white matter alterations or our inclusion of only early treated individuals may account for these discordant results.

Our results support findings by Weglage et al. (2001, 2002) who studied sibling pairs with disparate intellectual outcomes despite similar blood Phe concentrations. After Phe loading tests (100 mg/kg body weight), the children with the lowest IQ had the highest concentrations of Phe in the brain and higher degrees of white matter abnormalities. Although overall IQ did not correlate with brain Phe in our study, the one participant with intellectual disabilities (IQ = 64) had the highest brain Phe in white matter, a high concurrent blood Phe level, and the lowest blood Tyr level in the sample.

In previous studies using conventional MRS, spectral overlap prevented measurement of Tyr. In addition, these MRS methods encountered poor signal to noise ratios (SNRs), leading to relatively large error coefficients (see Pietz et al. (2002), with reply by Weglage et al. (2002)). Another limitation was the assumption that average brain Phe concentrations in large regions of interest reflected concentrations of brain Phe in smaller distinct regions. This assumption was based on work done by MacKean (1972) and had not been reevaluated. Finally, blood Phe and brain Phe appeared to be linearly related only at lower concentrations. Thus, Phe loading could have distorted the relationship between blood Phe and brain Phe due to potential saturation of Phe in the brain (Möller et al. 2000). Bik-Multanowski and Pietrzyk (2007) exposed many of these limitations in a study of brain MRS in 104 patients (ages 8–29 years) and ten healthy adult controls after an 8–12 h oral Phe load. Using the standard 1.5 T scanner, these researchers found that brain Phe intensity did not exceed the background signal in the majority of patients with blood Phe concentrations below 1,200 μmol/L. In the remaining cases, the intensity of signals was proportional to blood Phe concentrations, but could not be quantified due to the high variability of the background spectrum. These authors concluded, "Thus, although magnetic resonance spectroscopy allows semiquantitative assessment of brain phenylalanine signal, routine use of this method seems to be reasonable only in patients with massive hyperphenylalaninemia."

In 2009, Kreis et al. established a method for measuring brain Phe that addressed some of these concerns. Using the

1.5 T MR scanner (Signa; GE), 28 subjects with PKU (ages 11–55 years) were investigated and in 17 subjects, long-term reproducibility was analyzed. Results indicated determination of brain Phe with a variation in independent sessions of 7 μmol/kg Phe, or a coefficient of variance (CV) of 3%. SNR was optimized through long acquisition times and investigation of a large region of interest (ROI), as described by localization to the ROI performed with PRESS (echo time = 20 ms, repetition time = 2.0 s, acquisitions/spectrum = 256, supraventricular ROI approximately 70 cm^3, and phase rotation cycle = 16 steps). While an improvement over previous methods, the Kreis method requires a large voxel, which does not allow for differentiation between gray and white matter. Furthermore, this method was optimized only for the detection of Phe and not Tyr.

Our pilot study was limited in its sample size and scope. The validity of COSY in PKU needs further investigation through additional studies in humans (affected individuals and controls) that include COSY under fasting and non-fasting conditions, as well as scans repeated over several days. Attention needs to be given to possible effects related to the LAT1 gene related to transport of amino acids across the blood–brain barrier. Patients with higher values of the Michaelis constant (a lower affinity of the transporter for Phe) and smaller T_{max}/V_{met} ratios were found to have lower brain Phe concentrations at a given blood Phe level in the Möller (2003) study. Attention also needs to be given to the effects of long-term as well as concurrent blood Phe exposure. The PCG region was chosen for the current study because of the availability of control scans from another study. As noted above, future studies using COSY should include measurement of Phe and Tyr in the prefrontal cortex. Other LNAAs can and should be included in future studies.

In terms of neuropsychological outcomes, IQ appears to be too "blunt" of a measure for identifying the impact of brain biomarkers and is likely to reflect exposure to Phe during a critical period in early childhood rather than concurrent metabolic status (Waisbren et al. 2007). Rather than focusing on overall functioning, researchers should directly assess processing speed, auditory and visual memory, and executive functioning. Participants should complete questionnaires assessing anxiety and depression as well as health history forms that include prior and current treatments, including psychotherapy and psychotropic medications, since mood disorders appear to be a common symptom in PKU (Clacy et al. 2014) and may be particularly sensitive to brain metabolites.

Despite its limitations, this pilot study suggests hypotheses regarding pathological mechanisms in PKU. As noted, the higher the Phe level within brain tissue, the greater is the perturbation in some aspects of neuropsychological functioning. An important question is whether Phe is elevated in all cells to the same degree or only in those that are pathological targets of toxicity. In our study, white matter appears to be associated with a variety of neuropsychological functions, suggesting the possibility that the cellular target is an oligodendroglial cell and/or the axon itself. Another important question is whether the toxicity associated with elevated Phe in these cells is due to Phe per se or a consequence of increased influx of Phe at LAT-1 with inhibition of other LNAAs such as Tyr into the cell. With the new COSY methodology, these questions can begin to be answered.

But for now, we conclude that with further validation, COSY will improve measurement of brain Phe and Tyr. Our study suggests that these biomarkers correspond to indices of neuropsychological functioning and that effects of brain Phe and Tyr may vary depending on their concentrations in specific brain regions and the neuropsychological domains evaluated.

Synopsis

Correlated Spectroscopy (COSY) measures brain phenylalanine (Phe) and tyrosine (Tyr) in distinct brain regions and may provide a means for understanding the variability in neuropsychological outcomes found in phenylketonuria (PKU).

Details of the Contributions of Individual Authors

Susan E. Waisbren, PhD, led the research team in planning the study, recruiting participants, conducting neuropsychological evaluations, analyzing and interpreting the data, and drafting the manuscript for publication.

Sanjay P. Prabhu, MD, participated in planning the study, obtaining and interpreting MRI findings, analyzing the data, and drafting the manuscript.

Patricia Greenstein, MD, conducted neurological examinations and participated in planning the study, analyzing the data, and drafting the manuscript.

Carter Petty, MA, conducted statistical analyses and participated in interpreting the results and drafting the manuscript.

Donald Schomer, MD, conducted neurological examinations and participated in planning the study, analyzing the data, and drafting the manuscript.

Vera Anastasoaie participated in planning the study, collecting data, and critically reviewing the manuscript.

Kalin Charette participated in planning the study, collecting data, and critically reviewing the manuscript.

Daniel Rodriguez conducted the post-processing of the COSY data in controls, assisted in conducting the phantom studies, and contributed to the manuscript.

Sai Merugumala developed the software to reconstruct and quantify the COSY spectra, participated in the analyzing and interpreting of the data, and contributed to the manuscript.

Alexander P. Lin, PhD, participated in planning the study, obtaining MRI and COSY, conducting post-scanning analyses, analyzing and interpreting the data, and drafting the manuscript.

Dr. Waisbren serves as guarantor for the article.

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Competing Interest Statements

Dr. Waisbren consults to BioMarin Pharmaceuticals, Inc.

Vera Anastasoaie serves as research coordinator for studies supported by BioMarin Pharmaceuticals, Inc.

Kalin Charette serves as research coordinator for studies supported by BioMarin Pharmaceuticals, Inc.

Drs. Greenstein, Prabhu, Schomer, and Lin and Mr. Carter, Mr. Rodriguez, and Mr. Merugumala have no competing interests and nothing to declare.

This study was approved by the Committee on Clinical Investigations (Institutional Review Board) at Boston Children's Hospital (IRB-P00003864) and all participants provided written informed consent.

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RESEARCH REPORT

Table of Phenylalanine Content of Foods: Comparative Analysis of Data Compiled in Food Composition Tables

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Abstract *Background*: Knowing the phenylalanine (Phe) content of foods is essential for managing the diet of patients with phenylketonuria. Data on the Phe content of foods are scarce and sometimes vary between different Food Composition Tables (FCT). Brazil created its own table of the Phe contents of fruits and vegetables based exclusively on the chemical analysis of protein content, considering that proteins contain 3–4% Phe (TCFA/ANVISA). This study compared the protein and Phe contents of vegetables and fruits provided by the TCFA/ANVISA with those listed in international food composition tables.

Methods: The Phe content of 71 fruits and vegetables listed in TCFA/ANVISA was classified into four subgroups, and the Wilcoxon nonparametric test compared the Phe and mean protein contents provided by the FCTs. All tests considered the bilateral hypothesis, and the level of significance was set at 5%. The Spearman's correlation coefficient measured the statistical dependence between Phe and protein contents.

Results: The mean Phe content was <50 mg Phe/100 g for 15 fruits; >50 mg/100 g for 11 type-A vegetables; <50 mg/100 g for 8 type-B vegetables; ≤50 mg/100 g for 7 type-C vegetables. The percentage of Phe in protein varied from 3.13 ± 1.03% to 3.74 ± 2.55% in fruits; 3.33 ± 1.41 to 4.82 ± 1.17 in type-A vegetables; 3.46 ± 1.25% to 4.83 ± 2.46 in type-B vegetables; and 3.14% ± 1.49 to 4.62% ± 2.26 in type-C vegetables.

Conclusions: The Phe and protein contents provided by most FCTs were positively correlated, suggesting that it is possible to estimate the Phe content of fruits by multiplying its protein content by 3%. For type-A, -B, and -C vegetables, 4% may be used.

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Introduction

Food Composition Tables (FCTs) and searchable databases provide information on nutrients, but data about the amino acid content of foods are frequently either unavailable, scarce, or outdated (Pennington 2008; Nalin et al. 2010; MacDonald et al. 2010; Demirkol et al. 2011). This is one of the various factors that may explain the absence of the amino acid profile of fresh fruits and vegetables in several FCTs (Charrondiere et al. 2013; Blau et al. 2010; Feillet et al. 2010a, b; Guimarães and Lanfer Marquez 2002, 2005; Greenfield and Southgate 2003; Osmo et al. 2008).

Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism with a global prevalence ranging from 1:30,000 to 1:1,000 of live newborns, depending on country (Monteiro and Cândido 2006; Ahring et al. 2009;

Martins et al. 2009; Blau et al. 2010). Due to persistently high plasma Phe level, untreated PKU causes neurological impairment, intellectual disability, speech delay, convulsions, skin hypopigmentation, and eczema, among others (Brandalize and Czeresnia 2004; Feillet et al. 2010a, b; De Groot et al. 2010; Camp et al. 2012).

Reliable information on the Phe content of foods is indispensable for patients with phenylketonuria, since they require a diet low in this essential amino acid. Fresh fruits and vegetables are poor protein sources that do not contribute significantly to protein requirement. Although no consensus has been reached, some studies suggest that patients with phenylketonuria should be allowed to consume low-Phe fruits and vegetables. No significant negative impact on short-term metabolic control has been observed, but the literature encourages more research to confirm this finding (Mac Donald et al. 2003; Weetch and MacDonald 2006; MacDonald et al. 2011; Rohde et al. 2012; Zimmermann et al. 2012).

Therefore, it is crucial to know the Phe content of fruits and vegetables and their variability in international food tables and searchable food composition databases to guarantee that fruits and vegetables indeed contribute little to Phe intake. The present study compared the Phe content of fresh fruits and vegetables listed in the Brazilian PKU table (TCFA/ANVISA), with those listed in eight international FCTs. Additionally, the possibility of using the mean Phe content of the FCTs for some fruits and vegetables was investigated.

Methods

The project was approved by the Research Ethics Committee of the School of Health Sciences of the University of Brasilia (N. 389.679/2013).

Food Composition Tables and Study Design

Table of Phenylalanine Content of Foods of the Brazilian National Sanitary Surveillance Agency (TCFA/ANVISA)

Table 1 of TCFA/ANVISA lists 71 fresh fruits and vegetables, which were grouped into subgroups according to their edible parts: fruits ($n = 27$); type-A vegetables ($n = 18$): the edible parts of these vegetables are the leaves, flowers, buds, or stems; type-B vegetables ($n = 14$): the edible parts of these vegetables are fruits, seeds, or parts that develop on the ground; and type-C vegetables ($n = 12$): the edible parts of these vegetables are those that grow underground and palm trees.

The TCFA/ANVISA includes moisture, protein, and Phe contents. The protein content of all fruits and vegetables was given by their total N content, determined by Kjeldahl's method, which was then multiplied by 5.7 to obtain the crude protein content. The Phe content was estimated by multiplying the protein content by 4% and expressed as mg Phe/100 g food. Therefore, 1 g of fruit or vegetable protein has about 40 mg of Phe. The value of 4% was taken from studies that reported that fruits and vegetables contain about 40 mg of Phe per gram of protein, which is lower than the Phe content of other types of natural proteins, such as animal proteins, which seem to have a Phe content closer to 5% (50 mg/g protein) (Ahring et al. 2009; Weetch and MacDonald 2006; MacDonald et al. 2011; AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA 2013). In most countries including the United States (USA), multiplying plant protein content by 5% would force patients to eat less for fear of reaching the actual daily Phe intake tolerance.

