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RESEARCH REPORT

Difficulties in Daily Life and Associated Factors, and QoL of Children with Inherited Metabolic Disease and Their Parents in Japan: A Literature Review

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Abstract To assess the quality of life (QoL) of children in Japan with inborn errors of metabolism (IEM) as well as of their parents, we reviewed 23 previous studies published in Japanese and 1 published in English, focusing on the difficulties they encounter in daily life, the factors associated with these difficulties, and their QoL. We divided the difficulties and associated factors into three developmental stages. At the infant stage, individuals with IEM tend to be at high risk of hypercatabolism. Their parents suffered anxiety and distress because of the child's diet therapy and regarded the parents' support group as an essential presence, particularly given that IEM is a rare disease. At the school-age stage, as their sphere of social relationships expanded, children with IEM became nervous about being compared with healthy children of their own age because of their diet therapy. At the adolescence-to-adulthood stage, the children suffered medically, economically, and socially. Even in the absence of any IEM symptoms, the children's QoL was affected by the demands associated with the metabolic disorder, such as diet and treatment. The psychological health of their caregivers was also poor. To improve the QoL of children with IEM and of their parents, future comprehensive quantitative and qualitative studies of their QoL and of their subjective support needs are required. Additionally, the specific factors related to the QoL of such individuals need to be explored in large population-based statistical studies.

Abbreviations

AA	Argininosuccinic aciduria
CD	Citrin deficiency
GA	Glutaric acidemia
HM	Hypermethioninemia
IA	Isovaleric acidemia
IEM	Inborn error of metabolism
MPS	Mucopolysaccharidosis
MSUD	Maple syrup urine disease
OA	Organic acidemia
PA	Propionic acidemia
PKU	Phenylketonuria

Introduction

According to the Japanese Ministry of Health Labour and Welfare (2013), the number of patients in Japan with inborn errors of metabolism (IEM) was about 19,000 in 2013. The Ministry has been supporting several studies on the diagnosis and treatment of IEM (The Japanese Ministry of Health Labour and Welfare 2015). As a result of the expansion of the newborn tandem mass screening test, studies not only on diagnosis and treatment but also on the

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psychological and social aspects of the parents have been conducted (Sakoda et al. 2011; Abe 2012). At the same time, researchers have highlighted the importance of how patients with IEM and their families should be supported after diagnosis (Matsubara 2010).

Most patients with IEM have to follow a strict diet therapy throughout their life and to live with the permanent risk of metabolic crises (Zeltner et al. 2014). The diet therapy may affect many aspects of their daily life and quality of life (QoL), as well as of their family members'. In countries other than Japan, some studies have been conducted on the daily life and QoL of patients with IEM and of their parents (Lambert and Boneh 2004; Simon et al. 2008; Stockler et al. 2012; Cazzoria et al. 2014). As one of the factors associated with their QoL, children with IEM in Turkey with bad diet compliance reportedly had lower scores in two aspects of QoL assessment: school labeling and perception of disease (Eminoglu et al. 2013). However, only a few studies in Japan have been conducted and these focused mainly on the patients' and on their mothers' QoL (Kubo et al. 2008; Okano et al. 2013).

In this study, we sought to understand the problems encountered in the daily lives of Japanese children with IEM and of their parents by reviewing what has already been investigated in terms of their difficulties in daily life and the factors associated with those difficulties, and in terms of their QoL. And we considered future issues that need to be tackled to improve the QoL of these patients and their families.

Methods

Data Sources and Search Strategies

To identify eligible articles for this literature review, we conducted a search of the PubMed database and of the most popular database in the medical discipline in Japan – the Japan Medical Abstracts Society (version 5). The search was conducted using Japanese and English keywords such as "inborn error of metabolism," the names of particular types of IEM, and "daily life" or "QoL." At the initial search in March 2015, we retrieved 6,692 publications from the two databases. After reading the titles and abstracts, we excluded those articles focusing only on the diagnosis or the medical treatment or that were not about Japanese patients. After that, we conducted an additional search using quotations within quotations to ensure the comprehensiveness of the review. So as not to omit any reports on IEM studies in Japan, we included not only original articles but also feature articles and proceedings. Finally, we targeted 24 publications from 1987 to 2014. Only one of the articles was written in English, and the rest were written in Japanese.

Data Analysis

We intensively read the 24 articles and focused on the results related to difficulties faced by children with IEM and by their parents in daily life, the factors associated with those difficulties, and their QoL. We classified the results according to the child's developmental stages: infant stage, school-age stage, and adolescence-to-adulthood stage. And we summarized what has already been investigated in terms of their QoL. Finally, we considered future issues that need to be addressed to improve the QoL of these children and their parents.

Results

Table 1 shows the reviewed articles, and Fig. 1, the difficulties reported therein as experienced by children with IEM and their parents with regard to daily life, as well as the associated factors at each stage of the child's life. We have added the medical and clinical aspects of IEM to provide a more complete picture of their life. Other than those on MPS, most of the studies included only very small numbers of families, with many including only one family.

Difficulties in Daily Life and Associated Factors

Infant Stage

Newborn Mass Screening, Specialized Examinations, and Diagnosis In Japan, newborns who test positive for IEM on newborn tandem mass screening have to undergo several specialized examinations such as amino acid analysis, gas chromatography and mass spectroscopy (GC/MS), enzyme assay, and gene analysis. Doctors specializing in IEM then make a diagnosis (Endo et al. 2013).

Infants at High Risk of Hypercatabolism and Parents' Difficulty in Preventing the Child's Hypercatabolism Generally speaking, infants easily contract infections; thus, they are at high risk of hypercatabolism. In one case of maple syrup urine disease (MSUD), the child was admitted to hospital 30 times before the age of 10 years because of hypercatabolism caused by infections accompanied by diarrhea and fever (Fujiwara 2013). It is difficult to prevent infants with IEM from becoming sick in daily life. One mother of a child with *propionic acidemia (PA)* could not recognize the signs of severe acidosis in her child because she thought the child was just having another cold (Taketa et al. 1991). Families are also concerned about the risk of sickness for their child (Fujiwara 2013) because IEM causes, among others, hypercatabolism, psychomotor retardation, and intellectual disability (Yuhara et al. 1991).

Table 1 List of 24 eligible articles

Author (s)	Article type	Metabolic disease	*N*	Life stage[a]	Summary of articles focused on difficulties in daily life and associated factors, and QoL
Abe (2012)	Proceedings	PKU	1[b]	1	Importance of cooperation with families in caring for children
Fujiwara (2013)	Feature article	PKU, MSUD	5[b]	1, 2, 3	Difficulty in prevention of hypercatabolism caused by child's sickness, sharing their anxiety and distress related to raising children with IEM, and having people around them understand their child's diet therapy; importance of a parents' support group and genetic counseling to cope with anxiety and distress, worries about next pregnancy, and financial difficulties from adulthood related to diet therapy
Ishiyama et al. (1987)	Original article	PKU	4[b]	1, 2	Difficulty of management of diet therapy with developmental stages; psychological burden of child's diagnosis and diet therapy
Kashiwagi (2011)	Proceedings	OA	N/A	1, 2, 3	Parents' anxiety and distress related to home-rearing of children with IEM; lack of information on disease and treatment; social discrimination such as refusal of permission to enter nursery school
Kashiwagi (2012)	Proceedings	IMD	N/A	1, 2, 3	Difficulty associated with sharing their anxiety and distress about raising a child with IEM; difficulties with diet therapy; financial difficulties from adulthood related to diet therapy
Kato and Kashiwagi (2010)	Original article	MMA, PA, GA, IA, AA,	19[c]	N/A	Suffering from social discrimination when taking out insurance
Komatsu et al. (2011)	Original article	N/A	16[c]	N/A	Suffering from social discrimination when taking out insurance
Kubo (2007)	Original article	MPS	4[b]	1, 2	Positive impact of school as a chance to acquire the fundamental habit of daily life and to reduce mothers' stress and fatigue
Kubo (2010)	Original article	MPS	94(4)[b]	N/A	Lack of information on MPS; guilt feelings associated with the child's disease
Kubo and Tamura (2007)	Original article	MPS	11[b]	1, 2	Lack of information on MPS or child's care; necessity of a parents' support group for gathering information about MPS
Kubo et al. (2008)	Original article	MPS	94(10)[b]	1	Delayed diagnosis caused by medical professionals' lack of knowledge of MPS
Kubo and Tazaki (2008a)	Original article	MPS	94[b]	N/A	General Health Questionnaire 30: QoL of 28- to 70-year-old caregivers of children with MPS; the mean QoL score was higher than that for healthy parents, indicating poor psychological health; the associated factor of QoL was bathing assistance ($\beta = 0.363$, $r = 0.049$)
Kubo and Tazaki (2008b)	Original article	MPS	94[b]	N/A	General Health Questionnaire 30: QoL of 28- to 71-year-old caregivers of children with MPS; the significant factors of QoL were bathing assistance ($r = 0.465$) and impossibility of communication with their children ($r = 0.38$)
Manba et al. (1997)	Original article	PKU, HM, MSUD, AA	11[b]	1, 2	Most helpful role of parents' support group for families in the early phase after child's diagnosis; parents' support group as an inventory of their experience for families raising children for a long time; problems related to the child's expanding social life; suffering from social prejudice because of genetic disease
Matsumoto et al. (2014)	Proceedings	PKU	1[b]	1	Fulfillment from raising a child; expectation of a child's development
Nakata et al. (1998)	Original article	PA	1[b]	1	Difficulty of diet therapy because the child could not take enough milk
Nikaidou and Kosuga (2014)	Feature article	IEM	N/A	1	Psychological burden associated with the child's life and prognosis

(continued)

Table 1 (continued)

Author (s)	Article type	Metabolic disease	*N*	Life stage[a]	Summary of articles focused on difficulties in daily life and associated factors, and QoL
Okano (2011)	Feature article	PKU	N/A	1, 2, 3	Difficulty of management of the amount of milk and blood levels of phenylalanine; necessity of education about diet therapy from childhood; necessity of control during pregnancy of patients with PKU
Okano et al. (2013)	Original article	CD	52[b]	1, 2, 3	PedsQL Multidimensional Fatigue Scale, PedsQL Generic Core Scale: QoL of children with CD aged 1–22 years based on self-reports and proxy reports by their guardians; the mean scores of both were lower than those for healthy children
Shigematsu et al. (2000)	Original article	PA	1[b]	1	Difficulty of diet therapy because the child could not take enough milk; responsibility of the child's diet therapy concentrated on the mother; suffering from social discrimination from grandparents' generation
Taketa et al. (1991)	Feature article	PA	1[b]	1	Difficulty in preventing child's sickness; importance of education at discharge from hospital for the first time
Ueno et al. (1990)	Feature article	IA	2[b]	1	Struggles of coping with problems related to child's food restriction
Yoshino et al. (2010)	Feature article	PKU	N/A	3	Financial difficulty from adulthood associated with treatment
Yuhara et al. (1991)	Feature article	PKU	N/A	1, 2, 3	Realization and comparison with health children about diet; temptation to eat the restricted food; importance of special control of pregnancy

[a] Child's life stage: 1. infant stage 2. school-age stage 3. adolescence-to-adulthood transitional stage
[b] *N*: number of families
[c] *N*: number of insurance companies