International Food Composition Tables

The protein and Phe contents of foods provided by the TCFA/ANVISA were compared with those furnished by eight international FCTs listed in the International Food Composition Tables Directory (Table 2). The directory is maintained by the International Network of Food Data Systems (INFOODS) (www.fao.org/infoods). In addition to the FCTs listed by INFOODS, the Low Protein Food List for PKU (LPFL-PKU) was also included, due to its relevance for patients with phenylketonuria (Schuett 2010).

The international tables were selected based on the following criteria: free internet access; available in electronic format: such as Excel, Access, or PDF files, or online database; available in English, Portuguese, or Spanish; and containing the Phe content of fruits and vegetables with a protein content of 5% or less. The exclusion criteria were: restricted access; information not available in English or Spanish; Phe content not available for some or all items, or available only for fresh vegetables and fruits that do not have similar counterparts in the TCFA/ANVISA Table.

Moisture, protein, and Phe contents provided by the eight international FCTs were determined by different analytical techniques used specifically to construct the FCTs. Sometimes data were also obtained from analytical data published in the literature or compiled from other databases and FCTs (Table 2).

Fresh vegetables listed in the TCFA/ANVISA Table were compared with their counterparts in the international FCTs and identified by their popular and scientific names, by the edible parts, and also by their taxonomic description, including genus, species, and variety. Different varieties of fruits and vegetables of the same species, and fruits and vegetables without variety information were grouped, and

Table 1 Mean phenylalanine content of fruits and vegetables (mg/100 g) in TCFA/ANVISA

Fruits	Phe (mg/100 g)	Type-A vegetables[a]	Phe (mg/100 g)	Type-B vegetables[b]	Phe (mg/100 g)	Type-C vegetables[c]	Phe (mg/100 g)
Acai (*Euterpe oleracea mart.*)	27	Artichoke (globe) (*Cynara scolimus* L.)	96	Bur cucumber (*Cucumis anguria* L.)	49	Beets (*Beta vulgaris*)	54
Apple (*Pyrus malus*)	10	Arugula (*Eruca sativa Mill*)	97	Chayote (*Sechium edule*)	31	Carrot (*Daucus carota*)	41
Avocado (*Persea americana mil.*)	45	Butter green bean (*Phaseolus vulgaris* L.)	75	Cucumber (*Cucumis sativus* L.)	29	Cassava (*Manihot esculenta crantz manihot utilissima*)	43
Banana (*Musa* sp.)	40	Cabbage, white and red (*Brassica oleracea* L.)	35	Eggplant (*Solanum melogena* L.)	34	Garlic (*Allium sativum* L.)	236
Cashew (*Anacardium occidentale* L.)	27	Cauliflower (*Brassica oleracea* L.)	62	Okra (*Abelmoschus esculentus*)	82	Leeks (*Allium porrum* L.)	58
Fig (*Ficus carica* L.)	26	Celery (*Apium graveolens* L.)	38	Peas, green (*Pisum sativum* L.)	120	Onion (*Allium cepa* L.)	68
Grapes, red or green (*Vitis* sp.)	24	Chard, swiss (*Beta vulgaris* L.)	36	Peppers, sweet, yellow (*Capsicum annuum*)	42	Palm heart (*Euterpe edulis*)	98
Jackfruit (*Arctocarpus heterophyllus*)	49	Chicory (*Chichorim endivia* L.)	42	Peppers, sweet, green (*Capsicum annuum*)	33	Pupunha (palm heart) (*Bactris gasipaes kunth.*)	88
Kiwifruit (*Actinidia chinensis plack.*)	44	Chicory greens (*Cichorium intybus*)	62	Peppers, sweet, red (*Capsicum annuum*)	36	Potato (*Solanum tuberosum*)	71
Indian Cherry (*Malgiphia emarginata*)	31	Coriander (*Coriandrum sativum* L.)	173	Pumpkin (*Cucurbita* spp.)	46	Radish (*Raphanus sativus*)	26
Mango (*Mangifera indica*)	25	Dutch string bean (*Phaseolus vulgaris* L.)	98	Scarlet eggplant (*Solanum gilo Raddi.*)	40	Sweet Potato (*Ipomoea batatas* L.)	58
Melon (*Cucumis melo*)	22	Endive (*Cichorium endivia* L.)	66	Squash, summer (*Cucurbita pepo*)	42	Yam (*Dioscorea* spp.)	54
Papaya (*Carica papaya* L.)	18	Kale (*Brassica oleracea*)	96	Squash, winter (*Cucurbita maxima*)	87	Taro (*Colocasia esculenta*)	62
Passion fruit (granadilla) (*Passiflora edulis*)	138	Lettuce (*Lactuca sativa* L.)	40	Tomatoes (*Solanum lycopersicon* sp.)	38	Turnips (*Brassica rapa* L.)	41
Peaches (*Prunus persica*)	33	Onions, spring or scallions (*Allium fistulosum* L.)	57				
Pears (*Pyrus communis* L.)	20	Parsley, fresh (*Petroselinum sativum*)	211				
Pequi (*Caryocar brasiliense camb.*)	82	Spinach (*Tetragonia expansa Murr.*)	65				
Persimmon (*Diospyros kaki* L.)	21	String bean (*Phaseolus vulgaris* L.)	66				
Pineapple (*Ananas comosus L. merril*)	29	Watercress (*Nasturtium officinale* L.)	126				
Pomegranates (*Punica granatum* L.)	21						
Plums (*Prunus salicina Lindl.*)	43						
Soursop (*Annona muricata*)	56						
Strawberry (*Fragaria vesca* L.)	33						
Sugar apples (sweetsop) (*Annona squamosa*)	92						
Surinam cherry (*Eugenia uniflora* L.)	30						
Tamarinds (*Tamarindus indica* L.)	105						
Tangerines, mandarin oranges (*Citrus reticulata*)	29						

[a] The edible part of the vegetables consists of the leaves, flowers, buds, or stems
[b] The edible part of the vegetables consists of fruits, seeds, or parts that develop on the ground
[c] Consists of the parts that grow underground and palm trees

Table 2 Food composition tables included in the study

Name	Organization	Source of Phe contents
Brazilian table for PKU, 2013 (TCFA/ANVISA)	National Sanitary Surveillance Agency (ANVISA)	Estimated by multiplying their protein content by 4%
Food Amino-Acid Content of Foods and Biological Data on Proteins, 1970 (FAO-AA/FAO)	Food and Agriculture Organization of the United Nations	Analytical data published in the scientific literature, databases or other FCTs
Danish Food Composition Databank, 2009 version 7 (DTU FOOD)	National Food Institute/Technical University of Denmark (DTU)	Laboratory analyses conducted specifically for the FCTs and analytical data published in the scientific literature or databases, including the USDA-SR database and other FCTs
Food Composition and Nutrition Tables, 2008 (FCNT)	German Research Centre for Food Chemistry	Laboratory analyses conducted specifically for the FCTs and analytical data published in the scientific literature, databases, or other FCTs
Nutrient Tables – Food Standards Australia New Zealand (FSANZ), 2010 (NUTTAB)	Food Standards Australia New Zealand	Laboratory analyses conducted specifically for the FCTs
Health Canada – Canadian Nutrient File, 2015 (HCNT)	Health Canada	Analytical data published in the USDA database, SR 23–27
New Zealand Food Composition Database, FOODfiles 2014 Version 01 (NZFC)	The New Zealand Institute for Plant & Food Research Limited and the Ministry of Health (New Zealand)	Laboratory analyses conducted specifically for the FCTs and analytical data published in the scientific literature, databases, or other FCTs
Low Protein Food List for PKU, 2010 (LPFL-PKU)	Waisman Center in Madison, Wisconsin	Analytical data published in the USDA database (SR 22, 2009, SR 23, 2010, and SR 28, 2015)
USDA National Nutrient Database for Standard Reference, Release 28, 2015 (USDA-SR)	United States Department of Agriculture	Laboratory analyses conducted specifically for the FCTs

the mean Phe content was calculated. However, some fruits and vegetables, which are commonly found in Brazil, were not found in the international FCTs. Hence, they were maintained in the TCFA/ANVISA Table (Table 1), but were not compared.

The protein contents in the international FCTs were calculated using a conversion factor of 6.25 to transform total N into protein by considering that the protein fraction has a mean nitrogen content of 16%. However, all data from the TCFA/ANVISA Table used a conversion factor of 5.75, which seems to be closest to the actual protein content (Greenfield and Southgate 2003). Therefore, the protein level contents provided by the different tables could only be compared after recalculating all the data using the conversion factor of 5.75.

In addition to absolute Phe content, we calculated the percent contribution of Phe in the protein of each food to verify whether Phe content was relatively constant for each type of vegetable, therefore, possibly genetically determined. Then the percentage of Phe present in the proteins of the fruits and vegetables listed in the international FCTs were compared with the Phe content of 4% in vegetable proteins used by the TCFA/ANVISA Table.

Statistical Analysis

The Wilcoxon nonparametric test compared the protein and Phe contents provided by the TCFA/ANVISA Table and each of the eight international FCTs. All tests considered bilateral hypotheses and used a significance level of 5%. The correlation between Phe and protein contents was given by Spearman's correlation coefficient (Conover and Conover 1980).

Results

From the total number of fruits listed in the TCFA/ANVISA, 11 could not be compared because they were either not present in the majority of the other tables, or the Phe content was not provided. Some of these fruits are tropical, so analytical information is not easily available. The excluded fruits are: acai, cashew, Indian cherry, soursop, jackfruit, sweet passion fruit, pequi, sugar apple, Surinam cherry, pomegranate, and tamarind. Four type-A vegetables (butter green bean, Dutch string bean, endive, and string bean), two type-B vegetables (scarlet eggplant and bur cucumber), and two type-C vegetables (palm heart

and pupunha palm heart) also were not compared because they were not listed in the other tables.

Figure 1a–d shows the Phe content of fruits and vegetables listed in the nine FCTs, including the TCFA/ANVISA Table. Fruits have the lowest protein content and, as expected, the lowest Phe content. In Fig. 1a, 15 out of the 16 fruits had a mean Phe content smaller than 50 mg/100 g fruit, ranging from 8 to 49 mg/100 g. The only exception was avocado, which varied considerably, from 45 to 150 mg/100 g. The median Phe content of avocado, considering the eight FCTs and the TCFA/ANVISA Table, was 93 mg/100 g, which might be the most accurate Phe content of this fruit. In the TCFA/ANVISA Table, the Phe content of avocado is 45 mg/100 g, so this value should be reexamined to confirm the discrepancy in relation to the other FCTs.

In the international FCTs, the proportion of Phe in protein varied from 3.13 ± 1.03% to 3.74 ± 2.55% and was always lower than 4%, with significant differences between five international FCTs and the TCFA/ANVISA Table, which varied from 3.13% ± 1.03 to 3.28% ± 0.93 (Table 3a). Therefore, the results suggest that it is possible to estimate the Phe content of fruits by multiplying their protein content by 3%.

Vegetables have also very low Phe content, but in general and with some overlapping, they seem to have more Phe than fruits. Figure 1b shows the Phe content furnished by the FCTs for 15 study vegetables. On average, 11 out of the 15 type-A vegetables had a mean Phe content higher than 50 mg/100 g, ranging from 53 to 179 mg/100 g. The Phe content of proteins in these foods provided by the international FCTs ranged from 3.33 ± 1.41% to 4.82% ± 1.17, with no significant differences between the eight FCTs and the TCFA/ANVISA Table (Table 3a).

Not all 14 type-B vegetables listed in the TCFA/ANVISA Table were listed in the international FCTs, which resulted in a variable number of samples. Figure 1c shows the Phe content of 12 type-B vegetables. Of these, eight have a mean Phe content lower than 50 mg/100 g, ranging from 20 to 47 mg/100 g. The Phe content of peapods was variable and higher than 200 mg/100 g. Peapod is a legume with high protein content associated with degree of ripeness, as protein is synthesized in advanced maturation stages.

The mean Phe content in the protein fraction of type-B vegetables provided by the TCFA/ANVISA Table did not differ significantly from those provided by three of the eight international FCTs (FAO-AA, FCNT/Germany, and NUTTAB/Australia), whose percentages varied from 3.46 ± 1.25% (FAO-AA) to 4.07 ± 1.64% (FCNT/Germany). In the other FCTs, the mean percentages were significantly higher, varying from 4.28 ± 0.96% (LPFL-PKU/USA) to 4.83 ± 2.46% (HCNT/Canada) (Table 3a). However, the percentages of 27% Phe in the protein fraction of pumpkin provided by DTU FOOD (Denmark) and of roughly 11% in green pepper provided by HCNT (Canada) and USDA-SR (USA) may be incorrect because they differ greatly the percentages provided by the literature (3–5%) and do need confirmation (Greenfield and Southgate 2003; de Menezes et al. 2003).