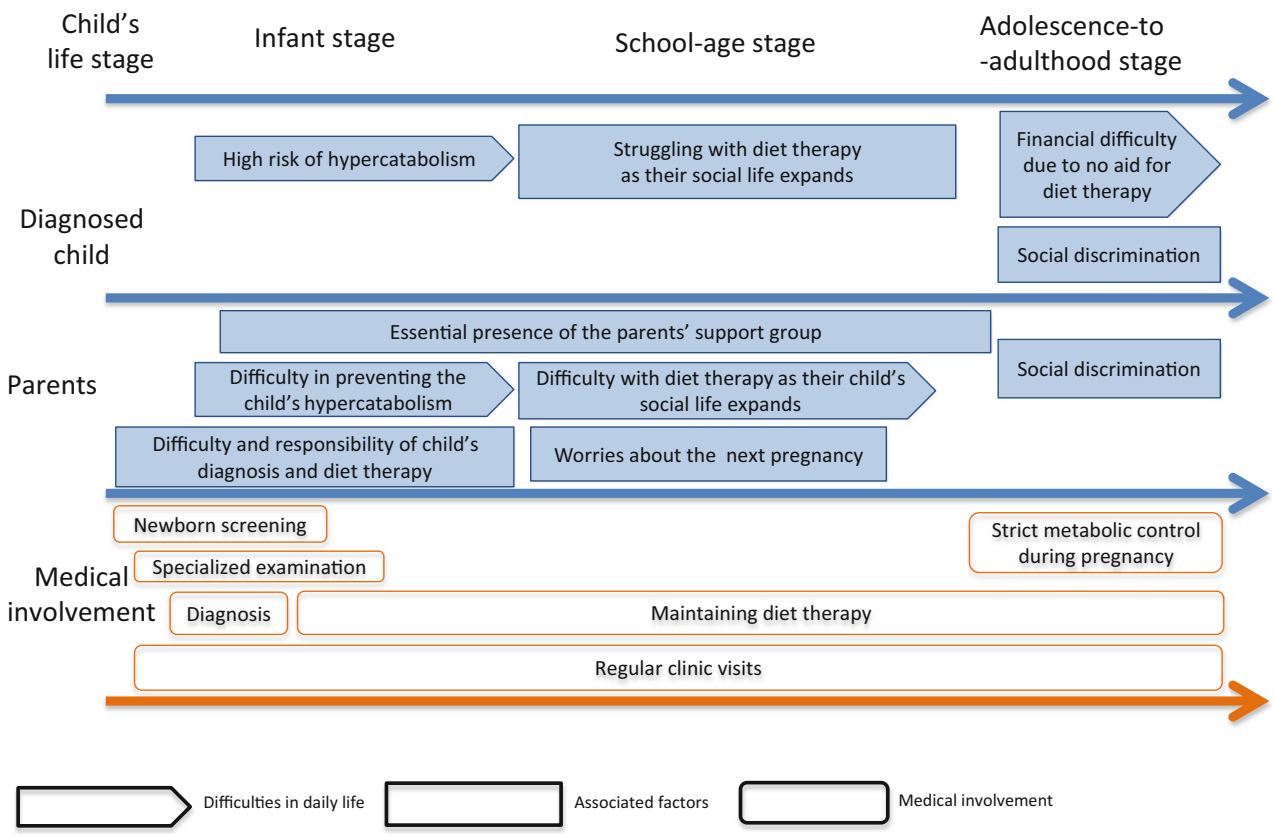

Fig. 1 Difficulties in daily life and associated factors, and medical involvement at each life stage of diagnosed children

Parents' Difficulty and Responsibility Concerning Their Child's Diagnosis and Diet Therapy Diet therapy is directly related to the daily life of children with IEM and their families (Okano 2011). A group interview study revealed that the mothers took about 2 months to face dealing with the diet therapy of their child; until then, they had been suffering from the psychological burden associated with the diagnosis and diet therapy of IEM (Ishiyama et al. 1987). On the other hand, one mother could face her child's diet therapy because her family cooperated in the care of her child (Abe 2012). One mother experienced a sense of fulfillment from taking care of her child with phenylketonuria (PKU) and looked forward to further developing their relationship (Matsumoto et al. 2014).

During the neonatal period, doctors instruct mothers of children with PKU to give them a certain amount of medical formula and as much breast milk as they want (Okano 2011). Starting with baby food, the children noticed the bad taste of medical formula and the good taste of baby food, and therefore, their mothers had difficulty in giving them the indicated quantity of medical formula (Fujiwara 2013). One mother, aware of the need to prevent ketoacidosis, experienced difficulty when her child with PA was not eating enough on a regular basis (Nakata et al. 1998; Shigematsu et al. 2000). For such mothers, deciding each day's combination of ingredients for the diet therapy was difficult (Ishiyama et al. 1987). They made every possible attempt so that their children could feel the same as their friends, for example, checking the school-lunch menu every day and adjusting the child's total protein intake (Ishiyama et al. 1987). Furthermore, mothers are often the ones who take responsibility for their child's diet therapy: one mother of a child with PA reported that she was dissatisfied with her husband's lack of understanding about the diet therapy (Shigematsu et al. 2000). Families carry the enormous burden of knowing that their child's life and prognosis is in their hands (Nikaidou and Kosuga 2014).

Essential Presence of the Parents' Support Group Because parents of children with IEM have few opportunities to meet parents in the same situation as themselves, they have difficulty in sharing their anxiety and distress (Kashiwagi 2012; Fujiwara 2013). Moreover, other than their primary doctor, they have no one with whom they can ask about the disease or talk about their distress (Kashiwagi 2012). Therefore, parents recognized their essential need for a parents' support group. To cope with their anxiety or distress, they participated in such a group or received counseling at hospital (Fujiwara 2013). They asked questions such as "What should we do when our child has symptoms?" For families at an early phase after their child's diagnosis, the parents' group was the most helpful place to hear about other families' actual experiences (Manba et al. 1997). And for those families raising a child with IEM for some years, it was a good opportunity to look back on their experiences (Manba et al. 1997).

Another important reason for their participation in the parents' support group was to gather information about the disease and care. Mothers of children with IEM complained about the lack of information available (Kubo and Tamura 2007). They asked the following such questions at the parents' group or on an Internet search: "Why did our child get this disease?" and "What are the things we can do for our child?" (Kubo and Tamura 2007). As regards *mucopolysaccharidosis (MPS)*, the medical professionals' lack of knowledge of MPS caused the diagnosis to be delayed until after the caregivers had noticed their child's abnormality (Kubo et al. 2008; Kubo 2010).

School-Age Stage

Children's Struggle and Parents' Difficulty with Diet Therapy as Children's Social Life Expands As children with IEM grow up and come into contact with more and more people, they tend to become nervous about being compared with healthy children of their own age; for example, this tension leads them to feel tempted to eat restricted food items (Yuhara et al. 1991). Children with isovaleric acidemia (IA) wanted to eat the same foods as their healthy siblings and friends; their family members also struggled with coping with these problems related to food restriction (Ueno et al. 1990). The representative of the PKU parents' support group reported that the main anxieties about diet therapy when their children were of school age were how to explain the diet therapy to the school staff and what to do about diet therapy when their child went on trips or sleepovers (Fujiwara 2013). In this way, as the children grow older, their families live with many new problems related to their social lives (Manba et al. 1997). On the other hand, studies on MPS revealed that most children with MPS attended school and that school life was good for them because it allowed them to settle into the normal routine of everyday life. Simultaneously, it was a good opportunity for their parents to feel less stress and fatigue (Kubo 2007).

Worries About the Next Pregnancy In the school-age stage, mothers of children with IEM were worried about their next pregnancy and about whether the next child would have the same disease, because IEM is a genetic disease (Fujiwara 2013).

Adolescence-to-Adulthood Stage

At this stage, new problems occur medically, economically, and socially (Yoshino et al. 2010; Kashiwagi 2011; Fujiwara 2013).

Strict Metabolic Control During Pregnancy IEM patients need strict metabolic control during pregnancy. For example, patients with PKU need to plan pregnancies well and to maintain their blood levels of phenylalanine within the safety threshold from about 3 months before the pregnancy (Yuhara et al. 1991; Okano 2011).

Financial Difficulty From adulthood, IEM patients in Japan cannot receive any aid for their diet therapy. So they experience financial difficulty because of their diet therapy, for example, having to buy therapeutic formula (Yoshino et al. 2010; Fujiwara 2013). Because the market is small, the pharmaceutical price of therapeutic formulas is high, and as it is now, companies selling therapeutic formulas carry those costs. In 1 case of MSUD, the over-20-year-old patient would have to pay about 680,000 yen (about US $5,600) per year for therapeutic formula if the companies stopped carrying the cost. Mothers cautioned that the expensive cost of treatment could lead patients to stop their treatments (Fujiwara 2013).

Social Discrimination Children with IEM in Japan suffer from social prejudice as a result of having a genetic disease. Generally, Japanese people do not have correct knowledge about IEM because it is a very rare disease (Manba et al. 1997). That study revealed that patients with IEM experience social discrimination from people of their grandparents' generation. After a mother told the child's grandparents about the child's diagnosis, they forced her not to disclose it to their neighborhood (Shigematsu et al. 2000). The results of a questionnaire survey revealed that about 52% of patients with IEM were discriminated against when they tried to take out private health insurance (Komatsu et al. 2011). The researchers indicated that even patients with mild IEM tend to suffer from discrimination regarding the insurance contract (Kato and Kashiwagi 2010).

QoL

Only two studies have been published in Japan about the QoL of children with IEM or of their families: one is about children with citrin deficiency (CD), and the other, about children with MPS. According to the study about children and young adults (1–22 years) with CD, the mean scores of the PedsQL® fatigue and generic core at the self-report and proxy report by their parents were both lower than those for healthy children (Okano et al. 2013). Even if there were no CD symptoms, the children felt fatigue due to compensation for metabolic failure, and it affected their QoL.

Of all the IEM types, MPS has the most clearly progressive symptoms, for example, intelligence disorders, movement disorders, and hearing loss (Endo et al. 2013). One survey study of parents of children with MPS revealed that they had low scores on the General Health Questionnaire (GHQ30) Japanese version (Kubo et al. 2008). The factors related significantly to the GHQ30 scores were burden of bathing assistance ($r = 0.465$) and impossibility of communication with one's children ($r = 0.38$) (Kubo et al. 2008). Multiple regression analysis also showed that the significant factor on the GHQ30 score was bathing assistance ($\beta = 0.363$, $p = 0.049$) (Kubo et al. 2008).

Discussion

Difficulties in Daily Life and Associated Factors

Infant Stage

Previous research has revealed that infants with IEM tend to be at high risk of hypercatabolism and that their parents are concerned about this high risk, particularly at the infant stage (Yuhara et al. 1991). As a preventive measure, at the first discharge from hospital after a child has been diagnosed with IEM, family members need to be educated about infection prevention and what swift action to take when the child falls sick. Referring to a study on the medical system for diabetic patients (Nagayama et al. 2011), and given the small number of IEM medical specialists in Japan, researchers suggested the importance of expanding education for primary-care physicians about provision of medical care for IEM patients, because they usually assume the immediate responsibility of caring for patients with IEM when they present with symptoms. Therefore, a system of cooperation between IEM specialists and primary-care physicians should be put in place for daily medical practice.

In the early phase after the child's discharge from hospital, mothers suffer from the psychological burden brought on by their child's diagnosis and the responsibility associated with diet therapy (Ishiyama et al. 1987). Specialists should support the families psychologically after understanding well the four aspects of IEM: that it is a rare, metabolic, chronic, and genetic disease (Sakoda et al. 2013). At the same time, the child's health condition depends on the care provided by the family at home. Therefore, medical professionals, especially nurses, have a role in supporting the family in feeling positive toward raising and caring for the child (Nakata et al. 1998).

The primary caregivers tend to assume the burden of care and responsibility of children with IEM. Because they have a role of management of the diet therapy and preparation of the family meals, and it is usually mothers. A case was reported, in which a mother complained about her husband's lack of understanding about diet therapy (Shigematsu et al. 2000). And mothers experience difficulty with their child's diet therapy (Ishiyama et al. 1987). Although it is a different disease, the

families of children with food allergies also have to follow the child's diet therapy, for example, eliminating eggs or nuts. It was reported that mothers of children with food allergies have anxiety about eating out, and this anxiety was affected by the diet therapy (Tatematsu and Ichie 2008), and mothers had lower QoL than did fathers (Warren et al. 2015). Researchers pointed out that the parental burden borne by mothers affected their parenting behavior (Ogata and Miyashita 2003). For IEM families, parenting behavior is related to the management of the child's diet therapy and affects the child's QoL. Therefore, not only the child's health status but also the mother's burden or stress should be assessed and investigated at regular clinic visits for total family care.

Mothers who have a child with chronic disease need to associate with mothers who have a similar experience (Ito et al. 2013). Parents' support groups fulfill a precious function of peer support for parents of children with rare diseases. It is a good place for parents to share their particular anxieties and distresses, their daily life, and their future (Manba et al. 1997).

School-Age Stage

As their social life expands, children with IEM struggle with being different from healthy children in terms of food restriction (Yuhara et al. 1991). Moreover, their parents face the difficulty of making the people around them understand the child's diet therapy (Fujiwara 2013). Casework is important for families with IEM children and should be conducted according to seven principles: individualization, acceptance, purposeful expression of feeling, controlled emotional involvement, nonjudgmental attitude, client self-determination, and confidentiality (Felix 1957; Kubota 2014). All medical professionals should offer their patients attentive hearing, empathy, and recipiency in daily clinical practice, focusing on each patient and his or her family, as well as understanding the features of IEM and these principles well.

The child's expanding social life is also associated with positive aspects, both for the child and for the parents. Going to school is a chance to acquire the fundamental routine of daily life and for the parents' stress and fatigue to be reduced. A previous study pointed out the benefit of parents' realizing the positive aspects of their child's development, thus increasing the mother's sense of fulfillment and competency (Kuno et al. 2006). To realize these positive aspects, it was efficient not only to calculate the child's protein intake but also to use a motherhood diary (Ueno et al. 1990). It is also important for parents that medical professionals recognize their daily efforts in raising their child at home, especially with regard to diet therapy, and at the same time to encourage them to also take a real look at the positive aspects, such as their child's development.

Adolescence-to-Adulthood Stage

For good health control, patients need to be educated about diet therapy from childhood (Okano 2011). They also need strict metabolic control during pregnancy (Yuhara et al. 1991). According to a previous study, education in self-care should be provided by all of the medical team with consideration for the child's growth and development, intelligibility, and sense of values, and also with praise for the child's efforts (Yoshida 2011). Other researchers suggested that it is necessary to make a model of self-care behavior for IEM patients and their families (Shigematsu et al. 2000). Therefore, medical professionals should consider these when cooperating with families in educating children with IEM from a young age. As regards parents' worries about their next pregnancy, it is important for them to receive genetic counseling (Okano 2011).

At this transitional stage from adolescence to adulthood, parents of children with IEM also have financial difficulty because of the costs of child's diet therapy (Yoshino et al. 2010). Fortunately, from July 2015, some IEM diseases have been accepted in Japan as specified intractable diseases. Some patients can receive medical aid in adulthood too, for example, patients with MSUD, PA, or IA.

Children with IEM and their families face the possibility of being discriminated against by the grandparents or when they take out life insurance (Shigematsu et al. 2000; Komatsu et al. 2011). Because Japanese generally form homogenous communities, they lack a basic awareness of social, ethnic, and economic diversity (Moriya et al. 2012). In addition, as discussed at a Eubios Ethics Institute seminar in 1992, the main anxiety of families of patients with genetic disease in Japan is the "honor of the family"; on the other hand, in the USA, it is the "well-being of patients" (Kubo 2010). However, that was nearly a quarter of a century ago; in the meantime, this unhealthy aspect of Japanese culture in terms of genetic disease has definitely been improving but has not completely disappeared. These social problems must be resolved immediately because social support affects the satisfaction with life of mothers who have a child with a chronic disease (Ohgino and Nakamura 2010). Other researchers emphasized the importance of social support for parents with IEM children (Gramer et al. 2014). Therefore, they should be helped to gain the support of the people around them, such as through diffusion of correct knowledge of IEM and its treatment for schoolteachers and neighbors.

QoL

CD patients tire easily owing to compensation for metabolic failure (Mutoh et al. 2008), and the features of this disease have an impact on their QoL (Okano et al. 2013). Furthermore, the results of a large international study

conducted in seven European countries showed that PKU affected the QoL of both the children and their parents (Bosch et al. 2015). Among the factors that strongly affected their QoL were the emotional aspects and anxiety about phenylalanine blood levels (Bosch et al. 2015). Parents of children with MPS have many more cares to contend with as the medical condition progresses: assisting bathing, eating, changing clothes, and so forth (Kubo et al. 2008). Even if children with MPS receive medical treatment such as enzyme replacement therapy, there is no way to stop the progress of this disease. This feature distinguishes MPS from other IEM types. Moreover, as the parents themselves grow older, their own burden and health problems also increase, thus affecting their own QoL (Yoshimoto et al. 1990). Therefore, research is needed on the QoL of not only the patients but also of their family members.

Until now, the lack of a registry of IEM patients in Japan has made recruitment of research participants difficult and probably explains the small number of studies conducted on the QoL of Japanese IEM patients. In 2013, the Japan Registration System for Metabolic and Inherited Diseases was established by the National Center of Child Health and Development (http://jasmin-mcbank.com). As the first step since its establishment, the factors associated with the QoL of a larger number of IEM patients must be explored, as well as each type of IEM. With these factors in mind, we should consider what types of intervention or education need to be provided for IEM patients and their families in Japan.

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Take Home Message

Both children with IEM and their parents have various difficulties in daily life; therefore, their QoL and the factors associated with it should be studied to determine how the QoL can be improved.

The Third Page

The corresponding author confirms that this work has not been published or submitted elsewhere. This article does not deal with any studies with human or animal subjects performed by any of the authors. All coauthors have seen the final version of the article.

Compliance with Ethics Guidelines

Conflict of Interest

I, Keiko Yamaguchi, have no conflict of interest to declare.

Rie Wakimizu received a research scholarship from the Pfizer Health Research Foundation.

Mitsuru Kubota received a research grant from the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development (AMED).

Details of the Contributions of Individual Authors

Keiko Yamaguchi planned this review article, drafted its concept and design, analyzed the contents of each eligible article, and had a central role in writing it.

Rie Wakimizu provided advice concerning the review's conception, design, and analysis, and also contributed to the revising of the draft as a professional researcher of child health nursing.

Mitsuru Kubota provided advice concerning the review's conception, design, and analysis, and also contributed to the revising of the draft as a clinical specialist of IEM.

References

Abe S (2012) The experience of the mother who had a child diagnosed by newborn mass-screening [in Japanese]. Jpn J Inherit Metab Dis 28:183

Bosch AM, Burlina A, Cunningham A et al (2015) Assessment of the impact of phenylketonuria and its treatment on quality of life of patients and parents from seven European countries. Orphanet J Rare Dis 10:80–94

Cazzoria C, Cegolon L, Burlina AP et al (2014) Quality of life assessment in a cohort of patients with phenylketonuria. BMC Public Health 14:1243–1252

Eminoglu TF, Soysai SA, Tumer L et al (2013) Quality of life in children treated with restrictive diet for inherited metabolic disease. Pediatr Int 55(4):428–433

Endo F, Yamaguchi S, Ooura T et al (2013) The handbook of inherited metabolic disease. Nakayama Shoten [in Japanese]

Felix PB (1957) The casework relationship. Loyola University Press. Ozaki A, Fukuda T, Harada K (2015) The Principles of a casework relationship: methodology of establishment of supportive relationship [in Japanese]. Seishin Shobo. New translation revised version. 20–21

Fujiwara K (2013) Suggestion for understanding of amino acid metabolism of nursing from heredity support group [in Japanese]. J Jpn Soc Genet Nurs 11(2):23–28

Gramer G, Haege G, Glahn ME et al (2014) Living with an inborn error of metabolism detected by newborn screening-Parents' perspectives on child development and impact on family life. J Inherit Metab Dis 37(2):189–195

Ishiyama S, Suzuki E, Nishimura A et al (1987) Diet therapy of children with phenylketonuria : analysis of 4 good control cases

and development of program of diet therapy [in Japanese]. Jpn J Child Nurs 10(12):1634–1640
Ito Y, Sirazaki E, Yamazaki Y (2013) Feeling of mothers have child with chronic disease: interview to mothers rearing child at home [in Japanese]. Collect Articles Jpn Nurs Soc Community Nurs 43:99–102
Kashiwagi A (2011) Mass-screening and support for patients: importance of follow-up [in Japanese]. Hokkaido J Public Health 25(1):26
Kashiwagi A (2012) The current situation and future issue of expanding newborn mass-screening [in Japanese]. J Jpn Soc Genet Nurs 10(2):33–36
Kato T, Kashiwagi A (2010) Rare diseases and reconstruction of neonatal screening: wishes of patients [in Japanese]. J Child Health 69(1):3–5
Komatsu S, Shintaku H, Hirata Y et al (2011) The current situation of contract of insurance of target disease of newborn mass-screening [in Japanese]. J Jpn Pediatr Soc 115(10):1573–1579
Kubo K (2007) A basic study on children with mucopolysaccharidoses (MPS) and their families: changes in school-age children and their relationships with doctors [in Japanese]. Annu Bull Dept Nurs 2:31–39
Kubo K, Tamura T (2007) A basic study on children with mucopolysaccharidoses (MPS) and their families: change in MPS children in their infancy based on observations by their parents. Bull Tokyo Gakugei Univ Sci Edu 58:387–395 [in Japanese]
Kubo K, Tazaki C (2008a) Mental condition of Caregivers of patients with mucopolysaccharidosis and factors related to their condition [in Japanese]. J Child Health 67(6):878–884
Kubo K, Tazaki C (2008b) The mind state of health of the family of the mucopolysaccharidosis child and connection with the everyday life care [in Japanese]. Annu Bull Dep Nurs Kyoritsu Women's Univ Jr Coll 3:103–107
Kubo K, Tasaki C, Oikawa Y (2008) Indication of physical features of infants with mucopolysaccharidosis [in Japanese]. Jpn J Mater Health 49(2):295–302
Kubo K (2010) Investigation of social and psychological problems of the caregiver or family of children with mucopolysaccharidosis [in Japanese]. J Child Health 69(1):63–39
Kubota M (2014) The problems on daily life of children with inherited metabolic disease [in Japanese]. Child Health 17(3):177–180
Kuno N, Yamaguchi K, Morita C (2006) The sense of burden of mother with severely disabled children at home and the related factors [in Japanese]. J Jpn Soc Nurs Res 29(5):59–69
Lambert C, Boneh A (2004) The impact of galactosaemia on quality of life: a pilot study. J Inherit Metab Dis 27(5):601–608
Manba T, Hori H, Takizawa M et al (1997) Family group of children with amino acid metabolism [in Japanese]. J Saitama Med Sch 13 (2):81–83
Matsubara Y (2010) The support system for new mass-screening [in Japanese]. Jpn J Inherit Metab Dis 26(1):55–56
Matsumoto Y, Tajima G, Matsumoto Y et al (2014) Analysis of the psychological process of mother who had a child with inherited metabolic disease diagnosed by newborn mass screening : a case of phenylketonuria [in Japanese]. J Jpn Soc Mass Screen 24;2:179
Moriya H, Ishibashi H, Wada K, Inoue I, Mizoguchi M (2012) Recognition on human diversity with mothers of preschool children. Jpn J Genet Nurs 10(2):3–9
Mutoh K, Kurokawa K, Kobayashi K et al (2008) Treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia with arginine and sodium pyruvate. J Inherit Metab Dis 31(2):343–347
Nagayama K, Kakisawa K, Ookawa Y et al (2011) Twenty-seven patients with hypoglycemic coma requiring hospitalization during oral hypoglycemic treatment [in Japanese]. J Jpn Diab Soc 54(4):271–276
Nakata M, Kurio R, Sugiura S (1998) The consideration on discharge education for children with propionic acidemia: focused on an education of tubal feeding [in Japanese]. Neonatal Care 11 (10):883–887
Nikaidou M, Kosuga M (2014) Is Tandem mass screening a genetic screening? [in Japanese] Jpn J Pediatr Med 46(4):452–455
Ogata K, Miyashita K (2003) Examination of the factors relevant to maternal parenting behavior [in Japanese]. Bull Fac Edu Chiba Univ 51:5–15
Ohgino A, Nakamura Y (2010) Factors affecting stress responses and satisfaction with life among mothers of children with chronic illness [in Japanese]. J Jpn Soc Child Health Nurs 19(1):1–7
Okano Y (2011) Phenylketonuria [in Japanese]. Jpn J Pediatr Med 43 (9):1509–1511
Okano Y, Kobayashi K, Ihara K et al (2013) Fatigue and quality of life in citrin deficiency during adaptation and compensation stage. Mol Genet Metab 109(1):9–13
Sakoda M, Yotsumot J, Yamamoto K et al (2011) A review of the psychological effects on parents who received positive/false-positive result in newborn screening [in Japanese]. Jpn J Genet Couns 32(4):157–167
Sakoda M, Yamamoto K, Yamaguchi S et al (2013) The life story of the mother who has children with propionic acidemia [in Japanese]. J Jpn Pediatr Soc 117(2):293
Shigematsu Y, Sasakawa Y, Iemori Y et al (2000) The first case with propionic acidemia found in newborn screening by tandem mass spectrometry in Japan: clinical management during infancy [in Japanese]. J Fukui Med Univ 1(2):275–282
Simon E, Schwarz M, Roos J et al (2008) Evaluation of quality of life and description of the sociodemographic state in adolescent and young adult patients with phenylketonuria [in Japanese]. Health Qual Life Outcomes 26(6):25–31
Stockler S, Moesilinger D, Herle M et al (2012) Cultural aspects in the management of inborn errors of metabolism [in Japanese]. J Inherit Metab Dis 35(6):1147–1152
Taketa M, Fujiwara Y, Endou M et al (1991) Nursing for children with propionic acidaemia need lifelong treatment-focusing on education to mother independently and continually [in Japanese]. Jpn J Child Nurs 14(11):1433–1437
Tatematsu S, Ichie K (2008) The feature of lifestyle of children with food allergy and their families, coordination of lifestyle by mothers and mothers' knowledge of allergy. Jpn J Child Nurs 31 (7):942–947
The Ministry of Health Labour and Welfare Equal Employment, Children Families Bureau, Maternal and Child health Division (2013) The implementation status of screening of inherited metabolic diseases [in Japanese]. http://www.jsms.gr.jp/contents04-01.html
The Ministry of Health Labour and Welfare (2015) Research Grant for Research on Measures for Intractable Diseases [in Japanese]. http://www.mhlw.go.jp/seisaku nitsuite/bunya/hokabunya/kenkyujigyou/hojokin-koubo-h26/gaiyo/13.html
Ueno K, Tochibora Y, Kado C et al (1990) Nursing of children with isovaleric acidemia: looking back to discharge education [in Japanese]. Jpn J Child Nurs 13(9):1153–1160
Warren CM, Gupta RS, Sohn MW et al (2015) Differences in empowerment and quality of life among parents of children with food allergy. Ann Allergy Asthma Immunol 114(2):117–125
Yuhara H, Fujino Y, Sekiya T et al (1991) A metabolic disease (phenylketonuria) and breast feeding [in Japanese]. Jpn J Perinat Care 10(6):477–485
Yoshimoto M, Nishiuchi S, Nishina K et al (1990) The study of function and role and position of home-nursing visit related to QoL of children with severe motor and intellectual disabilities