Figure 1d shows the Phe content of 12 type-C vegetables even though not all of them were listed in the international FCTs, resulting in the comparison of a variable number of samples. On average, seven of these foods had a Phe content of 50 mg/100 g or less, ranging from 22 to 50 mg/100 g. The mean Phe content of the other four type-C vegetables varied from 73 to 88 mg/100 g, except for garlic, which had a mean Phe content of 190 mg/100 g. The Phe content in the protein of 50% of these foods varied significantly, from 3.14 ± 1.49% to 4.62 ± 2.26% in the international FCTs, but the contents were not significantly different from those provided by the TCFA/ANVISA Table (Table 3a).

Phe and protein contents were positively correlated in all FCTs (Table 3b). The correlation for fruits was statistically significant in all eight international FCTs, and the Spearman's correlation coefficient varied from $\rho = 0.692$ to 0.972 ($p < 0.05$). For type-C vegetables the correlation was significant in seven of the eight FCTs ($\rho = 0.664$ to 0.995; $p < 0.05$). The correlation for type-A vegetables was significant in six FCTs (Spearman, $\rho = 0.879$ to 1.000; $p < 0.05$). The correlation for type-B vegetables was significant in three FCTs ($\rho = 0.900$ to 1.000; $p < 0.05$).

Discussion

The present study compared the Phe content of fresh fruits and vegetables listed in nine food composition tables. The Brazilian table (TCFA/ANVISA) was used as reference for the number of samples compared. The possibility of using a mean Phe content for each food group was also investigated.

Based on our results about 70% of the fresh fruits and vegetables listed in the FCTs have similar Phe content: apple, artichoke, arugula, aubergine, banana, cassava, cauliflower, chayote, chicory, cucumber, endive, fig, grape, kiwi, leek, lettuce, mandarin, mango, melon, okra, onion, papaya, peach, pear, persimmon, pineapple, plum, squash summer, strawberry, taro, turnip, yellow pepper, radish, red pepper, white and red cabbage, and tomato.

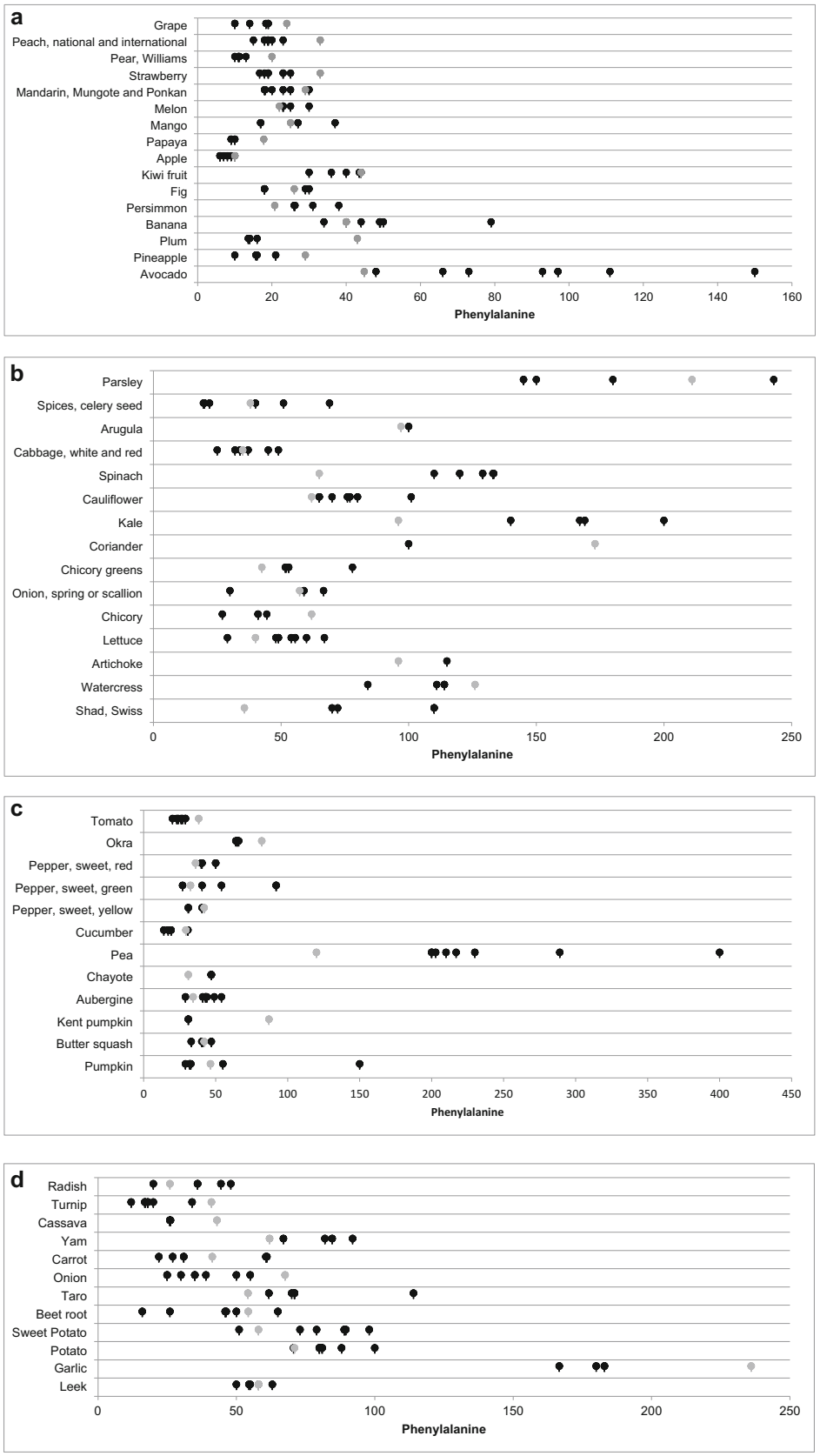

Fig. 1 Phenylalanine content (mg/100 g) of fruits (**a**) and type-A, -B, and -C vegetables (**b**, **c**, **d**) provided by TCFA/ANVISA (*gray dots*) and eight international food composition tables (*black dots*): DTU FOOD/Denmark, FAO-AA, FCNT/Germany, HCNT/Canada, LPFL-PKU/USA, NUTTAB/Australia, NZFC/New Zealand, USDA-SR/USA. *Legend for fruits*: Grape, several varieties (Thompson, White,

The Phe content of about 30% of the study fresh fruits and vegetables differ considerably between the nine FCTs: avocado, beet root, carrot, celery seed, coriander, garlic, green pepper, kale, onion spring or scallion, parsley, pea, potato, pumpkin, kent pumpkin, spinach, swiss chard, watercress, sweet potato, and yam.

The differences may stem from several factors, such as accuracy of protein and Phe estimates, origin of the food, genetic variability, climate, degree of maturation, time of harvest, and even a transcription error of raw data, which cannot be omitted. Therefore, an adequate number of samples of these vegetables should be reanalyzed to obtain more accurate Phe and protein estimates, and consequently explain or correct the different protein and Phe contents provided by the FCTs. Result dispersion may be minimized by analyzing a representative number of samples of the species and varieties of fruits and vegetables cultivated and consumed in a country, taking into account factors that may affect nutrient composition (de Menezes et al. 2003). The sampling design, analytical method, expression of the results, and data treatment should also be considered.

According to the nine FTCs, 58 and 84% of the 55 fresh study fruits and vegetables had a mean Phe content of 50 mg/100 g or less, and 100 mg/100 g or less, respectively. Since clinical trials suggest that fruits and vegetables with Phe content of 50 mg to 100 mg/100 g are safe for patients with phenylketonuria, these fruits and vegetables could be classified as unrestricted for these individuals (Mac Donald et al. 2003; Weetch and MacDonald 2006; MacDonald et al. 2011; Rohde et al. 2012; Zimmermann et al. 2012).

We understand that the data in the FCTs, some based on chemical analyses and some on the literature, and the small number of analyzed samples are study limitations. Therefore, caution is advised when using the Phe contents provided by the FCTs, since many factors contribute to different or incorrect results.

Although chemical analyses are recommended to obtain more accurate Phe content data, the positive correlation found between the protein and Phe contents of the study fruits and vegetables indicates that Phe content can be reliably estimated from protein content.

Pimentel et al. (2014) made a similar attempt by analyzing the protein and amino acid contents of 16 vegetable and fruit preparations usually included in the diet of Portuguese patients with phenylketonuria. The Phe and protein contents of these preparations were highly correlated. Lanfer Marquez et al. (1997) studied the chemical composition of cereal flakes and found a linear correlation between the samples' total nitrogen and Phe contents.

Hence, given the scarcity of analytical Phe data and the present comparative analysis, the Phe content of fresh fruits and vegetables can be estimated from their protein content, despite the limitations associated with result accuracy. Comparative analyses indicated that 3% seems to be the most appropriate multiplier to calculate the Phe content in the fruit protein contents provided by most FCTs.

For type-A, type-B, and type-C vegetables, the results support the possibility of estimating the Phe content of leaf vegetables by multiplying their protein content by 4%, as performed by the TCFA/ANVISA Table (Brazil) and most FCTs. These results confirm the literature reports that Phe contributes with 3–5% of the total amino acid content of these foods (Weetch and MacDonald 2006; Bremer et al. 1996). However, when the percentage of 3 or 4% is used for estimating Phe content, the result is lower than 50 mg/g of protein. When the percentage of 5% is used, the result exceeds 50 mg/g of protein.

Processed plant-based foods, such as fruit and vegetable juices, and jams, should also be studied to determine whether the correlation between the Phe and protein contents of fresh vegetables and fruits also applies to these products. If so, the concentrations of 3–5% Phe in proteins could be used to estimate the Phe content of these products. Vegetable and fruit preparations should also be chemically analyzed because processing may change the Phe content of the food (Weetch and MacDonald 2006; Pimentel et al.

Fig. 1 (continued) Niagara) (*Vitis* sp.); Peach, Brazilian and international (*Prunus persica*); Pear, Williams (*Pyrus communis* L.); Strawberry (*Fragaria vesca* L.); Mandarin, Mungote and Ponkan (*Citrus reticulata* "Murgote"); Melon (*Cucumis melo*); Mango (*Mangifera indica* L.); Papaya (*Carica papaya* L.), Apple (*Pyrus malus*); Kiwi fruit (*Actinidia chinensis Plack.*); Fig (*Ficus carica* L.); Persimmon (*Diospyros kaki* L.); Banana (several varieties) (*Musa* sp.); Plum (*Prunus salicina Lindl.*); Pineapple (*Ananas comosus*); Avocado (*Persea americana mil.*). *Legend for type-A vegetables*: Parsley (*Petroselinum sativum*), Spices, celery seed (*Apium graveolens* L.), Arugula (*Erucata sativa*), Cabbage, white and red (*Brassica oleracea* L.), Spinach (*Tetragonia expansa Murr.*), Cauliflower (*Brassica oleracea* L.), Kale (*Brassica oleracea* L.), Coriander (*Coriandrum sativum* L.), Chicory greens (*Cichorium intybus* L.), Onions spring or scallions (*Allium fistulosum* L.), Chicory (*Chicorium endivia*), Lettuce (*Lactuca sativa* L.), Artichokes, globe (*Cynara scolimus* L.), Watercress (*Nasturtium officinale* L.), Chard, Swiss (*Beta vulgaris L. var. cicla*). *Legend for type-B vegetables*: Tomatoes, several varieties (*Solanum lycopersicon Mill., Lycopersicon* sp.), Okra (*Hibiscus esculentus*), Pepper, sweet, red (*Capsicum annuum*), Pepper, sweet, green (*Capsicum annuum*), Pepper, sweet, yellow (*Capsicum annuum*), Cucumber (*Cucumis sativus* L.), Peas, green (*Pisum sativum* L.), Chayote (*Sechium edule*), Aubergine (*Solanum melogena* L.), Kent pumpkin (*Cucurbita maxima*), Butter, squash, (*Cucurbita pepo*), Pumpkin (*Cucurbita* spp.). *Legend for type-C vegetables*: Radish (*Raphanus sativus*), Turnip (*Brassica rapa* L.), Cassava (*Manihot esculenta Crantz Manihot utilissima*), Yam (*Colocasia esculenta*), Carrot (*Daucus carota* L.), Onion (*Allium cepa* L.), Taro (*Dioscorea* spp.), Beet root (*Beta vulgaris*), Sweet potato (*Ipomoea batatas* L.), Potato (*Solanum tuberosum*), Garlic (*Allium sativum* L.), Leek (*Allium porrum* L.)