and their families rearing at home: focused on the survey of their needs, for systemization of a network of care of children with disabilities [in Japanese]. J Sever Motor Intellect Disabil. 24 (1):53–62

Yoshino M, Watanabe Y, Ohira T et al (2010) Phenylketonuria: toward a better carry-over care [in Japanese]. Jpn J Clin Med 68 (1):123–126

Yoshida Y (2011) Education for improving self-care ability on pediatric nursing field: looking back to involvement with early adolescent suffered from bronchial asthma repeatedly [in Japanese]. Kawasaki Hosp Res Bull 13:5–8

Zeltner NA, Huemer M, Baumgartner MR et al (2014) Quality of life, psychological adjustment, and adaptive functioning of patients with intoxication-type inborn errors of metabolism: a systematic review. Orphanet J Rare Dis 9(1):159–177

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RESEARCH REPORT

Swallow Prognosis and Follow-Up Protocol in Infantile Onset Pompe Disease

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Abstract Oro-pharyngeal dysphagia commonly occurs in patients with infantile onset Pompe disease (IOPD), which is a rare recessive neuromuscular disorder caused by deficiency of the lysosomal enzyme acid alpha-glucosidase. Without treatment, death occurs by 1 year of age from cardiorespiratory failure. Enzyme replacement therapy (ERT) has been used to increase life expectancy, however emerging developmental and medical morbidities have become apparent. A case file review of the feeding outcomes of 12 patients with IOPD, managed at a single tertiary centre, was undertaken. Two types of assessment had been completed: clinical feeding assessment (CFA) and instrumental videofluoroscopy swallow study (VFSS). A rating of functional oral intake at every Speech and Language Therapy feeding assessment from initial diagnosis to the most recent assessment was applied using the functional oral intake scale (FOIS).

Results indicate, initial diagnosis VFSS predicts long-term feeding outcomes. Even if a patient had an improvement in oral feeding after diagnosis, over a period of time their oral intake returned to the initial diagnosis VFSS level or below. All patients (8/8) who required non-oral feeding support under 6 months of age went on to require non-oral feeding support, even if they had periods of full oral feeding. CRIM negative status predicted significant oral feeding difficulties. An evidence-based follow-up protocol was developed. The information is used at diagnosis to counsel families regarding feeding prognosis and consideration of early gastrostomy when cardiac status allows safe anaesthesia. The results reinforce that feeding changes over time and patients require on-going dysphagia monitoring.

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Introduction

Pompe disease (glycogen storage disease type II, acid maltase deficiency, OMIM 232300) is a rare recessive neuromuscular disorder caused by deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA, EC 3.2.1.20). Patients with the most severe form, infantile onset Pompe disease (IOPD), typically present with symptoms within the first 6 months of life. Symptoms are characterised by progressive muscle weakness, manifesting as hypotonia, motor developmental delay, hypertrophic cardiomyopathy, respiratory difficulties, and feeding and swallowing problems. If untreated, death by 1 year of age ensues from cardiorespiratory failure (Chakrapani et al. 2010). Enzyme Replacement Therapy (ERT) has been available since 2006 in this population. Alglucosidase alfa (Myozyme, Genzyme, Cambridge, MA) is a recombinant enzyme which significantly improves survival rates, cardiomyopathy, and motor development (Kishnani et al. 2007; Reuser and van der Ploeg 2012).

Since patients are now surviving longer, impacts on functional capacities and the development of other co-morbidities are being identified. Respiratory compromise, reduced mobility, speech and language delay/disorders, and

oro-pharyngeal dysphagia have all been documented (van Gelder et al. 2011; Chakrapani et al. 2010). Oro-pharyngeal dysphagia commonly presents in typically affected children, ranging from mild to severe impairment, characterised by weak oral stage skills, delayed swallow initiation, and residue in the pharynx post-swallow (Jones et al. 2009; Prater et al. 2012; van Gelder et al. 2011). Oro-pharyngeal dysphagia can lead to aspiration of food or fluid into the airway, causing chest infections, lung damage, poor nutrition, and dehydration (Wallis and Ryan 2012; Young 1993). Currently, there are two published long-term follow-up studies regarding swallow prognosis in this population, both indicating an improvement in swallow function once starting ERT and over time (Fecarotta et al. 2013; van Gelder et al. 2011). The reports included a single-case study followed for a period of 3 years and the assessment of 6 patients over time. There are no international or national guidelines for swallowing management.

In the authors' clinical practice it was anecdotally observed that patients were experiencing more significant dysphagia and poorer long-term outcomes than previously reported in the literature. Thus this study had two aims: to document the progression of oral feeding skills in a cohort of children with IOPD from a single centre and to determine the frequency and modes of dysphagia follow-up that are required in the optimal management of such patients.

Methods

The medical records of 12 patients with IOPD currently managed in a single tertiary centre in the UK were reviewed. The study was approved by the local Research and Audit team as a retrospective review of case file documents that did not require patient consent. Inclusion criteria were defined as any active patient with a confirmed diagnosis of IOPD (on the basis of abnormal GAA enzyme activity in bloodspot or leucocyte assay, compatible *GAA* genotype, elevated urine tetrasaccharide, and presence of typical vacuolated lymphocytes on blood film), and receiving ERT.

Each Speech and Language Therapy feeding assessment, from diagnosis until May 2015, documented in the medical records was reviewed. Inpatient and outpatient assessments were included. Two different types of swallowing assessment were completed: clinical feeding assessment (CFA) and instrumental videofluoroscopy swallow study (VFSS). No data from any other centres were used.

CFAs were completed by one of three speech and language therapists. Every CFA involved the completion of a detailed case history, oro-motor examination, and direct observation of the patient eating and/or drinking while the speech and language therapist used cervical auscultation (Leslie et al. 2004). During this assessment, clinical signs of dysphagia were identified and the amount and type of oral intake were documented. The outcome of the CFA was used to guide oral feeding management and make recommendations for VFSS (Arvedson and Christensen 1993). VFSS was also carried out as routine assessment at initial diagnosis/start of ERT and 3–6 months after commencing ERT.

VFSS was completed by a speech and language therapist with expertise in dysphagia alongside a consultant radiologist. A VFSS is a radiological assessment of the swallowing process, by which a patient is observed drinking and/or eating using fluoroscopic screening with the addition of a contrast material, e.g. E-Z-Paque. During the assessment, oro-pharyngeal structure and function were assessed to identify aspiration and risk of aspiration. In addition, different strategies were trialled in order to optimise oral feeding and inform clinical management. The oesophageal phase was routinely reviewed by the radiologist.

The paediatric version of the functional oral intake scale (FOIS) was used to rate the oral intake of each patient at every assessment (Crary et al. 2005). The tool is a 7-point scale providing information on what the patient is eating and drinking, but does not describe aspiration (Table 1).

Baseline demographic data, age of diagnosis, *GAA* genotype, cross-reactive immunologic material (CRIM) assay status, and treatment regimen were determined from medical records for all patients. Serial echocardiographic studies were reviewed and degree of cardiac hypertrophy estimated by calculation of the left ventricular mass index

Table 1 Functional oral intake scale (FOIS) paediatric version (Crary et al. 2005)

Level 1—Nothing by mouth.
Level 2—Tube dependent with minimal attempts at food or liquids. For example, dummy dips, tastes of puree or small specified volumes of certain textures.
Level 3—Tube dependant with consistent intake of food or liquids, e.g. one or more textures ad lib but still requires tube feeding.
Level 4—Total oral intake of a single consistency. This classification is not used in paediatrics.
Level 5—Total oral intake with multiple consistencies but requiring special preparation or compensations, e.g. thickened formula, puree with syrup thick fluids.
Level 6—Total oral diet with multiple consistencies without special preparation, but with specific food limitations, e.g. no mixed consistencies.
Level 7—Total oral diet with no restrictions.

(LVMI) derived from M-mode echocardiograms (Khoury et al. 2009) at the time of initial diagnosis and at the most recent assessment. Requirement for respiratory support (supplemental oxygen, non-invasive or invasive ventilation) and gross motor function at the same time points was determined.

Results

Patients

Twelve subjects, three females and nine males, of different ethnicities were included in the study. No patient currently on the caseload was excluded from the study. Table 2 outlines patient information and age at initial VFSS and the most recent assessment. 11/12 patients were diagnosed at the centre. One patient (patient 5) was transferred with a diagnosis and receiving treatment from an overseas centre as an older child. The median age at diagnosis was 4.9 months and the median age of ERT initiation was 5.0 months. The age range at the most recent assessment was 19–144 months of age with a mean age of 61.4 months.

Treatment

Four patients were CRIM negative and were treated with a standardised immunomodulation protocol (rituximab and methotrexate) at time of initiation of ERT. Eight patients were CRIM positive, of whom one received immunomodulation. All patients were treated with a standardised ERT schedule with a 12-week induction with weekly intravenous alglucosidase alfa 20 mg/kg/dose followed by alternate week dosing.