Table 3 Mean, standard deviation, and comparative analysis of the percentage of phenylalanine in the protein portion of foods (a) and association between the Phe and protein contents (Spearman's correlation) (b) of foods listed in the study food composition tables and the Table of Phenylalanine Content of Foods ofANVISA/Brazil

(a)

	Fruits			Type-A vegetables			Type-B vegetables			Type-C vegetables		
International FCT	Number of foods	FCT[x]	TCFA/ ANVISA[y]	Number of foods	FCT[x]	TCFA/ ANVISA[y]	Number of foods	FCT[x]	TCFA/ ANVISA[y]	Number of foods	FCT[x]	TCFA/ ANVISA[y]
DTU FOOD	16	3.19±0.9[a]	3.97±0.2[b]	11	4.31±1.31[a]	4.01±0.07[a]	10	6.18±7.47[a]	4[b]	11	3.14±1.49[a]	3.98±0.04[a]
FAO-AA	9	3.28±0.93[a]	4±0.22[b]	6	4.82±1.17[a]	3.98±0.04[a]	5	3.46±1.25[a]	4[a]	9	4±1.07[a]	3.99±0.03[a]
FCNT	7	3.61±1.29[a]	3.96±0.21[a]	8	4.34±1.33[a]	4.01±0.08[a]	6	4.07±1.64[a]	4[a]	7	3.41±1.41[a]	4[a]
HCNT	16	3.74±2.55[a]	3.97±0.2[a]	10	4.3±1.29[a]	3.99±0.03[a]	11	4.83±2.46[a]	4[b]	9	4.62±2.26[a]	3.98±0.04[a]
LPFL-PKU	15	3.16±1.03[a]	3.97±0.2[b]	15	4.46±1.07[a]	4.01±0.06[a]	11	4.28±0.96[a]	4[b]	12	4.36±1.57[a]	3.98±0.04[a]
NUTTAB	7	3.56±1.3[a]	3.96±0.21[a]	4	3.33±1.41[a]	3.98±0.05[a]	4	3.53±0.67[a]	4[a]	6	3.93±0.99[a]	4[a]
NZFC	10	3.13±1.03[a]	3.94±0.17[b]	3	4.53±1.17[a]	4[a]	1	4.9	4	5	3.62±0.91[a]	3.98±0.04[a]
USDA-SR	16	3.25±1.04[a]	3.97±0.2[b]	12	4.28±1.19[a]	4.01±0.07[a]	12	4.72±2.38[a]	4[b]	12	4.28±1.72[a]	3.98±0.04[a]

(b)

	Fruits		Type-A vegetables		Type-B vegetables		Type-C vegetables	
International FCT	Number of foods	Spearman's correlation	Number of foods	Spearman's correlation	Number of foods	Spearman's correlation	Number of foods	Spearman's correlation
DTU FOOD	16	0.841*	11	0.918*	10	0.390**	11	0.664*
FAO-AA	9	0.883*	6	0.771**	5	0.900*	9	0.908*
FCNT	7	0.889*	8	0.905*	6	0.986*	7	0.714**
HCNT	16	0.692*	10	0.879*	11	0.221**	11	0.764*
LPFL-PKU	15	0.814*	15	0.900*	11	0.528**	12	0.673*
NUTTAB	7	0.857*	4	1.000*	4	1.000*	6	0.899*
NZFC	10	0.848*	4	0.400**	1	–	5	0.900*
TCFA/ANVISA	16	0.972*	15	0.997*	12	0.998*	12	0.995*
USDA-SR	16	0.747*	12	0.923*	12	0.377**	12	0.722*

Statistical analysis: different letters in lines between FCTs and TCFA/ANVISA indicate significant differences ($p < 0.05$)
*$p < 0.05$; **$p > 0.05$
[x] Mean Phe content±standard deviation in the protein fraction provided by international FCTs
[y] Mean Phe content±standard deviation in the protein fraction provided by TCFA/ANVISA

2014). Weetch and MacDonald (2006) found that the Phe content of different potato varieties prepared in different ways resulted in a mean Phe content of 28 mg/g of protein. For cooked potatoes, the Phe content varied from 44 mg to 109 mg/100 g. The vegetable preparations analyzed by Pimentel et al. (2014) had Phe contents of 12 mg to 33 mg/g of protein, and Phe and protein contents were highly correlated.

Conclusion

For fruits, 3% seems to be the best multiplier. For type-A, -B, and -C vegetables, 4% may be used. Analysis of the Phe and protein contents of the 55 fruits and vegetables listed in the nine FCTs indicated that it is possible to calculate mean Phe content from the amounts of Phe and protein provided by the various FCTs. In the absence of analytical Phe data, it is possible to estimate the Phe content of fresh fruits and vegetables from their protein content, despite the limitations associated with the accuracy of this method.

These findings may be useful for updating FCTs for patients with phenylketonuria, assisting dietitians in their practice and patients in estimating the Phe content of their diet. This information may increase the number of dietary options that best fit patients with phenylketonuria's daily routine.

It is important to create national FCTs with Phe data in the local language, accessible to the population, and to include in local FCTs regional fruits, vegetables, and preparations that would not normally be found in international FCTs.

Knowing Phe content variability in fruits and vegetables may help to reduce uncertainty, provide more reliable Phe contents, and expand the dietary guidelines of foods for patients with phenylketonuria.

Take-Home Message

The Phe content of most fresh fruits and vegetables listed in Brazilian PKU table is similar to those listed in other food composition tables.

Compliance with Ethics Guidelines

Ana Claudia Marquim Araújo, Wilma M. C. Araújo, Ursula M. Lanfer Marquez, Rita Akutsu, and Eduardo Y. Nakano declare that they have no conflict of interest.

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

Authors' Contributions

A.C.M.A. designed and conducted the research, analyzed samples, performed the statistical analyses, and wrote the paper; W.M.C.A. designed the research and wrote the paper; U.M.L.M. designed the research and reviewed the manuscript; R.A. reviewed the manuscript; E.Y.N. performed the statistical analyses.

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RESEARCH REPORT

Inhaled Sargramostim Induces Resolution of Pulmonary Alveolar Proteinosis in Lysinuric Protein Intolerance

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Abstract Pulmonary alveolar proteinosis (PAP) is a potentially fatal complication of lysinuric protein intolerance (LPI), an inherited disorder of cationic amino acid transport. The patients often present with mild respiratory symptoms, which may rapidly progress to acute respiratory failure responding poorly to conventional treatment with steroids and bronchoalveolar lavations (BALs). The pathogenesis of PAP in LPI is still largely unclear. In previous studies, we have shown disturbances in the function and activity of alveolar macrophages of these patients, suggesting that increasing the activity and the number of macrophages by recombinant human GM-CSF (rhuGM-CSF) might be beneficial in this patient group.

Two LPI patients with complicated PAP were treated with experimental inhaled rhuGM-CSF (sargramostim) after poor response to maximal conventional therapy. BAL fluid and cell samples from one patient were studied with light microscopy and transmission electron microscopy.

Excellent response to therapy was observed in patient 1 with no compliance problems or side effects. Macrophages with myelin figure-like structures were seen in her BAL sample. Slight improvement of the pulmonary function was evident also in patient 2, but the role of sargramostim could not be properly evaluated due to the complicated clinical situation.

In conclusion, inhaled rhuGM-CSF might be of benefit in patients with LPI-associated PAP.

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Introduction

Lysinuric protein intolerance (LPI) is a rare inherited amino acid transport disorder leading to decreased plasma concentrations and increased urinary excretion of cationic amino acids (CAAs) arginine, ornithine and lysine by defective y^+LAT1 transporter. The patients present with a variety of symptoms including failure to thrive, postprandial hyperammonaemia and haematological and immunological abnormalities, the exact mechanism of which is still unclear. The patients may remain asymptomatic for decades with a carefully planned low-protein diet and supplementation with low-dose oral L-citrulline and ammonia-scavenging drugs. Unfortunately, the current treatment regime does not seem to protect the patients from long-term complications including renal disease and pulmonary alveolar proteinosis (PAP), a rare condition characterized by accumulation of lipoproteinaceous material in the alveoli (Parto et al. 1993; Santamaria et al. 1996). Acute episodes

of PAP may occur even in those patients with excellent treatment compliance and no previous respiratory symptoms. Unlike in idiopathic PAP, granulocyte-macrophage colony-stimulating factor (GM-CSF) antibodies have not been detected in the LPI patients, and the pathogenesis of PAP in LPI patients has thus remained unclear.

The LPI PAP patients often present initially with mild respiratory symptoms, which may progress rapidly and lead to pulmonary insufficiency and multi-organ failure. To date, the only treatment options, i.e. repeated bronchoalveolar lavations (BALs) and systemic corticosteroids, have shown limited effectiveness (Santamaria et al. 2004; Ceruti et al. 2007). During the last 10 years, four Finnish paediatric patients from our cohort of over 40 Finnish LPI patients have been diagnosed with acute PAP. Three of them died despite immediate management in an intensive care unit (Table 1). The incidence of PAP has been even larger in other LPI cohorts. Valimahamed-Mitha and colleagues have reported a series of 14 paediatric patients with LPI, out of which ten fulfilled the diagnostic criteria of PAP and six died of pulmonary failure (Valimahamed-Mitha et al. 2015). In a group of nine Italian LPI patients reported by Santamaria and colleagues, one patient died of respiratory insufficiency, and five other patients had signs of lung involvement when studied with high-resolution computed tomography (HRCT) imaging despite being asymptomatic at the time of the study (Santamaria et al. 1996).

Although the exact pathogenesis of PAP in LPI has been unclear, disturbances in the function and phagocytic activity of monocyte-derived macrophages have been demonstrated (Barilli et al. 2010, 2012; Kurko et al. 2015). Thus, accumulation of proteinous material into the lungs may be caused by insufficient clearance of proteins by poorly functioning alveolar macrophages. Inhaled granulocyte-macrophage colony-stimulating factor (GM-CSF) is used off-label in patients with idiopathic PAP with anti-GM-CSF antibodies to activate and attract monocyte-derived macrophages into the lungs. We hypothesized that increasing the activity and the number of alveolar macrophages in the alveolar fluid by recombinant human GM-CSF (rhuGM-CSF) inhalation could promote the resolution of PAP also in LPI. Barilli et al. (2010) have previously reported one Italian patient diagnosed with LPI-associated PAP at the age of 15 years, whose respiratory condition and CT showed marked improvement after rGM-CSF treatment. However, the authors were naturally unable to draw conclusions on the efficacy of rGM-CSF in LPI patients based on a single patient case.

Here, we describe two Finnish LPI patients, one child and one adult, with complicated PAP treated with experimental inhaled rhuGM-CSF (sargramostim, Leukine®, Genzyme) after already receiving maximal conventional therapy.

Table 1 Summary of the four Finnish paediatric patients with LPI-related PAP followed up at Turku University Hospital between years 1997 and 2014

	Age at diagnosis	Age at PAP	Presenting signs of PAP	Treatment for PAP	Result
1.	1 year	1. 12 years 2. 16 years	1. Dyspnoea, dry cough 2. Poor exercise tolerance, progressive hypoxia	1. Large-dose corticosteroids, BAL, supplementary oxygen, saline inhalations, salbutamol 2. BAL, peroral steroids and antibiotics, saline inhalations, sargramostim	1. Partial recovery (decreased exercise tolerance, need for regular lung lavations) 2. Normal pulmonary function, able to participate to sports activities normally 1.5 years after the last episode of PAP
2.	1 year	16 years	Fever, cough, epistaxis, haematemesis	Intravenous antibiotics, large-dose corticosteroids, ventilatory support	Multi-organ failure and death 2 days after admission to hospital
3.	3 months	3 months	Dry cough, poor eating	Supplemental oxygen, corticosteroids, BAL	Died at the age of 12 months
4.	8 months	4 years 8 months	Fever, fatigue, poor appetite, enlarged liver and spleen, enlarged lymph nodes	Intravenous antibiotics, large-dose corticosteroids, BAL	Died 10 days after admission to hospital

Patient no. 1 is patient 1 described in the "Results" section
BAL bronchoalveolar lavation

Patients and Methods

Patients

The research was conducted according to the principles of the Declaration of Helsinki. A written informed consent was obtained from the patients before the initiation of the experimental rhuGM-CSF treatment.

Methods

BAL Fluid and Cell Sample Collections

BAL fluid was collected routinely from patient 1. After Cyto-Tek and Cytospin cytocentrifugations of the BAL sample, routine Papanicolaou, May-Grünwald-Giemsa, Prussian blue and periodic acid-Schiff (PAS)-stained slides were prepared and studied under a light microscope. In addition, a part of the BAL fluid was filtered through a sterile gauze. The filtered cells were centrifuged 250*g* for 10 min, washed with ice-cold HBSS and suspended in the RPMI-1640 medium with a GlutaMAX supplement (Invitrogen Life Technologies, Carlsbad, CA, USA) and 10% FBS before the following experiments.

Transmission Electron Microscopy

The centrifuged cell pellet was fixed with 5% glutaraldehyde overnight, and osmium tetroxide was added to fix the sample for 2 h. The sample was then dehydrated with ethanol and embedded with propylenoxid in epoxy resin. Ultrathin sections contrasted with uranyl acetate and lead citrate were studied under the Jeol JEM-1400Plus transmission electron microscope (Jeol, Tokyo, Japan).