Swallow Assessments

10/12 patients had an initial baseline VFSS at time of diagnosis or when starting ERT. Of the two patients who did not have a baseline VFSS, one (patient 2) was fully orally fed since birth and has remained fully orally feeding with no concerns, and with no clinical indications to complete a VFSS. The other (patient 5) arrived in the UK as an older child and had already been receiving treatment. 5/10 patients had a repeat VFSS, which was completed on average 3.4 months after the initial VFSS (range from 2 to 4 months).

Serial FOIS scores for each patient are summarised in Table 3. When comparing the most recent assessment to the initial diagnosis VFSS ($n = 10$ patients), 50% of patients maintained the same level of oral feeding and 50% of patients deteriorated. No patient demonstrated increased oral intake over time. No clinically significant improvement in oral intake was seen after starting ERT. Only 1/5 patients (patient 4) had an increase in oral intake after starting ERT, as shown on VFSS; however, this was not maintained as seen at the most recent assessment.

One patient (patient 9) who was fully orally feeding with no restrictions at the initial diagnosis VFSS was able to maintain full oral feeding. 100% of patients (8/8) who required non-oral feeding support before 6 months of age went on to require non-oral feeding support at their most recent assessment even if they had periods of full oral feeding.

CRIM negative status predicted significant oral feeding difficulties. All patients who were CRIM negative ($n = 4$) had long-term poor prognosis with limited oral intake. No patient had an FOIS score over level 2 at the most recent assessment. CRIM positive patients ($n = 8$) presented with a range of oral feeding skills, predicted at initial diagnosis VFSS.

At the most recent assessment all patients who were nonambulatory had an FOIS score of 2, however there was wider variability in feeding status among patients with preserved ambulation. Similarly, all patients requiring invasive or non-invasive ventilation had an FOIS score of 2, with wider variability among those not requiring ventilation. All patients showed at least partial resolution of cardiac hypertrophy, hence this did not appear to be a major factor driving feeding issues.

At the most recent assessment, 75% of patients were tube dependant, requiring a nasogastric tube or gastrostomy tube to meet their nutrition and hydration needs. All patients had some oral feeding, even if only small amounts, e.g. lip swipes/tastes of puree. Three patients were fully orally feeding. Two of those patients were feeding with no oral restrictions. One patient required thickened fluids.

Discussion

The long-term oral feeding outcomes for patients with IOPD in this study were poor and differ to findings in the existing literature. 75% of the cohort is dependent on non-oral enteral feeding, with all patients displaying significantly limited oral intake, predominantly due to oropharyngeal dysphagia. The data suggest that initial diagnosis VFSS is prognostic in determining long-term oral intake.

The results of this study are in contrast to previous published work that reported an improvement in oral feeding once commencing ERT (Fecarotta et al. 2013; van

Table 2 Patient information

Patient	Sex	Age at diagnosis (months)	*GAA* genotype	CRIM status (Pos/Neg)	Age ERT started (months)	Immunomodulation (Y/N)	Age at initial diagnostic VFSS (months)	Age at the most recent assessment (months)	Cardiac hypertrophy (LVMI, g/m^2)		Respiratory support		Gross motor status	
									Start of ERT	Recent assessment	Start of ERT	Recent assessment	Start of ERT	Recent assessment
1	F	4.9	c.784G > A / c.784G > A	Pos	5	N	5.1	56	613	60	Oxy	Nil	Hypotonia	Walking independently
2	M	0.4	c.2744A > C / c.2744A > C	Pos	0.4	N	N/A	89	96	48	Nil	Nil	Normal	Walking independently
3	M	0.2	c.877G > A / c.877G > A	Pos	2.2	Y	3.7	55	50.6	36	Nil	nNIV	Hypotonia	Antigravity UL
4	F	6.2	c.2078dup / c.2078dup	Neg	6.9	Y	6.6	24	773	55.7	Oxy	cNIV	Hypotonia	Antigravity UL
5	M	6.9	n/a	Pos	NK	N	N/A	82	n/a	55.6	Nil	Nil	Hypotonia	Walk with support (left hemiplegia from neonatal stroke)
6	M	3.1	c.1933G > A / c.1933G > A	Pos	3.4	N	7	40	355	178.6	NIV	Nil	Hypotonia	Walking independently
7	M	4.6	c.1933G > A / c.1933G > A	Pos	4.8	N	5.3	20	114.3	49.8	Nil	Nil	Hypotonia	Walking independently
8	M	6.2	c.2608C > T / c.2608C > T	Neg	6.7	Y	6.3	19	367	128	Oxy	cNIV	Hypotonia	UL + LL antigravity movements
9	M	6.4	c.1798C > T / c.2105G > T	Pos	9.2	N	10.1	124	n/a	49.1	Nil	Nil	Hypotonia	Bottom shuffling
10	F	5	c.2560C > T / c.2560C > T	Neg	5.1	Y	4.9	40	665	69	Nil	Nil	Hypotonia	Walking independently
11	M	3.6	c.525del / c.2481 + 102_c.2646 + 31del535	Neg	4	Y	4	44	343	60.2	Nil	Vent	Hypotonia	Minimal movement UL
12	M	31.1[a]	c.1082C > T / c.1933G > A	Pos	31.2	N	29.8	144	140.4	70	nNIV	nNIV	Walk with support	Walking independently

F female, *M* male, *ERT* enzyme replacement therapy, *VFSS* videofluoroscopy swallow study, *CRIM* cross-reactive immunologic material, *NK* not known, *N/A* not applicable, *LVMI* left ventricular mass index, *Oxy* oxygen, *nNIV* nocturnal non-invasive ventilation, *cNIV* continuous non-invasive ventilation, *vent* invasive ventilation, *UL* upper limbs, *LL* lower limbs

[a] Patient 12, classified as infantile onset as symptomatic with hypotonia, heart failure from 10.2 months age

Table 3 FOIS scores over time

Patient	FOIS score at initial diagnosis VFSS	FOIS score at VFSS approx. 3–6 months post-ERT	FOIS score at CFA approx. 3–6 months post-ERT	Most recent FOIS score (CFA or VFSS)
1	3	N/A	2	2
2	N/A	N/A	7	7
3	3	N/A	2	2
4	2	3	3	2
5	N/A	N/A	N/A	6
6	3	N/A	7	2
7	2	2	2	2
8	2	2	2	2
9	7	N/A	7	7
10	3	3	3	2
11	2	2	2	2
12	5	N/A	3	2

FOIS functional oral intake scale, *VFSS* videofluoroscopy swallow study, *ERT* enzyme replacement therapy, *CFA* clinical feeding assessment, *N/A* not applicable

Gelder et al. 2011). The data presented here represents the first large longitudinal cohort study examining oral feeding over time, capturing a current caseload of all ERT-treated patients at this tertiary centre. Patients reported here have benefited from a routinely high level of speech and language therapy involvement, having routine assessments for an average of 3:8 years (range 0:9–9:6 years) resulting in accurate and complete clinical data in all patients. The cohort described here included four CRIM negative patients, who are known to have worse clinical outcomes in other areas such as mobility and respiratory function (Chakrapani et al. 2010). This study has shown that CRIM negative status is also associated with poorer feeding outcomes.

Although long-term oral feeding prognosis is poor the results indicate that over time oral intake fluctuates and patients require on-going monitoring by a dysphagia trained speech and language therapist to ensure safe management. Subsequently, a tertiary level care pathway was developed (Fig. 1). The care pathway outlines management from initial diagnosis to transition to adult services. Routine VFSS at diagnosis is completed to provide prognostic information and accurate dysphagia management. This information is used to educate and counsel parents about long-term oral feeding outcomes and non-oral feeding options. When no dysphagia is identified in the VFSS, patients continue with a full oral diet with no restrictions. They are then reviewed every 6 months at the tertiary centre and no referral to local Speech and Language Therapy feeding services are completed.

Modified diet and/or non-oral feeding support are recommended to those patients presenting with dysphagia in the VFSS. Dysphagic patients are then routinely seen every 6 months and as clinically indicated at the tertiary centre for monitoring. Patients requiring non-oral feeding support prior to 6 months of age will be referred for gastrostomy when medically stable. All dysphagic patients will be referred to local Speech and Language Therapy feeding services for community follow-up, to ensure liaison with school/nursery. VFSS is completed as clinically indicated for all patients.

Further long-term studies of feeding outcomes are required to ensure robust longitudinal data are gathered, and to validate the proposed care pathway. A multi-centre study should be considered in order to increase the number of participants. It is also recommended that national and international guidelines are developed to ensure evidence-based standardised care is available.

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Single Sentence Synopsis

Oro-pharyngeal dysphagia is common among patients with IOPD and oral intake at diagnosis predicts long-term feeding outcomes.

Conflict of Interest

Gyani Swift, Maureen Cleary, Stephanie Grunewald, Sonia Lozano, Martina Ryan, and James Davison declare that they have no conflict of interest.

Compliance with Ethics Guidelines

This article does not contain any studies with human or animal subjects performed by any of the authors. The study was approved by the local Research and Audit team as a retrospective review of case file documents that did not require patient consent.

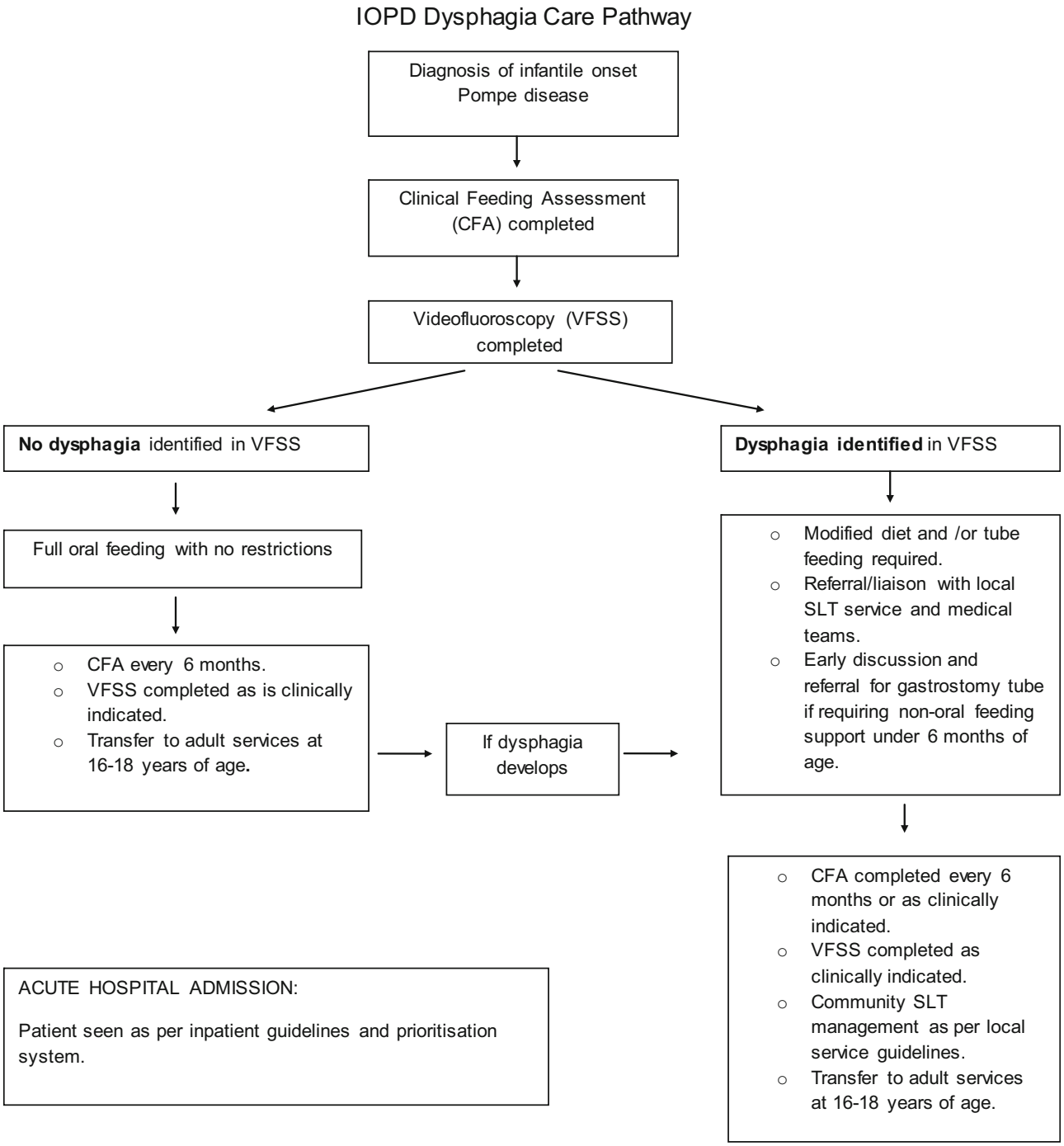

Fig. 1 Tertiary level care pathway. *IOPD* infantile onset Pompe disease, *VFSS* videofluoroscopy swallow study, *CFA* clinical feeding assessment, *SLT* speech and language therapy

Contribution of Authors

GS: Responsible for the study's conception and design, collated and analysed all data and wrote the manuscript.

MC: Involved in critical revision and editing of the article.

SG: Involved in critical revision and editing of the article.

SL: Involved in the study's conception, design, analysis of data, critical revision, and editing.

MR: Involved in critical revision and editing of the article.

JD: Oversaw the critical revision and editing of the article.

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References

Arvedson J, Christensen S (1993) Instrumental evaluation. In: Arvedson JC, Brodsky L (eds) Pediatric swallowing and feeding: assessment and management. Singular Publishing Group, California, pp 293–326

Chakrapani A, Vellodi A, Robinson P, Jones S, Wraith JE (2010) Treatment of infantile Pompe disease with alglucosidase alpha: the UK experience. J Inherit Metab Dis 33(6): 747–750

Crary M, Carnaby Mann GD, Groher ME (2005) Initial psychometric assessment of a functional oral intake scale for dysphagia in stroke patients. Arch Phys Med Rehabil 86:1516–1520

Fecarotta S, Ascione S, Montefusco G et al (2013) Improvement in dysphagia in a child affected by Pompe disease treated with enzyme replacement therapy. Ital J Pediatr 39:30–35

Jones H, Muller C, Lin M et al (2009) Oropharyngeal dysphagia in infants and children with infantile Pompe disease. Dysphagia 25(4):277–83

Khoury PR, Mitsnefes M, Daniels SR, Kimballs TR (2009) Age-specific reference intervals for indexed left ventricular mass in children. J Am Soc Echocardiogr 22:709–714

Kishnani PS, Corzo D, Nicolino M et al (2007) Recombinant human acid alpha-glucosidase: major clinical benefits in infantile-onset Pompe disease. Neurology 68:99–109

Leslie P, Drinnan MJ, Finn P, Ford GA, Wilson JA (2004) Reliability and validity of cervical auscultation: a controlled comparison using videofluoroscopy. Dysphagia 19:231–240

Prater SN, Banugaria S, De Armey PA-C et al (2012) The emerging phenotype of long-term survivors with infantile Pompe disease. Genet Med 14(9):800–810

Reuser AJJ, van der Ploeg T (2012) Pompe disease. In: Mehta A, Winchester B (eds) Lysosomal storage disorders: a practical guide. Wiley, West Sussex, pp 101–106

van Gelder CM, Van Capelle CI, Ebbink BJ et al (2011) Facial-muscle weakness, speech disorders and dysphagia are common in patients with classic infantile Pompe disease treated with enzyme therapy. J Inherit Metab Dis 35(3):505–511

Wallis C, Ryan M (2012) Assessing the role of aspiration in pediatric lung disease. Pediatr Allergy Immunol Pulmonol 25(3):132–142

Young C (1993) Nutrition. In: Arvedson JC, Brodsky L (eds) Pediatric swallowing and feeding: assessment and management. Singular Publishing Group, California, pp 157–208

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RESEARCH REPORT

Clinical and Genetic Characteristics of Romanian Patients with Mucopolysaccharidosis Type II

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Abstract *Background*: Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is a rare X-linked disorder caused by deficiency of iduronate-2-sulfatase (I2S) enzyme, which leads to the accumulation of partially digested glycosaminoglycans (GAGs) in the lysosomes and induces multisystemic alteration (coarse facial features; skeletal dysplasia; hepatosplenomegaly; joint stiffness and contractures; heart, lung, vision, and hearing disability; profound neurological decline).

The purpose of this study is to present the clinical and genetic characteristics of Romanian patients with Hunter syndrome and the genotype–phenotype correlation.

Material and Methods: 15 unrelated patients, with MPS II ranging from mild (4 subjects) to severe phenotype (11 subjects) aged 2 to 20 years, were evaluated clinically, cognitive development, enzyme assay and molecular analysis.

Results: The molecular analysis of the 15 unrelated Romanian MPS II patients has identified 15 different mutations (2 major genetic defects (13%) and 13 minor genetic defects (87%)): microdeletions and point mutations (missense, nonsense), seven of them described for the first time—deletion encompassing 3 to exon 7; c823G>T, pD275Y; c.1600A>C (pN534H); c.102_10delAG (p.D5Cfs*11); c.448_471del (p.P150_P157del); c.421delA (p.I141Yfs*72); and c.419-1G>C. The major genetic defects were correlated with a severe course of disease.

Conclusion: This is the first study on the clinical and molecular characterization of the MPS II Romanian patients. This study supports the evidence of the mutational heterogeneity of the I2S gene as well as the difficulty to correlate genotype and phenotype in the patients with MPS II.

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Introduction

Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is a rare X-linked disorder caused by alterations in the iduronate-2-sulfatase (I2S) gene (Wraith et al. 2008). This enzyme degrades glycosaminoglycans (GAGs), such as heparan sulfate and dermatan sulfate. The deficiency of the enzyme activity leads to the accumulation of partially digested GAGs in the lysosomes and induces multisystemic alteration. The gene responsible for IDS synthesis is located on chromosome Xq27–q28 and includes nine coding exons. IDS pseudogene is located in a distance of 20 kb of IDS's telomere and includes identical sequence of exon 2 and intron 2 and chimeric sequence of intron 3 and intron 7 which is responsible for homologous recombination of the active gene and pseudogene (Timms et al. 1995). IDS gene encodes the synthesis of 550-amino acid protein. The IDS alterations include large gene deletions, rearrangements, and small gene alterations (Froissart et al. 1993). According to the Human Genome Mutation Database (www.hgmdtrial.biobase-international.org), to date, more than 500 distinct mutations of the IDS gene have been identified, most of them point mutations (52%) or small deletions (17%).

Two types of disease have been described: the severe form with progressive neurologic alteration and the mild

form without or with minimal neurological dysfunction. This classification is challenged by more and more experts, because the severity of cardiac, respiratory, skeletal, and visceral damage and the term of attenuated form of disease are accepted (Wraith et al. 2008; Scarpa et al. 2011).

The patients with Hunter syndrome present wide allelic and phenotypic heterogeneity (Flomen et al. 1992). The genotype–phenotype correlation is present only in large deletions or large rearrangements of the active gene and pseudogene, which lead to inactive enzyme synthesis (zero enzyme activity), with severe forms of disease (Bunge et al. 1992). The genotype–phenotype correlation is not valid in small, point mutations and missense, nonsense mutations, which, in most cases, are "private" mutations. There are cases where the same mutation induces different clinical forms of severity. The relationship between I2S activity and clinical phenotypes was noted.

The clinical characteristics of MPS II are macrocephaly, coarse facial features, hepatosplenomegaly, hernia, stiff joints, respiratory infections, recurrent otitis, deafness, cardiac valve disease, and neurologic impairment, in two-thirds of cases (Beck 2011; Giugliani et al. 2014). The onset of the disease is at age of 12–18 months (severe form) and at age of 2–4 years (attenuated form) (Wraith et al. 2008).

Because there are no studies on MPS II patients in Romania, the objective of this study is to present the clinical and genetic characteristics of Romanian patients with Hunter syndrome and the genotype–phenotype correlation.

Subjects and Methods

Fifteen patients with MPS type II, from unrelated families, aged 3.16–31.25 years, were enrolled. All these patients were specifically diagnosed (enzyme and molecular determination), and they represent all Romanian patients diagnosed with this disease. The study protocol was approved by the hospital ethics committee, and written, informed consent was obtained from participants or their guardians prior to the enrollment in the study. Clinical assessment of the patients included standard auxological assessment, according to www.who.int/childgrowth/standards (using Seca Vogel and Halke Hamburg 702 device), bone radiographs, goniometry, neurological and psychological evaluation, otorhinolaryngology examination with audiogram, ophthalmological examination, cardiology evaluation (ECG, Doppler echocardiography), and spirometry. Hepatic and splenic volumes were determined by ultrasonography (Patlas et al. 2002), and the cutoff values accepted were 2.5% of the body weight for the liver and 0.2% of the body weight for the spleen (Weinreb et al. 2002). The values obtained at ultrasonography were expressed as multiple of normal value. Iduronate-2-sulfatase activity was determined by variable methods which were available at different times, plasmatic activity, or dried blood spot by fluorometry, based on the fluorometric determination of methylumbelliferone (Centogene Laboratory Rostock, Germany). All genetic procedures were done in accordance with the ethical standards on human experimentation, of the hospital committee and with the Helsinki Declaration of 1975, as revised in 2000. The written, informed consent was obtained from participants or their guardians prior to the genetic analysis. The molecular analysis was realized at Centogene Laboratory Rostock. IDS gene was analyzed by PCR and sequencing of both DNA strands of the entire coding region and the highly conserved exon–intron slice junctions. The reference sequence of the IDS gene is NM_000202.5/NM_006123.4.

Novel, unreported mutations were analyzed by Alamut 2.2 software and Polyphen-2, SIFT, Mutation Taster, and Align-GVGD in order to reproduce the effect of each mutation on enzyme activity.

Results

Clinical characterization of the patients is presented in Table 1. The height of the patients was variable, depending on the age (between +3.4 and −4.66 SD): the mean height was 1.8 ± 1.06 SD in the group of patients under 5 years, −2 ± 2.12 SD in the group of 5–10 years, and −3.71 ± 0.25 SD in the group >10 years.

All the patients presented coarse facial features, osteoarthropathy, stiff joints, umbilical hernia, and hepatosplenomegaly (mean hepatic volume was 1.7 ± 0.79 × normal volume; mean spleen volume was 4 ± 1.45 × normal volume).

Cardiac involvement consisted in variable grades of left ventricular hypertrophy (73.33% of patients) and valve involvement. All the patients presented mitral valve thickening with different grades of regurgitation, depending of the age at diagnosis. Eight patients (53%) presented aortic regurgitation. Hearing loss was present in 11 patients (73%). Neurological and psychological assessment revealed four patients (26.66%) with normal intellectual development and 11 patients with a variable neuro-intellectual impairment, from mild retardation to dementia.

The clinical onset was at 1.73 ± 0.84 years; the unspecific diagnosis was established at the age of 4.36 ± 2.6 years, and specific diagnosis was established at 5.14 ± 3.2 years. We described an important gap (3.4 ± 2.36 years) between the clinical onset and specific diagnosis in this group (the limits: 0.9 and 10.1 years).