Histology

The cells for histopathological examination were fixed in 10% buffered formalin, centrifuged and pre-embedded in agar. Then, the agar blocks were further embedded in paraffin, and routine 4 μm thick histological sections were cut on slides. The sections were stained with haematoxylin and eosin, and PAS, and studied under a light microscope.

Results

In the BAL sample of patient 1, a total of 520 million cells per litre were detected. Of these, 55% were macrophages, 42% lymphocytes and the remaining 3% neutrophils. Cytological bronchoalveolar preparations showed macrophages which contained PAS-positive granules. Similar granules were also seen around the cells. In addition, the histological sections contained macrophages with PAS-positive granules (Fig. 1). In a sample studied with electron microscopy, several macrophages containing lysosomes as well as myelin figure-like structures were observed (Fig. 2).

Patient 1

The patient is a girl who was diagnosed with LPI at the age of 1 year after her older sister had been diagnosed with LPI. At that time, she had no clinical findings except for the characteristic urinary amino acid profile and elevated serum ferritin. Her treatment regime consisted of oral L-citrulline (100–200 mg/kg/day), sodium benzoate (125–250 mg/kg/day) and protein-restricted diet (1–1.2 g/g/day) supplemented with calcium carbonate, vitamins, L-lysine hydrochloride (13–17 mg/kg/day) and commercial carbohydrate energy supplements. She had mild growth retardation, mild proteinuria and recurrent urinary tract infections but was

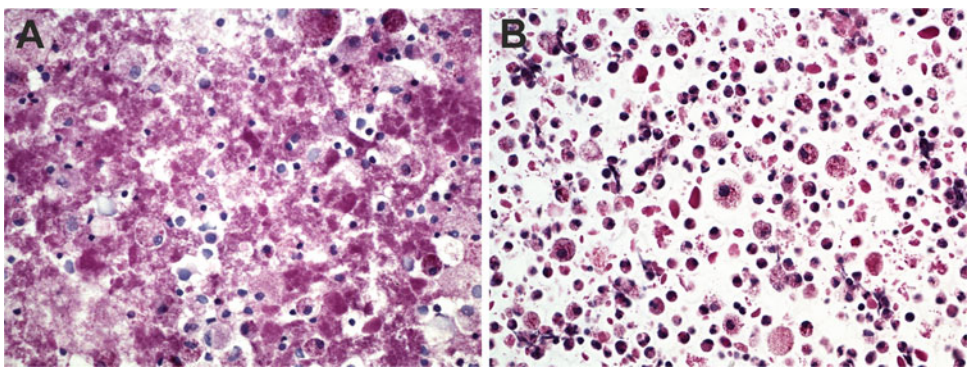

Fig. 1 Bronchoalveolar lavage fluid with macrophages containing PAS-positive granules. The cells from the patient 1 were stained with periodic acid-Schiff and examined with a light microscope using a 400× enlargement (**a**). Microscope preparations of filtered and washed cells in an agar/paraffin block obtained from the patient 1 were stained with periodic acid-Schiff and examined with a light microscope using a 400× enlargement (**b**)

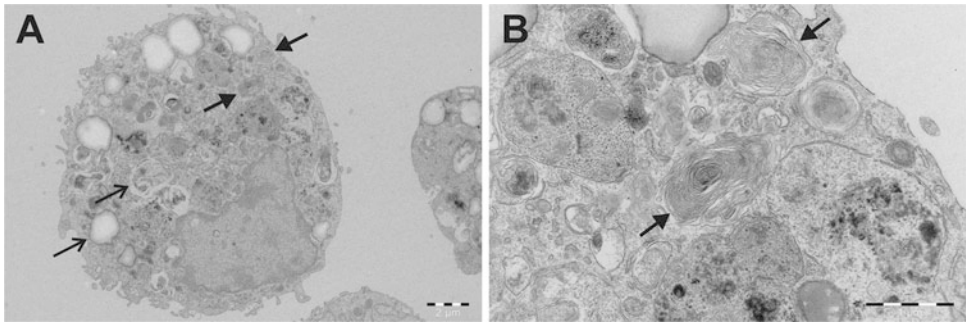

Fig. 2 An alveolar macrophage containing lysosomes and myelin figures. The filtered and washed cells obtained from the broncho-alveolar lavage fluid of the patient 1 were examined with an electron microscope. A 3,000× enlargement shows lysosomes indicated by *narrow arrows* and myelin figures indicated by *thick arrows* (**a**). A 10,000× enlargement reveals more closely the myelin figure-like surfactant structures indicated by *thick arrows* (**b**)

previously otherwise quite healthy and regularly participated in sports activities.

At the age of 12 years, she had an acute episode of dyspnoea and cough without symptoms of a respiratory infection. HRCT imaging suggested acute PAP. She was treated with repeated BALs and high-dose corticosteroids with a partial response but she remained dependent on supplemental oxygen. Hypertonic saline and salbutamol inhalations were not of significant benefit. After several large-volume BALs, her respiratory function gradually improved, and supplemental oxygen was discontinued 3 months after the initial episode. However, only a couple of months later, her pulmonary function started to worsen again and she developed dyspnoea and cough. A new BAL was performed. Three weeks later, the patient developed symptoms of pneumonia, and a PCR test performed on the lavation fluid was shown to be positive for human herpesvirus 6 (HHV-6) and *Mycoplasma pneumoniae*. She was treated in an intensive care unit with intravenous antibiotics and BALs which were of benefit, and her condition improved. However, regular BALs were continued as a prophylactic treatment.

During the following years, her condition remained relatively stable; she was able to participate in sports activities to some extent, and the glucocorticoid treatment was gradually weaned off. At the age of 16, her pulmonary function again decreased rapidly and she developed progressive dyspnoea and poor exercise tolerance. The response to high-dose corticosteroids was poor. HRCT revealed an acute episode of PAP. BAL was performed several times and surfactant was given to prevent atelectasis after the procedure. Chest physiotherapy was also initiated to improve lung function. After 6 months, her condition had not improved and she remained dependent on supplemental oxygen. Due to a poor overall prognosis, an experimental treatment with inhaled rhuGM-CSF (sargramostim) was offered.

The treatment started with 125 μg of rhuGM-CSF in 2 ml of saline given twice a day with PARI BOY nebulizer every other week for 12 weeks. Inhalation was well tolerated. Blood leucocyte counts and liver enzymes were followed up weekly, but no adverse effects were observed during the treatment. After no more than 2 weeks on rhuGM-CSF, her lung function had significantly improved and the radiographic findings had ameliorated (Figs. 3 and 4). After three courses (1 week each) with rhuGM-CSF, supplemental oxygen was discontinued and tapering off corticosteroids was initiated. Two years after the treatment, the pulmonary function has remained normal and no relapses have occurred. The patient is currently able to participate normally in sports activities and inhaled asthma medications have been discontinued. The respiratory function before and after the rhuGM-CSF treatment is summarized in Table 2.

Patient 2

The patient was a 58-year-old woman who was diagnosed with LPI in her early childhood. Her treatment regime consisted of a protein-restricted diet (about 1 g/kg/day) together with oral L-citrulline (about 2,500 mg/day), L-lysine (750 mg/day), sodium benzoate (2,000 mg/day) and calcium and vitamin supplements. At the age of 49 years, a statin therapy was initiated for combined hyperlipidaemia, and mild renal insufficiency was diagnosed at the age of 50 years. She also had a tendency to bruising and nosebleeds. At the age of 57 years, she complained of prolonged cough and dyspnoea. A BAL was performed twice, but the symptoms persisted and the patient also had an episode of pneumonia. Radiological findings were consistent with PAP. The patient was given oral and inhaled steroids without any effect. The patient became dependent on supplementary oxygen and was subsequently hospitalized. HRCT and pulmonary function tests could not be

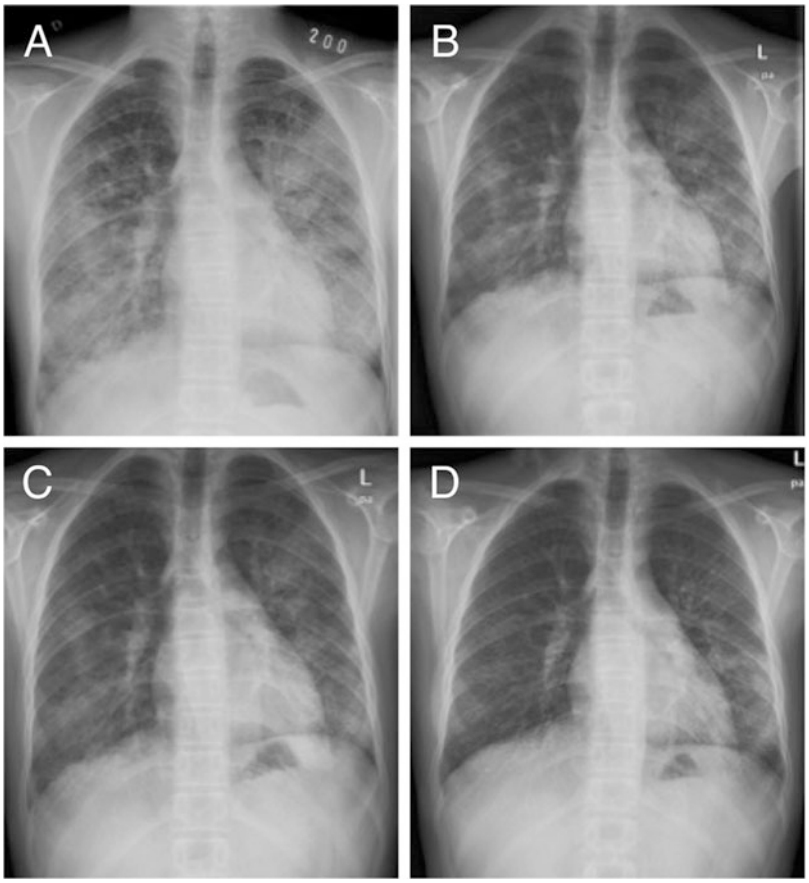

Fig. 3 Chest radiographs of patient 1 (**a**) before treatment, (**b**) after the first cycle, (**c**) after the second cycle, and (**d**) after the last (6th) cycle of sargramostim

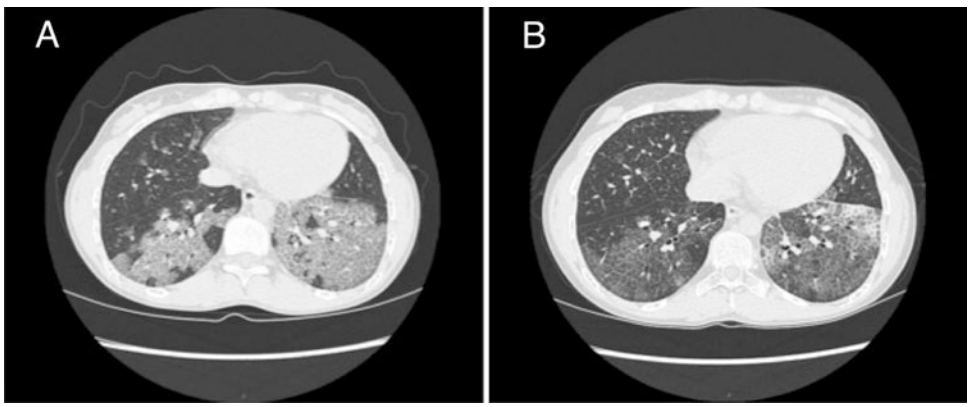

Fig. 4 Computed tomography images of patient 1 (**a**) before treatment and (**b**) after the third cycle of sargramostim

performed due to her poor general condition. Inhaled rhuGM-CSF treatment (125 μg) was initiated with the protocol described above, along with oral prednisolone (40 mg × 1), furosemide (40 mg × 1) and antibiotics (oral doximycin and intravenous moxifloxacin). The clinical condition of the patient improved enough for her to be released from hospital although still dependent on supplementary oxygen, and significant radiographic improvement was also observed (Fig. 5). Two weeks later, she was readmitted because of severe dyspnoea and elevated CRP (90 mg/L). A treatment with intravenous cefuroxime was initiated and the prednisolone dose was doubled to 40 mg × 2. A chest radiograph showed mildly increased alveolar opacity of the left lung. Five days after hospital admission, the patient developed severe epistaxis, which led to severe anaemia requiring red blood cell transfusions.