Enzyme replacement therapy was started at the age of 7.45 ± 5.28 years (limits: 2.1–20.25 years). Iduronate-2-

Table 1 Clinical characteristics of MPS II patients

Nr.	Age					SDS for height	Hernia	Liver volume (×N)	Splenic volume (×N)	Valvulopathy				Cardiomyopathy	Osteoarthropathy	Hypoacusia	Neurological involvement
	Onset	Unspecific diagnosis	Specific diagnosis	Starting therapy	Current					MI	MS	AI	AS				
H1	1	5	11.83	20.25	31.25	−4.66	+	1.26	1.88	++	++	–	–	+	+	+	–
H2	3	5.75	6	12.5	15.91	−3.7	+	1.2	5	+	+	–	–	+	+	+	–
H3	2	5.83	6.9	11.25	14.58	−3.1	+	1.45	3.8	++	+	+	–	+	+	+	–
H4	1.5	3	3.5	10.16	13.41	−2.8	+	1.23	5.38	+	+	++	–	+	+	+	+++
H5	1.5	11.5	11.6	12	12.16	−4.30	+	5.45	7.27	++	–	++	–	+	+	+	+++
H6	2	3.5	4.16	8	11.25	−3.5	+	1.2	4	++	–	++	–	+	+	+	+++
H7	4	6	6.8	7.8	8.33	−2	+	1.58	3.25	+	–	++	–	+	+	+	–
H8	2	6.83	6.9	–	7.33	−2.22	+	1.26	1.3	++	+	++	–	+	+	+	++
H9	1	2	2.25	3.25	6.66	+1	+	1.33	3.61	++	–		–	+	+	+	++
H10	1.5	3.9	4	4.1	5.25	+1.4	+	1.6	3.5	+	–	+	–	+	+	–	++
H11	2	2.9	3	3.25	5.08	−0.1	+	1.75	3.55	++	–	–	–	–	+	–	++
H12	1	2.5	2.6	3.25	4.91	+3.4	+	2.37	4	++	–	+	–	+	+	+	++
H13	1.5	3.75	3.9	4.25	3.75	+2.2	+	1.24	5.1	++	–	–	–	–	+	+	+
H14	1	1.5	1.9	2.1	3.66	+1.2	+	4.21	4.86	++	–	–	–	–	+	–	+
H15	1	1.5	1.8	2.16	3.16	+2.5	+	1.4	3.5	+	–	–	–	–	+	–	++

H Hunter patient, *SDS* standard deviation score, *N* normal, *MI* mitral valve insufficiency, *MS* mitral valve stenosis, *AI* aortic valve insufficiency, *AS* aortic valve stenosis

Table 2 Iduronat sulfate–sulfatase activity

Patient range	Enzyme activity	Normal range	Unit of measure	Sample type
H1	0.0	300–800	mMol/l/4 h*	Plasma
H2	4.5	300–800	mMol/l/4 h*	Plasma
H3	17.452	300–800	mMol/l/4 h*	Plasma
H4	1.5759	300–800	mMol/l/4 h*	Plasma
H5	<0.8	≥5.6	μmol/l/h	DBS
H6	3.409	300–800	mMol/l/4 h[a]	Plasma
H7	3.6	300–800	nM/4h/ml	DBS
H8	0	≥5.6	μmol/l/h	DBS
H9	1	>4	μmol/l/h	Plasma
H10	<0.8	≥5.6	μmol/l/h	DBS
H11	<0.8	≥5.6	μmol/l/h	DBS
H12	1.2	>2	μmol/l/h	DBS
H13	<0.8	≥5.6	μmol/l/h	DBS
H14	1.1	>4	μmol/l/h	DBS
H15	0	0.02–0.25	μmol/spot[a]/ 21 h	DBS

H Hunter patient, *DBS* dried blood spot, * none

sulfatase activity was markedly reduced in all patients (Table 2).

Molecular analysis (Table 3) revealed major genetic defects in two cases (13%) and minor genetic defects, such as deletions and point mutations (nonsense, missense mutations), in 13 patients (87%).

The major genetic defects consisted in deletion of the segment exon/intron 3–7 in one patient and complete deletion of the exon 8 in another case. Both mutations correlated with a severe course of disease.

The minor genetic defects affected the exons in nine cases and the introns in four cases. In six patients point mutations of the exons 3, 6, 7, and 9 were identified, and two novel mutations were described. Three patients presented mutations of the exon 9.

Exonic Mutations

We found a previously unreported hemizygous variant in *exon 6 (c823G>T, pD275Y).* It is located at a highly conserved nucleotide and amino acid position, with physiochemical differences between the amino acids aspartate and tyrosine. The second previously unreported hemizygous mutation was in *exon 9 (c.1600A>C, pN534H).* Both mutations indicate an attenuated phenotype.

In three patients microdeletions were identified. In one patient, with attenuated form of disease, a previously unreported hemizygous variant in *exon 1 (c.102_10delAG, p.D5Cfs*11)* was described. It creates a shift in the reading frame starting from codon D35. The new reading frame ends in a stop codon 10 position downstream, which is very likely to result in a truncated protein production.

Two novel microdeletions were identified at *exon 4: c.448_471del (p.P150_P157del)* and *c.421delA (p.I141Yfs*72).* Both mutations determined a severe course of the disease. We detected two hemizygous mutations in exon 4 of the IDS gene: c.448_471del (p.P150_P157del), which creates the loss of eight residues, which is very likely to result in a shortened protein that may function improperly, and c.421delA (p.I141Yfs*72), which creates a premature stop codon which is very likely to result in a truncated protein or loss of protein production. These mutations were the first time detected in Centogene's internal mutation/variation database (CentoMDTM).

Intronic Mutations

In four patients the mutations were located in the introns, and all mutations were correlated with severe forms of disease with early clinical onset and progressive neurological impairment. *Intron 4* was affected in two patients and *intron 2* and *intron 3* in one patient, respectively. We detected a previously unreported hemizygous mutation in *intron 3 (c.419-1G>C*). It is located in a highly conserved region within the acceptor splice site of intron 3. Five patients presented mutation c.438C>T (pT146T) in intron 4, and one patient presented mutation c.418 + 12T>C in intron 3. These mutations are considered as polymorphisms, without effect on the enzyme activity.

Discussion

This is the first study on the clinical and molecular characterization of the MPS type II Romanian patients. All patients presented coarse facial features, organomegaly, arthropathy, cardiac involvement, and respiratory difficulties, but variable neurological impairment. In the first years of life, the height of most patients with MPS II is normal; subsequently, the growth velocity decreases with age (according Patel et al. 2014).

Eleven patients (73.4%) presented a severe form of disease, which exceeds the rate of approximately two-thirds reported by Martin et al. (2008), Beck (2011), and Giugliani et al. (2014). Two of them presented major genetic defects (deletion of exon/intron 3–7 and deletion of exon 8), and nine patients presented minor genetic defects. The incidence of large deletions (13.3%) in our group is according to results obtained by Goldenfum et al. (1996), Lissens et al. (1997), Vafiadaki et al. (1998), and Froissart et al. (1998), but in contrast to 19–29% reported by Brusius-Facchin et al. (2014), Hartog et al. (1999), and Zhang et al. All the gross deletions caused severe course of disease accordingly with

Table 3 Molecular characteristics of the MPS II patients

Patient range	Location	Nucleotide change	Amino acid change	Reference	Mutation effect	Clinical form
H4	Exon03/exon 7	Deletion encompassing 3 to exon 7		–	Low enzyme activity	Severe
H6	Exon 08	Deletion		Zhang et al. (2011)	Low enzyme activity	Severe
H2	Exon 01	c.102_103delAG>T	p.D35Cfs*11	–	Low enzyme activity	Attenuated
	Exon 04	c.438C>T	p.T146T	Rs.1141608	SNP	–
H1	Exon 03	c.253G>A	p.A85T	Rathmann et al. (1996)	Low enzyme activity	Attenuated
H8	Exon 04	c.448_471del	p.P150_P157del	–	Low enzyme activity	Severe
H13	Exon 04	c.421delA	p.I141Yfs*72	–	Low enzyme activity	Severe
H3	Exon 06	c.823G>T	p.D275Y	_	Low enzyme activity	Attenuated
H11	Exon 07	c.998C>T	p.S333L	Flomen et al. (1992)	Low enzyme activity	Severe
H7	Exon 09	c.1600A>G	p.N534H	–	Low enzyme activity	Attenuated
	Exon 04	c.438C>T	p.T146T	Rs.1141608	SNP	–
H14	Exon 09	c.1294T>C	p.C432R	Lualdi et al. (2006)	Low enzyme activity	Severe
	Exon 04	c.438C>T	p.T146T	Rs.1141608	SNP	–
H5	Exon0 9	c.1402C>T	p.R468W	Crotty et al. (1992)	Low enzyme activity	Severe
	Intron 03	c.418+12T>C		rs148419392	SNP	–
H10	Intron 02	c.241-3C>G	–	Gort et al. (1998)	Low enzyme activity	Severe
H9	Intron 03	c.419-1G > C	–	–	Low enzyme activity	Severe
H12	Intron 04	c.419-2A > G	–	Bunge et al. (1993)	Low enzyme activity	Severe
	Exon 04	c.438C>T	p.T146T	Rs.1141608	SNP	–
H15	Intron 04	c.507+1G>A	–	Karsten et al. (1998)	Low enzyme activity	Severe
	Exon 04	c.438C>T	p.T146T	Rs.1141608	SNP	–

H Hunter patient, *SNP* single nucleotide polymorphism

Zhang, but contrary to Bonuccelli et al. (1998) who found an association with an intermediate form of the disease.

Minor genetic defects (9/11 patients with neurological involvement, 81.81%) consisted in point mutations and small deletions in six and three patients, respectively. The intronic splice mutations in the present study (26%) are more frequent than those reported by Bertoli et al. (10.9%), but less frequent than those reported by Alvares (36%).

The missense mutation S333L (c.998C>T), described by Flomen et al. (1992) in a Korean patient with severe form of disease, was also present in our study and has induced a similar evolution.

Mutation c.1402C>T (R468W) located on exon 9 was described for the first time by Crotty in 1992 in a patient with attenuated form of disease (IQ = 115). This mutation was associated in our study with a severe form of disease in a boy who has lost his cognitive acquisitions at the age of 12 years. Zhang et al. (2011) also reported the absence of genotype–phenotype correlation of this mutation in three patients. These data suggests that there could be other mechanisms involved in the clinical course of this disease.

Mutation c.419-1G>C, located on intron 3, reported for the first time in this study, was present in a patient with severe form of disease.

Missense mutation c.1294T>C (C423R), located on exon 9, described for the first time in an Italian patient with "intermediate" form of disease who died at the age of 23 years, was identified in our study in a 21-month-old patient who presented mild neuro-intellectual retardation.

Another patient with mild neurological impairment presented mutation c.241-3C (deletion of 44bp) which was reported by Gort in 1998 in a Spanish patient with intermediate form of disease.

Mutation c.507+1G>A located on intron 4 was correlated with a severe phenotype, as Karsten described in 1998.

Four patients presented an attenuated form of disease. In three cases, new "private" mutations were identified. These novel mutations were located in exons 1, 6, and 9, respectively. In one patient with attenuated form of disease, the point mutation c.253G>A, P.A85T (described by Rathmann in 1996) was identified, located on exon 3.

Conclusions

This is the first study on the clinical and molecular characterization of the MPS II Romanian patients. Fifteen different mutations of which seven novel unreported mutations were identified. The results of this study support the evidence of the mutational heterogeneity of the IDS gene as well as the difficulty to correlate genotype and phenotype in the patients with MPS II.

Contributions of Individual Authors

Camelia Alkhzouz: study design, acquisition, analysis and interpretation of data, writing, and literature search

Cecilia Lazea: cardiac assessment of the patients and writing

Simona Bucerzan: acquisition, analysis, and interpretation of data

Ioana Nascu: acquisition, analysis, and interpretation of data

Eva Kiss: acquisition, analysis, and interpretation of data

Carmencita Lucia Denes: ultrasound assessment of the patients

Paula Grigorescu-Sido: study design; acquisition, analysis, and interpretation of data; and writing

Name of One Author Who Serves as Guarantor

Camelia Alkhzouz

The Conflict of Interest Statements

1. *Camelia Alkhzouz* declares she has received speaker honorarium and travel and accommodation funding from Shire and Genzyme companies in the past 5 years.
2. *Cecilia Lazea* declares she has received travel and accommodation funding from Shire and Genzyme companies in the past 5 years.
3. *Simona Bucerzan* declares she has received travel and accommodation funding from Shire and Genzyme companies in the past 5 years.
4. *Ioana Nascu* declares she has received speaker honorarium and travel and accommodation funding from Shire and Genzyme companies in the past 5 years.
5. Eva Kiss has nothing to declare in the past 5 years.
6. Carmencita Lucia Denes.
7. Paula Grigorescu-Sido has nothing to declare in the past 5 years.