Table 2 Respiratory function of patient 1 before and after the sargramostim treatment

Date	VC	VC % Ref	FVC (L)	FVC % Ref	FEV1 (L)	FEV1% Ref	FEV1/ FVC	FEV1/FVC %Ref	PEF (L/min)	PEF % Ref
Day −82	1.69	55	1.68	53	1.59	56	94	105	7.5	133
Day −70	1.75	56	1.65	51	1.53	52	92.61	101	5.56	97
Day −69 BAL										
Day −34	1.56	51	1.52	48	1.39	49	92	102	6.06	107
Day −28	1.64	53	1.62	51	1.5	52	92	103	5.78	102
Day −27 BAL										
Day −7	1.7	55	1.59	50	1.5	52	94	105	5.89	104
Day 0	1.66	53	1.59	49	1.49	51	93.24	104	6.15	107
Day +14	1.62	52	1.62	51	1.5	53	93	104	6.52	116
Day +28	1.58	51	1.56	49	1.43	50	91	102	6.38	113
Day +42	1.71	55	1.68	53	1.55	54	92	103	6.38	113
Day +51	1.76	52	1.71	52	1.59	55	93.39	104	6.65	116
Day +78	2.08	67	1.93	60	1.73	61	90	100	6.61	117

BAL bronchoalveolar lavation, *VC* vital capacity, *FVC* forced vital capacity, *FEV1* forced expiratory volume in 1 s, *PEF* peak expiratory flow

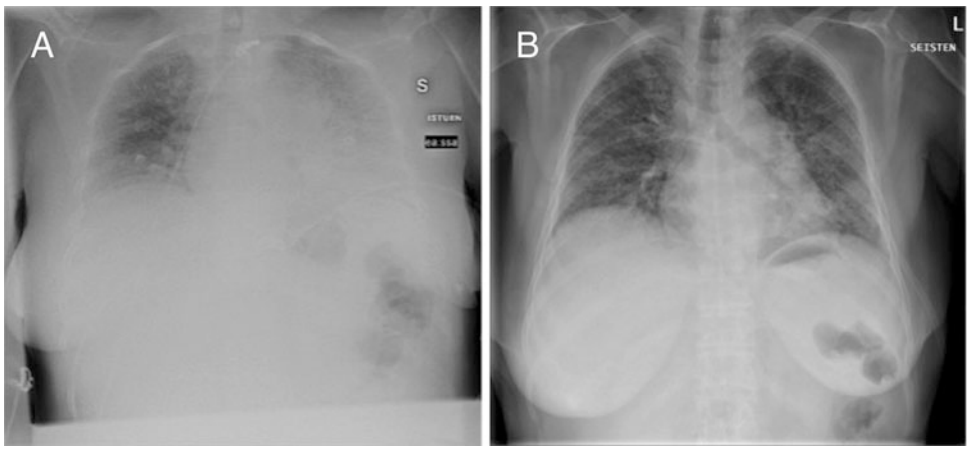

Fig. 5 Chest radiographs of patient 2 before (**a**) and after (**b**) the first cycle of sargramostim

After 2 weeks, the patient was again released from hospital as her CRP had normalized. The rhuGM-CSF dose was increased to 250 μg × 1, and inhaled corticosteroid was also initiated. With this regime, her condition seemed to remain stable. However, 2 months later she was again admitted to hospital due to severe epistaxis, and posterior tamponade was performed. Shortly afterwards, she was diagnosed with fulminant staphylococcal septicaemia, of which she subsequently died.

Discussion

Pulmonary alveolar proteinosis is a relatively poorly known clinical entity, which can develop with a variety of mechanisms including genetic defects of the GM-CSF protein or its receptor, neutralizing anti-GM-CSF antibodies, infections and malignancies (Campo et al. 2012). Patients with LPI are predisposed to secondary PAP, the prognosis of which seems to be especially poor in

paediatric patients (Parto et al. 1993, 1994). The initial symptoms of PAP may be very subtle, making the diagnosis challenging. However, the disease can rapidly progress to a life-threatening multi-organ failure, and a careful monitoring of any LPI patient with dyspnoea, unexplained fatigue or fever in the absence of other signs of an infection is therefore warranted.

Toll-like receptor signalling, especially a response to viral DNA, has been shown to be impaired, and nitric oxide (NO) production decreased in LPI monocyte-derived macrophages reinforcing the role of macrophages in the development of secondary complications of LPI (Kurko et al. 2015). We hypothesized that, in contrast to the earlier suggestions by other groups (Sebastio et al. 2011; Ogier de Baulny et al. 2012), arginine reservoirs and the subsequent NO levels may actually be diminished in LPI macrophages as a result of reduced arginine influx by the defective y^+LAT1 transporter, known to be the most important transporter of arginine in macrophages, including those of alveolar origin (Barilli et al. 2011; Rotoli et al. 2007). In addition, the defective CAA transport also results in impaired phagocytic activity of LPI macrophages (Barilli et al. 2012). This may, in turn, lead to an impaired clearance of phospholipoproteinaceous material from the alveoli. It is interesting that iNOS synthesizing NO from arginine is expressed in the airway epithelial cells where the gene encoding y^+LAT1 is also expressed (Thomassen and Kavuru 2001; Rotoli et al. 2005). Therefore, NO synthesis in the LPI airway cells could be distorted affecting, for example, the production of cytokines in alveolar macrophages. Since NO is known to be involved in several inflammatory lung diseases (Thomassen and Kavuru 2001), it would be reasonable to measure NO levels in the airway aspirate or bronchoalveolar lavage fluid of the LPI patients with PAP.

In the study by Douda and colleagues, a supplementation with surfactant protein D (SP-D) and GM-CSF increased the uptake of protein and dying cells ex vivo, but GM-CSF increased the number of spontaneously generated granulomas (Douda et al. 2009). The authors therefore suggested that GM-CSF might not be a suitable mode of treatment for patients with LPI-associated PAP. The 2-year-old LPI patient in their report had large amounts of cholesterol, cholesterol crystals and lipid-laden macrophages in the airways, and the authors suggested that therapies aiming to decrease the amount of cholesterol in the airways might be beneficial instead. On the other hand, in the study by Ohashi and colleagues, the GM-CSF inhalation therapy improved lung clearance and decreased the amount of protein in BAL fluid in a patient with autoimmune alveolar proteinosis (Ohashi et al. 2012). Furthermore, in 2014, Yu and colleagues reported a patient with autoimmune PAP treated successfully with a combination of whole lung lavage and inhaled GM-CSF (Yu et al. 2014). It is therefore logical to assume that rhuGM-CSF might also be useful in patients with LPI-associated PAP. New, noninvasive therapeutic options would be warmly welcomed as the effectiveness of corticosteroids and whole lung lavage has proven to be inadequate in this patient group. Although repeated whole lung lavage has been shown to alleviate symptoms and reduce hypoxia in PAP, it does not actually stop the progression of the underlying disease (Gao et al. 2014). Also, general anaesthesia is required to perform the whole lung lavage, which may present a challenge due to the pre-existing respiratory failure.

Our experiences based on two patient cases together with the case previously reported by Barilli et al. (2010) suggest that inhaled rhuGM-CSF may be useful in LPI-associated pulmonary alveolar patients. In patient 1, an excellent response to therapy was observed with no compliance problems or side effects. In patient 2, the role of rhuGM-CSF could not be properly evaluated due to the complicated clinical situation, but slight improvement in the pulmonary function was observed also in this patient. However, comparing the outcomes is complicated by the marked age difference between the two patients.

In conclusion, the data on these two patients suggest that inhaled rhuGM-CSF might be of benefit in patients with LPI-associated PAP responding inadequately to high-dose corticosteroids and lung lavations. Such PAP often carries a dismal prognosis, especially in paediatric patients. However, further studies on this subject are warranted.

Take Home Message

Inhaled sargramostim induces resolution of pulmonary alveolar proteinosis associated with lysinuric protein intolerance.

Contributions

LT collected the clinical data. LT, JK and HA drafted the manuscript. JK and MT performed the laboratory analyses. HA performed the electron microscopy imaging and provided Figs. 1 and 2. JM, KN-S and HN supervised the study. KN-S, HN and HL were responsible for the treatment and follow-up of the patients and provided clinical data. HL planned the treatment protocol. All the authors commented on and approved the manuscript.

Guarantor

Laura Tanner

Compliance with Ethics Guidelines

Conflicts of Interest

Laura Tanner, Johanna Kurko, Maaria Tringham, Heikki Aho, Juha Mykkänen, Kirsti Näntö-Salonen, Harri Niinikoski and Heikki Lukkarinen declare that they have no conflict of interest.

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The Ethics Committee of the Hospital District of Southwest Finland has approved the follow-up study concerning the pathogenesis of long-term complications of LPI and informed consent has been obtained from all the patients or their parents. A written informed consent was also obtained from the patients before the initiation of the experimental rhuGM-CSF treatment. 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.

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CASE REPORT

COXPD9 an Evolving Multisystem Disease; Congenital Lactic Acidosis, Sensorineural Hearing Loss, Hypertrophic Cardiomyopathy, Cirrhosis and Interstitial Nephritis

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Abstract We present the second report of combined oxidative phosphorylation deficiency-9. The infant presented in the neonatal period with poor feeding, lactic acidosis and sensorineural hearing loss. He subsequently developed a lethal hypertrophic cardiomyopathy during infancy. Cirrhosis and interstitial nephritis were identified at autopsy. Exome sequencing has detected compound heterozygous mutations in the *MRPL3* gene which encodes a large mitochondrial ribosome subunit protein. We identified a known heterozygous variant NM_007208 c.950>G (Pro317Arg) in the *MRPL3* gene and a novel heterozygous mutation NM_007208 c.49delC p.(Arg17Aspfs*57). Mutations in *MRPL3* have previously been shown to alter ribosome assembly and cause abnormal function of multiple respiratory chain complexes. Our case adds to the evolving knowledge of disorders of mitochondrial translation.

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Introduction

Mitochondrial disorders comprise a clinically and genetically diverse group of diseases affecting cellular energy production through mitochondrial oxidative phosphorylation. Mutations in mitochondrial DNA and nuclear genes encoding the proteins which comprise the five complexes of the mitochondrial respiratory chain are well characterised. The mechanisms and disorders of mitochondrial DNA maintenance, replication and transcription are less well understood. Mitochondrial translation involves greater than one hundred proteins which are nuclear gene encoded (Pearce et al. 2013; Ottl et al. 2016).

The mitochondrial ribosome (mitoribosome) synthesises 13 components of the mitochondrial oxidative phosphorylation complex and consists of over 80 interconnected proteins. Mitoribosomes are composed of a large (39S) subunit containing a 16S ribosomal RNA (rRNA) and a small (28S) subunit containing a 12S rRNA. The small subunit contains approximately 30 proteins and the large subunit contains approximately 50 proteins (Pearce et al. 2013).

To date, pathogenic mutations in only six genes encoding mitochondrial ribosomal proteins have been reported in association with clinical phenotypes (see Table 1), i.e. *MRPL3* gene (MIM 607118, phenotype combined

Table 1 Mitochondrial ribosomal disorders: major features

Gene	*MRPL3*	*MRPS7*	*MRPS16*	*MRPS22*	*MRPL44*	*MRPL12*
Sensorineural hearing loss	1/5	2/2	0/1	0/5	0/4	0/1
Lactic acidosis	5/5	2/2	1/1	5/5	3/4	1/1
Structural brain anomalies	2/5 cortical hypergyria	0/2	Corpus callosal agenesis, ventricular dilatation	2/5 corpus callosum and other abnormalities (3 not imaged)	1/4	1/1
Cardiomyopathy	5/5	0/2	0/1	4/5	4/4	0/1
Hepatic involvement	4/5	1/2	1/1	1/5 (multiple organ dysfunction)	4/4	1/1 (hepatomegaly)
Renal involvement	1/5	2/2	0/1	4/5 (tubulopathy, multiple organ dysfunction)	1/4	0/1
Developmental delay	2/5	0/2	Neonatal demise	1/1 surviving the neonatal period	1/4	1/1
Lethality	3/5	2/2	1/1	4/5	1/4	1/1
Reference	3 and current case	9	7	8,10,11	5,6	4

oxidative phosphorylation deficiency-9 (COXPD9 MIM 614582) (Galmiche et al. 2011), *MRPL12* (MIM602375) (Serre et al. 2013), *MRPL44* (MIM641849, phenotype COXPD16 MIM615395) (Carroll et al. 2013; Distelmaier et al. 2015), *MRPS16* (MIM 610498) (Miller et al. 2004), *MRPS22* (MIM605810; phenotype COXPD5 MIM 611719) (Smits et al. 2011; Baertling et al. 2015) and *MRPS7* (MIM 611974)) (Menezes et al. 2015). Pathogenic mutations in *MRPL3* are responsible for the clinical phenotype of COXPD9 in which severe hypertrophic cardiomyopathy is a central presentation. Our patient expands the phenotype of COXPD9 to also include neonatal lactic acidosis, sensorineural hearing loss (SNHL), cirrhosis and interstitial nephritis.

Case Report

Our patient is a male infant born at 39 weeks' gestation via elective lower uterine segment caesarean section. Apgar scores were 9 at 1 and 5 min post birth. His initial physical exam was unremarkable and he was transferred to the postnatal ward. Over the ensuing 24 h, he was noted to be lethargic and feed poorly and hypoglycaemic via glucometer testing. Formal bloods demonstrated a true blood glucose of 1.4 mmol/L and a metabolic acidosis with a bicarbonate level of 7 mmol/L and an elevated anion gap of 28. Subsequent routine bloods revealed mildly deranged transaminases which peaked on day 4 (gamma-glutamyl transferase GGT 280 U/L (0–70), alanine aminotransferase ALT 164 U/L (0–45), aspartate aminotransferase AST 941 U/L (0–41)), lactate of 10.7 mmol/L, blood pyruvate 563 umol/L and an elevated lactate/pyruvate ratio of 19. Creatine kinase was mildly elevated at 580 U/L (0–190). A urine metabolic screen (organic acids and amino acids) demonstrated lactic acid, ketoacidosis and mildly elevated pyruvate and fumarate, all suggestive of mitochondrial dysfunction. Magnetic resonance imaging (MRI) scan in the neonatal period revealed a structurally normal brain, and magnetic resonance spectroscopy (MRS) demonstrated a lactate peak in the basal ganglia. The infant was commenced on a trial of thiamine and co-enzyme Q10 supplementation which reduced the lactic acidosis from a peak of 16.1 mmol/L pre-therapy to a range of 4–5 mmol/L within 1 week post-therapy.

The patient failed newborn hearing screening in both ears and was referred for formal audiological assessments. Physiological testing (optoacoustic emissions, auditory brain stem response, auditory steady-state response) confirmed a severe sloping bilateral SNHL, and he was fitted with bilateral Naida Q 70SO hearing aids at 1 month of age. At 7 months of age, he was reassessed as part of the cochlear implant candidacy programme. Tympanography demonstrated normal middle ear pressures and compliance. Physiological testing with and without bone conductors again confirmed a severe sloping audiogram in keeping with profound SNHL. Bilateral cochlear implants were inserted at 9 months of age without incident. At this point in time, he was progressing normally from a developmental perspective although his growth was suboptimal.

Echocardiography in the newborn period showed a structurally normal heart with a patent foramen ovale and normal left ventricular function. Follow-up echocardiography at 8 months of age revealed hypertrophic cardiomyopathy, with the myocardial thickness having increased from 3 mm in the newborn period to 7 mm (z-score = +2.6).

Systolic function remained normal and there was no left ventricular outflow tract obstruction.

At the age of 11 months, the patient developed bronchiolitis and rapidly deteriorated, with hypotension, hypoglycaemia and severe metabolic acidosis. Cardiac arrest ensued and he was unable to be resuscitated. An autopsy was conducted, with the cause of death thought to be cardiac decompensation due to an unidentified respiratory virus. Microscopic examination of the liver revealed mild periportal steatosis and mild lymphoplasmacytic infiltration with minimal fibrosis. A chronic inflammatory cell infiltrate surrounded the renal tubules in keeping with evolving interstitial nephritis. The glomeruli were normally formed. Cardiac histology demonstrated cardiac muscle fibre disarray and cytomegaly in keeping with hypertrophic cardiomyopathy. Myocardial thickness had increased to 15 mm.

Genetic testing for common mitochondrial DNA point mutations and deletions including the three common *POLG* mutations (MIM174763) was negative. Subsequently next-generation sequencing was performed at the Australian Genome Research Facility. After enrichment of all the coding and flanking intronic regions, sequencing analysis was performed using an Illumina HiSeq platform. Quality criteria required at least 10 reads per base; however, 97.7% of targeted regions achieved ×100 coverage and 99.7% achieved ×10 coverage. Only sequence variations with an allele frequency <1% were considered pathogenic. The following *MRPL3* sequence variants were identified, NM_007208 c.950C>G (Pro317Arg) and NM_007208 c.49delC p.(Arg17Aspfs*57). These mutations were confirmed by Sanger sequencing. The patient's father was found to be heterozygous NM_007208 c.950C>G (Pro317Arg) and his mother heterozygous NM_007208 c.49delC p.(Arg17Aspfs*57). No other candidate genes were identified. There were no sequence variants identified in genes known to be associated with SNHL.

The *MRPL3* NM_007208 c.950C>G p.(Pro317Arg) variant is located on chromosome 3 at position 131,181,664 bp. It overlaps the coding sequence of at least one transcript of gene *MRPL3*. The reference allele for this variant is G, whereas the alternative allele is C. This variant is predicted to be a missense mutation which alters the protein's amino acid from proline (Pro) to arginine (Arg). The prediction for p.(Pro344Arg)/p.(Pro86Arg)/p.(Pro212Arg)/p.(Pro317Arg) is based on four annotated transcripts for that gene locus. The BLOSUM62 substitution matrix reports a score of −2 for this alteration. The mutation has been described in patients with COXPD9 deficiency (Galmiche et al. 2011) and classified as pathogenic (ID RCV000023618). There was no alternative allele frequency listed for this variant in the 1,000 genomes data set. There was no minor allele frequency listed for this variant in the NHLBI GO Exome Sequencing Project (ESP6500) data set. This variant overlaps with evolutionary constrained element (detected using SiPhy-ω and SiPhy-π statistics). The conservation across 28 species is described with PhyloP (score: 2.44). GERP identifies constrained elements in multiple alignments by quantifying substitution deficits (score: 5.26).

The *MRPL3* NM_007208 c.49delC p.(Arg17Aspfs*57) heterozygous deletion is located on chromosome 3 at position 131,221,616 bp. It overlaps the intronic region of gene *MRPL3*. It overlaps the coding sequence of at least one transcript of gene *MRPL3*. The reference allele for this variant is CG, whereas the alternative allele is C. This variant is predicted to be a nonsense mutation which alters the protein's amino acid sequence and leads to a premature stop codon. The prediction for p.(Arg17Aspfs*57) is based on one annotated transcript for that gene locus. This variant is predicted to be a nonsense mutation which alters the protein's amino acid sequence and leads to a premature stop codon. The variant was not previously reported in dbSNP. There has been no clinical classification available for this novel variant. There was no alternative allele frequency listed for this variant in the 1,000 genomes data set. There was no minor allele frequency listed for this variant in the NHLBI GO Exome Sequencing Project (ESP6500) data set. The amino acid substitution is predicted to be damaging (SIFT score: −1.00). The accuracy of these tools is unknown. Variant overlaps with evolutionary constrained element (detected using SiPhy-ω and SiPhy-π statistics). The conservation across 28 species is described with PhyloP (score: 2.31). GERP identifies constrained elements in multiple alignments by quantifying substitution deficits (score: 3.61).

Discussion

This is only the second report of an individual with COXPD9 due to pathogenic *MRPL3* mutations. The previous cases were four siblings from the same family (Galmiche et al. 2011), who are compound heterozygotes for a maternally derived missense mutation (identical to one detected in our patient) and a paternally inherited 255 kb deletion (Galmiche et al. 2011). The clinical features of the five known COXPD9 patients are detailed in Table 2. Persistent elevation of hepatic transaminases is reported in 4/5. All of the COXPD9 patients have demonstrated failure to thrive and a lactic acidosis, although our patient is the only one noted to have presented with a lactic acidosis in the neonatal period. Our current report expands the clinical spectrum as he is the only COXPD9 patient to have SNHL diagnosed during life, with hepatic cirrhosis and interstitial nephritis described at autopsy. The renal manifestations of

Table 2 Compares the features of the case described here with the four siblings (patients 1–4) previously described by Galmiche et al. in 2011

	Case 1	Case 2	Case 3	Case 4	Current case
HCM age at diagnosis	11 months	12 months	9 months	9 months	8 months
Hepatomegaly	+		+	+	
Elevated liver enzymes	+		+	+	+
Lactic acidosis	+	+	+	+	+
FTT	+	+	+	++	+
SNHL					+ (cochlear implant)
MRI/MRSbrain	Not reported	Not reported	Cortical hypergyria	Cortical hypergyria	Lactate peak
MRPL3 genotype	c.950>G (Pro317Arg) 255 kbp contiguous gene deletion	c.950>G (Pro317Arg) 255 kbp contiguous gene deletion	c.950>G (Pro317Arg) 255 kbp contiguous gene deletion	c.950>G (Pro317Arg) 255 kbp contiguous gene deletion	c.950>G (Pro317Arg) c.49delC p.(Arg17Aspfs*57)
MRC studies	Decreased complex I, IV, V	Decreased complex I, IV, V	Decreased complex I, IV	Decreased complex I, IV, V	Not performed
Outcome	Cardiac death at 17 months	Cardiac death at 15 months	Alive at 3 years, stable cardiomyopathy, failure to thrive, psychomotor retardation	Alive at 3 years, stable cardiomyopathy, severe failure to thrive, psychomotor retardation	Cardiac death at 11 months. Cirrhosis and interstitial nephritis
Additional findings at autopsy					Hepatic cirrhosis Interstitial nephritis

HCM hypertrophic cardiomyopathy, *FTT* failure to thrive, *SNHL* sensorineural hearing loss, *MRI* magnetic resonance imaging, *MRS* magnetic resonance spectroscopy, *MRC* mitochondrial respiratory chain, + present

mitochondrial diseases most commonly involve glomerular (e.g. focal segmental glomerulosclerosis, nephrotic syndrome) or tubular pathology (e.g. Fanconi syndrome, renal tubular acidosis) (Emma et al. 2012; O'Toole 2014). However interstitial nephritis has been reported in a range of mitochondrial disease including mtDNA defects (e.g. the common MELAS mutation m.A3243G in the *MTTL1* gene (MIM 590050)), respiratory chain assembly factors (e.g. the complex-III assembly factor *BCSL1* (MIM 603647)), defects of post-translational modification of mitochondrial aminopeptidases (e.g. *XPNPEP3* MIM613513) and most commonly disease processes associated with elevated excretion of methylmalonic acid (Emma et al. 2012; O'Toole 2014). While the interstitial nephritis in our patient was identified at autopsy, he was not exposed to any known medications or infectious agents associated with interstitial nephritis. We postulate that his evolving multisystem disease process would have included a significant renal burden if his cardiac disease hadn't intervened.

COXPD-9 deficiency due to pathogenic mutations in the *MRPL3* gene can be predicted to produce a severe multisystem disorder as observed in our patient. The advent of accessible next-generation sequencing meant that we didn't move to mitochondrial respiratory chain biopsies in our patient. The original report by Galmiche et al. demonstrated a combined decrease in activity of mitochondrial respiratory complexes I, III, IV and V, with a mild decrease in complex II on fibroblasts and muscle samples. This highlights the critical role of MRPL3 in the early steps of mitochondrial translation. Galmiche et al. also demonstrated alteration in the stability of MRPL3 and defective assembly of the large ribosomal subunit resulting from the *MRPL3* NM_007208 c.950C>G p.(Pro317Arg) variant.

The mitoribosome is a protein complex which is active within the mitochondria whose function is to facilitate the translation of mtDNA-encoded proteins. Thus the mitoribosome synthesises 13 components of the mitochondrial oxidative phosphorylation complex, which contribute to all of the mitochondrial respiratory chain complexes with the exception of complex II. At the time of writing, there are 18 patients reported with clinical phenotypes secondary to mitochondrial ribosomal subunit mutations in the *MRPL3*, *MRPL12*, *MRPL44*, *MRPS16*, *MRPS22* and *MRPS7* genes, which are summarised in Table 1. They share common

clinical features to our patient including cardiomyopathy, lactic acidosis and renal involvement. Cardiomyopathy is a common component to the phenotype mitochondrial ribosomal protein defects, having been present in all of the reported *MRPL3* and *MRPL44* cases and almost all of the reported *MRPS22* cases (Galmiche et al. 2011; Serre et al. 2013; Carroll et al. 2013; Distelmaier et al. 2015; Saada et al. 2007; Smits et al. 2011; Baertling et al. 2015). The mitochondrial ribosomal subunit disorders appear to have a severe phenotype, with death in the neonatal period in five cases and before 3 years in a further five patients (including our case). Of the remaining eight patients, one succumbed to hepatic failure aged 14, one was very severely disabled aged 5.5 years, and two had significant impairment at age 3 (Serre et al. 2013; Carroll et al. 2013; Distelmaier et al. 2015; Miller et al. 2004; Saada et al. 2007; Menezes et al. 2015; Smits et al. 2011; Baertling et al. 2015).

Perturbations in the mitoribosome translational machinery produce significant disruption to mitochondrial function, which is manifested in the severe and often lethal clinical phenotypes as demonstrated by the current cohort of COXPD9 deficiency patients.

Compliance with Ethics Guidelines

Conflict of Interest

David Coman, Carolyn Bursle, Anna Narendra, Raymond Chuk, Bruce Lewis, Rob Justo, and John Cardinal declare that they have no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (Carroll et al. 2013). Informed consent was obtained from all patients for being included in the study.

Author Contributions

Dr. Carolyn Bursle has driven the manuscript development and been involved in patient care.

Dr. Anna Narendra has driven the manuscript development and been involved in patient care.

Dr. Raymond Chuk has driven the manuscript development and been involved in patient care.

Dr. Bruce Lewis is a senior paediatrician coordinating the patients care and contributed to the manuscript development.

Dr. Rob Justo is a paediatric cardiologist involved in the patient's care and contributed to the manuscript development.

Dr. John Cardinal is a medical scientist who has been involved in the manuscript development.

Professor David Coman is a metabolic physician coordinating care of patient and has coordinated the manuscript development and design.

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RESEARCH REPORT

Incidence and Geographic Distribution of Succinic Semialdehyde Dehydrogenase (SSADH) Deficiency

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Abstract The incidence of succinic semialdehyde dehydrogenase (SSADH) deficiency, an autosomal recessive inherited disorder of GABA degradation, is unknown. Upon a recent diagnosis of a new family of affected fraternal twins from the Punjabi ethnic group of India, case ascertainment from the literature and our database was done to determine the number of confirmed cases along with their geographic distribution. The probands presented with global developmental delay, infantile onset epilepsy, and a persistent neurodevelopmental disorder upon diagnosis at 10 years of age with intellectual disability, expressive aphasia, and behavioral problems most prominent for hyperactivity. Gamma-hydroxybutyric aciduria and homozygous ALDH5A1 c.608C>T; p.Pro203Leu mutations were confirmed. Identification of all available individual cases with clinical details available including geographic or ethnic origin revealed 182 patients from 40 countries, with the largest number of patients reported from the USA (24%), Turkey (10%), China (7%), Saudi Arabia (6%), and Germany (5%). This study provides an accounting of all published cases of confirmed SSADH deficiency and provides data useful in planning further studies of this rare inborn error of metabolism.

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Summary Statement: This study provides the worldwide geographic distribution of SSADH deficiency from all identified published cases and subjects in the investigators' database.

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Introduction

Succinic semialdehyde dehydrogenase (SSADH) deficiency (OMIM 271980, 610045) is a rare autosomal recessive disorder of GABA degradation initially described in 1981 (Jakobs et al. 1981). GABA-transaminase (GABA-T) converts GABA to succinic semialdehyde, which is metabolized to succinic acid by SSADH. In SSADH deficiency, there is an excessive buildup of GABA and γ-hydroxybutyrate (GHB). High levels of GABA and GHB have been implicated in the neurological manifestations of SSADH deficiency, and recent evidence suggests multiple metabolic perturbations may be associated with the pathophysiology including markers of oxidative stress, dysregulation of autophagy including the mTOR pathway, and accumulation of the semialdehyde intermediate.

SSADH deficiency does not typically feature intermittent deterioration or neurodevelopmental regression and may be relegated to a nonprogressive encephalopathy or atypical neurobehavioral syndrome with either a missed diagnosis or identification late into adulthood (Lapalme-Remis et al. 2015). We report affected twins from the second family reported from India and the first since the early report of three affected siblings from 1997 that added

23 new cases to the literature and described the phenotype (Gibson et al. 1997). We subsequently reviewed the literature to identify all reported cases since the initial description of urinary gamma-hydroxybutyric aciduria (Jakobs et al. 1981) and added patients from our database with a confirmed diagnosis.

Methods

Clinical and genetic confirmation of SSADH deficiency of a Punjabi family from Northern India came to our attention as the second affected family reported from India and the first in 20 years. We reviewed the literature and our database for confirmed cases of SSADH deficiency and report on the geographic origin of reported patients. Collection and reporting of subjects from our database was approved by the Boston Children's Hospital Institutional Review Board.

Case Reports and Results

Ten-year-old dizygotic twin boys born to a North Indian couple of the Punjabi ethnic group presented for evaluation of developmental disability and a history of seizures. There was no known history of consanguinity or family history of neurodevelopmental disorders.

Twin A presented with cognitive impairment, profound deficit of expressive language, and incoordination. Behavior was notable for hyperactivity and, at times, aggressiveness. Developmental assessment indicated moderate intellectual deficiency with WISC-III Full Scale IQ measurement of 43, gross motor skill level approximating age 4 years, and fine motor skills at 14 months. Activities of daily living were affected such that the patient could not dress independently or drink from a cup without spillage. The proband had onset of convulsive seizures at 3 months of age. Delayed developmental with hypotonia was noted then. The past medical history disclosed no gestational or perinatal problems, but acquisition of developmental milestones was notable for head control at 12 months, independent sitting 30 months, independent standing at 36 months, and walking at 42 months. On neurological examination, the sparse vocabulary used was very inarticulate and there was decreased attention. Motor examination revealed hypotonia and chorea, and deep tendon reflexes were hypoactive.

Twin B similarly presented with cognitive impairment and hyperactive behavior. Developmental assessment showed moderate intellectual disability with a WISC-III Full Scale IQ measurement of 38 and impairment of motor coordination along with hyperactive behavior. This patient had infantile onset of convulsive seizures at 3 months of age, treated with phenobarbital and without a recurrence since the age of 4 years. Phenobarbital was tapered off after two seizure-free years. Levetiracetam was initiated recently when the breakthrough seizure occurred in the twin brother.

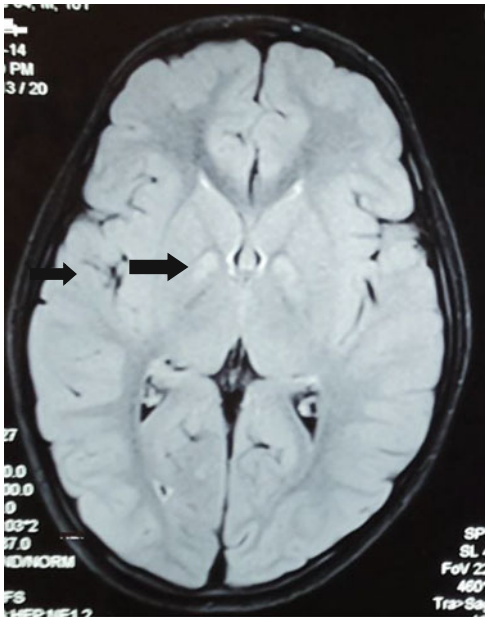

Fig. 1 Magnetic resonance imaging of the brain. Axial MRI FLAIR sequence with hyperintensities of globus pallidi (*arrowhead*)

There had been no gestational or perinatal complications. The developmental history similarly showed that hypotonia was noted in infancy at the time of seizure onset and there was delayed acquisition of milestones. Neurological examination showed minimal expressive and disarticulate language, hypotonia, and hyporeflexia.

Brain MRI was obtained in Twin A and showed symmetrical hyperintensities of the globus pallidi on T2-weighted and FLAIR sequences (Fig. 1). Urine was sent on both twins for organic acid analysis, and gas chromatography-mass spectrometry (GC-MS) revealed significantly increased levels of 4-hydroxybutyric acid and 4,5-dihydroxyhexanoic acid. These findings led to ALDH5A1 sequencing which revealed homozygous c.608C>T; pPro203Leu mutations, previously described as disease causing, in both subjects. The parents were confirmed as heterozygous as were two siblings.

Case ascertainment dating back to the initial report of gamma-hydroxybutyric aciduria identified 91 unique patients with clinical details provided (Aoshima et al. 2002; Bekri et al. 2004; Brown et al. 1987; Dayan et al. 2006; Deng et al. 2011; Divry et al. 1983; Escalera et al. 2010; Gogou et al. 2016; Haan et al. 1985; Ishiguro et al.

Table 1 Ethnicity/geographic distribution of reported SSADH deficiency cases

Country	Total
United States	43
Turkey	18
China	13
Saudi	10
Germany	9
Australia	7
Netherlands	7
UK	7
Pakistan	6
Greece	5
Spain	5
Iran	4
Japan	4
India	3
Ireland	3
Israel	3
Italy	3
Afghanistan	2
Argentina	2
Bulgaria	2
Canada	2
France	2
Korea	2
Lebanon	2
Lifu	2
Taiwan	2
Albania	1
Algeria	1
Belgium	1
Denmark	1
Inuit	1
Luxemburg	1
Malaysia	1
Sicily	1
Sweden	1
Syria	1
Tunisia	1
UAE	1
Uruguay	1
Yemen	1
Total	182

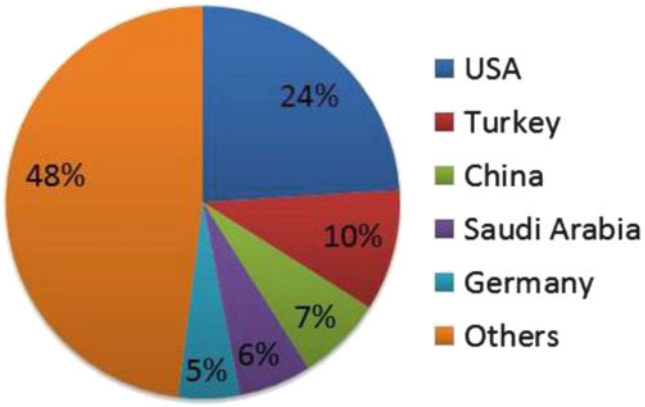

Fig. 2 Countries reporting cases of SSADH deficiency ($N = 40$)

2001; Jakobs et al. 1981; Jiang et al. 2013; Kratz 2009; Kwok et al. 2012; Lemes et al. 2006; Li et al. 2015; Lin et al. 2015; Liu et al. 2016; Neu et al. 2002; Niemi et al. 2014; O'Rourke et al. 2010; Onkenhout et al. 1989; Pearl et al. 2003; Peters et al. 1999; Puttmann et al. 2013; Racaru et al. 2010; Rashed et al. 1994; Rating et al. 1984; Saronwala et al. 2008; Spilioti et al. 2013; Tay et al. 2015; Wang et al. 2016; Yamakawa et al. 2012; Zhao et al. 2003; Ziyeh et al. 2002). We furthermore identified 91 additional subjects with confirmed SSADH deficiency in our database published in aggregate (Parviz et al. 2014). Overall, we were able to identify 182 unique cases of SSADH deficiency. The ethnicity of the patients is shown in Table 1; when only geographic residence was provided, this was used instead. There were a total of 40 ethnicities or countries represented in the patient population (Fig. 2). The countries with the greatest number of patients reported were the USA (24%), Turkey (10%), China (7%), Saudi Arabia (6%), and Germany (5%). Together, these five countries accounted for approximately half of all reported patients.

Discussion

The median age at diagnosis of SSADH deficiency is 2 years, but underdiagnosis is suspected. Nearly 80% of reported patients are diagnosed by the age of 5 years although 10% of patients are diagnosed after the first decade (Lapalme-Remis et al. 2015). An adult was recently diagnosed at age 62 during a terminal illness marked by repeated status epilepticus, although his family described a typical childhood course yet without a specific diagnosis or suspicion of a metabolic disorder (Lapalme-Remis et al. 2015). The phenotype typically is neurodevelopmental impairment with intellectual deficiency, marked expressive language impairment, and epilepsy. Typical neurological features include hypotonia, ataxia, and hyporeflexia with some patients manifesting chorea. The neuropsychiatric profile includes often disabling obsessive compulsive disorder and anxiety in addition to inattention, hyperactive behavior, and sleep disturbances (Gibson et al. 2003; Pearl et al. 2003).

This is the first report of a family from India since the relatively large series of patients that added 23 new cases to the literature in 1997 (Gibson et al. 1997). The previously reported family had three affected children, the oldest diagnosed posthumously based on a consistent phenotype with death at 13 years. The siblings in that family were diagnosed at ages 9 and 3 years, with laboratory detection using urine organic acids and confirmation based on enzyme activity determination.

The twins in the present report had a delay in diagnosis to 10 years of age and otherwise presented with delay of acquired infantile milestones, early hypotonia, intellectual deficiency, and marked impairment of expressive language. The infantile onset of epilepsy with remission in early childhood is less typical but does occur. Epilepsy often appears during later childhood or adolescence and is more prevalent in the adult than pediatric cohort (Lapalme-Remis et al. 2015). The findings on examination of hypotonia, chorea, and hyporeflexia are typical of the disorder. Globus pallidus hyperintensities on T2-weighted MRI and gamma-hydroxybutyric aciduria along with elevated 4,5-dihydroxy-hexanoic acid are diagnostic markers of the disorder (Pearl et al. 2009). Epileptiform discharges on EEG tend to be generalized but focal spikes have been described (Pearl et al. 2009). More than 35 ALDH5A1 mutations including missense, nonsense, and splice mutations have been reported. The c.608C>T; p.Pro203Leu mutation has been previously reported and affects an amino acid highly conserved across all known mammalian species and was undetected in approximately 1,300 control alleles (Akaboshi et al. 2003).

We were able to identify 182 subjects with SSADH deficiency based on the literature and our database, from 40 countries with five countries reporting half of all patients. The nonspecific phenotype of the disorder, usually following the pattern of a nonprogressive encephalopathy that may have superimposed epilepsy, mandates a high index of clinical suspicion for a metabolic disorder, and we suspect these are the countries with centers more likely to pursue metabolic diagnostic studies in this clinical scenario. Underdiagnosis, late diagnosis, and disproportional geographic representation are common as with other neurometabolic disorders.

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