Details of Funding

All the authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

Details of Ethics Approval

The research study was approved by the hospital's ethics committee.

Patient Consent Statement

The written, informed consent was obtained from participants or their guardians prior to the genetic analysis. All genetic procedures were done in accordance with the ethical standards on human experimentation, of the hospital committee, and with the Helsinki Declaration of 1975, as revised in 2000.

Approval from the Institutional Committee for Care and Use of Laboratory Animals

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

References

Beck M (2011) Mucopolysaccharidosis type II (Hunter syndrome): clinical picture and treatment. Curr Pharm Biotechnol 12:861–866

Bonuccelli G, Regis S, Filocamo M et al (1998) A deletion involving exons 2–4 in the iduronate-2-sulfatase gene of a patient with intermediate Hunter syndrome. Clin Genet 53:474–477

Brusius-Facchin AC, Schwartz IVD, Zimmer C et al (2014) Mucopolysaccharidosis type II: Identification of 30 novel mutations among Latin American patients. Mol Genet Metab 111:133–138

Bunge S, Steglich C, Beck M et al (1992) Mutation analysis of the iduronate-2-sulfatase gene in patients with mucopolysaccharidosis type II (Hunter syndrome). Hum Mol Genet 1:335–339

Bunge S, Steglich C, Zuther C et al (1993) Iduronate-2-sulfatase gene mutations in 16 patients with mucopolysaccharidosis type II (Hunter syndrome). Hum Mol Genet 2:1871–1875

Crotty PL, Braun SE, Anderson RA et al (1992) Mutation R468W of the iduronate-2-sulfatase gene in mild Hunter syndrome (mucopolysaccharidosis type II) confirmed by in vitro mutagenesis and expression. Hum Mol Genet 1(9):755–757

Flomen RH, Green PM, Bentley DR et al (1992) Detection of point mutations and a gross deletion in six Hunter syndrome patients. Genomics 13:543–550

Froissart R, Blond JL, Maire I et al (1993) Hunter syndrome: gene deletions and rearrangements. Hum Mutat 2:138–140

Froissart R, Maire I, Millat G et al (1998) Identification of iduronate sulfatase gene alterations in 70 unrelated Hunter patients. Clin Genet 53:362–368

Giugliani R, Villarreal ML, Valdez CA et al (2014) Guidelines for diagnosis and treatment of Hunter syndrome for clinicians in Latin America. Genet Mol Biol 37:315–329

Goldenfum SL, Young E, Michelakakis H et al (1996) Mutation analysis in 20 patients with Hunter disease. Hum Mutat 7:76–78

Gort L, Chabás A, Coll MJ (1998) Hunter disease in the Spanish population: molecular analysis in 31 families. J Inherit Metab Dis 21(6):655–661

Hartog C, Fryer A, Upadhyaya M (1999) Mutation analysis of iduronate-2-sulphatase gene in 24 patients with Hunter syndrome: characterisation of 6 novel mutations. Hum Mutat 14(1):87

Karsten SL, Voskoboeva E, Tishkanina S et al (1998) Mutational spectrum of the iduronate-2-sulfatase (IDS) gene in 36 unrelated Russian MPS II patients. Hum Genet 103(6):732–735

Lissens W, Seneca S, Liebaers I (1997) Molecular analysis in 23 Hunter disease families. J Inherit Metab Dis 20(3):453–456

Lualdi S, Di Rocco M, Corsolini F et al (2006) Identification of nine new IDS alleles in mucopolysaccharidosis II. Quantitative evaluation by real-time RT-PCR of mRNAs sensitive to nonsense-mediated and nonstop decay mechanisms. Biochim Biophys Acta 1762(4):478–484

Martin R, Beck M, Eng C et al (2008) Recognition and diagnosis of mucopolysaccharidosis II (Hunter syndrome). Pediatrics 121 (2):377–386

Patel P, Suzuki Y, Maeda M et al (2014) Growth charts for patients with Hunter syndrome. Mol Genet Metab Rep 1:5–18

Patlas M, Hadas-Halpern I, Abrahamov A, Zimran A, Elstein D (2002) Repeat abdominal ultrasound evaluation of 100 patients with type I Gaucher disease treated with enzyme replacement therapy for up to 7 years. Hematol J 3:17–20

Rathmann M, Bunge S, Beck M, Kresse H, Tylki-Szymanska A, Gal A (1996) Mucopolysaccharidosis type II (Hunter syndrome): mutation "hot spots" in the iduronate-2-sulfatase gene. Am J Hum Genet 59(6):1202–1209

Scarpa M, Almássy Z, Beck M et al (2011) Mucopolysaccharidosis type II: European recommendations for the diagnosis and multidisciplinary management of a rare disease. Orphanet J Rare Dis 6(1):72

Timms KM, Lu F, Shen Y et al (1995) 130 kb of DNA sequence reveals two new genes and a regional duplication distal to the human iduronate-2-sulfate sulfatase locus. Genome Res 5 (1):71–78

Vafiadaki E, Cooper A, Heptinstall LE et al (1998) Mutation analysis in 57 unrelated patients with MPS II (Hunter's disease). Arch Dis Child 79(3):237–241

Weinreb NJ, Charrow J, Andersson HC et al (2002) Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: a report from the Gaucher Registry. Am J Med 113:112–119

Wraith JE, Scarpa M, Beck M et al (2008) Mucopolysaccharidosis type II (Hunter syndrome): a clinical review and recommendations for treatment in the era of enzyme replacement therapy. Eur J Pediatr 167(3):267–277

Zhang H, Li J, Zhang X et al (2011) Analysis of the IDS gene in 38 patients with Hunter syndrome: The c.879G > A (p.Gln293Gln) synonymous variation in a female create exonic splicing. PLoS One 6(8), e22951. doi:10.1371/journal.pone.0022951

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RESEARCH REPORT

Gastrointestinal Health in Classic Galactosemia

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Abstract Classic galactosemia (CG) is an autosomal recessive disorder of galactose metabolism that affects approximately 1/50,000 live births in the USA. Following exposure to milk, which contains large quantities of galactose, affected infants may become seriously ill. Early identification by newborn screening with immediate dietary galactose restriction minimizes or prevents the potentially lethal acute symptoms of CG. However, more than half of individuals with CG still experience long-term complications including cognitive disability, behavioral problems, and speech impairment. Anecdotal reports have also suggested frequent gastrointestinal (GI) problems, but this outcome has not been systematically addressed. In this study we explored the prevalence of GI symptoms among 183 children and adults with CG (cases) and 190 controls. Cases reported 4.5 times more frequent constipation (95% CI 1.8–11.5) and 4.2 times more frequent nausea (95% CI 1.2–15.5) than controls. Cases with genotypes predicting residual GALT activity reported less frequent constipation than cases without predicted GALT activity but this difference was not statistically significant. Because the rigor of dietary galactose restriction varies among individuals with galactosemia, we further tested whether GI symptoms associated with diet in infancy. Though constipation was almost four times as common among cases reporting a more restrictive diet in infancy, this difference was not statistically significant. These data confirm that certain GI symptoms are more common in classic galactosemia compared to controls and suggest that future studies should investigate associations with residual GALT activity and dietary galactose restriction in early life.

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Introduction

Classic galactosemia (CG) results from profound deficiency of galactose-1-phosphate uridylyltransferase (GALT) activity and affects approximately 1/50,000 live births in the USA (Pyhtila et al. 2015). Following exposure to milk, which contains large quantities of galactose, affected infants can become seriously ill and die if not immediately switched to a low-galactose formula (Berry 2014). Early identification by newborn screening and rapid dietary intervention generally prevents or resolves the potentially lethal acute symptoms of CG (Berry 2014).

Despite early diagnosis and intervention, most individuals with CG experience long-term complications that can include multiple developmental disabilities (Kaufman et al. 1995; Waggoner et al. 1990). The majority of girls and women with CG also experience primary or premature ovarian insufficiency (Fridovich-Keil et al. 2011; Kaufman et al. 1979; Spencer et al. 2013; Waggoner et al. 1990). For years, anecdotal reports of increased gastrointestinal (GI) health problems in CG have been shared by families but not

investigated formally. To determine whether children and adults with CG indeed experience increased prevalence of GI symptoms, we performed a systematic survey of GI health among 183 individuals with CG (cases) and 190 controls. To address possible genetic and environmental modifiers of GI outcome in CG we also gathered *GALT* genotype and retrospective diet information for each case.

More than 300 different *GALT* variants have been reported (http://arup.utah.edu/database/GALT/GALT_display.php; Calderon et al. 2007) and this allelic heterogeneity has been a suspected modifier of outcomes (e.g., Tyfield et al. 1999). Recently, trace residual GALT activity predicted from a yeast model system for specific genotypes was associated with both improved scholastic (Ryan et al. 2013) and ovarian outcomes (Spencer et al. 2013), suggesting that residual GALT activity might also modify GI outcomes in CG.

Another potential modifier of GI outcomes in CG is diet. While the majority of healthcare providers recommend lifelong dietary restriction of milk and other dairy products for their patients with CG, some also recommend restriction of non-dairy foods that contain low levels of galactose (Gleason et al. 2010; van Calcar et al. 2014). As a result, rigor of dietary galactose restriction varies among individuals with CG.

Using GI health outcomes, *GALT* genotype, and retrospective diet information collected for volunteers in our study we sought to address: (1) whether cases reported more frequent GI problems than controls, (2) whether presence of predicted residual GALT activity associated with frequency of GI symptoms among cases, and (3) whether rigor of dietary galactose restriction in infancy associated with frequency of GI symptoms among cases.

Materials and Methods

Study Volunteers

Children and adults with classic galactosemia were ascertained by referral from healthcare professionals or self-referral, often following interactions facilitated by the Galactosemia Foundation (www.galactosemia.org). Controls were recruited in two ways. First, unaffected siblings of CG volunteers participating in the study were recruited as "related controls." Second, "unrelated controls" were recruited by posting an IRB-approved flyer to the Centers for Disease Control (CDC) parents' email listserv (a widely subscribed electronic mailing list). All study volunteers completed informed consent prior to joining this IRB-approved study (Emory IRB00024933, PI: JL Fridovich-Keil).

Gastrointestinal Health Parent- or Self-Report Survey

We developed the gastrointestinal (GI) health survey used in this study to assess how frequently each study volunteer experienced different GI symptoms including abdominal pain, constipation, diarrhea, heartburn, nausea, and vomiting (see Supplemental data). The survey was administered online via Emory's HIPAA-compliant Feedback Server in 2013 and 2014. Surveys were completed by parent/guardians for their children, or by adults for themselves. Symptoms of each GI outcome were rated by frequency: "never," "less than once a month," "at least once a month," "weekly," or "daily." We classified problems that were experienced more than once a month as "frequent" and problems experienced less than or equal to once a month as "infrequent."

In addition to measures of GI health, we also gathered data on potential covariates including probiotic/antibiotic usage within the prior 6 months, date of birth, gender, race, and ethnicity. Our study design did not allow calculation of an overall response rate because the survey distribution routes used prevented us from knowing how many eligible people received the invitation to participate.

Dietary Restriction Parent-Report Survey

Our diet survey was developed to assess historical dietary information retrospectively. For individuals with classic galactosemia, this included which food groups were restricted in infancy to avoid galactose exposure. Like the GI health survey, our diet survey was administered online via Emory's Feedback Server. One hundred fourteen cases who responded to the diet survey also completed the GI health survey. We scored dietary restriction of milk/dairy only or milk/dairy plus legumes as "moderate" and restriction of milk/dairy, legumes, plus other food groups (e.g., some fruits or vegetables) as "strict."

Predicted Residual GALT Activity

We collected all available *GALT* genotype information for cases and calculated predicted GALT enzyme activity using results from a previously described yeast expression system (Fridovich-Keil and Jinks-Robertson 1993; Riehman et al. 2001). Cases were classified as having either $\geq$0.4% predicted residual GALT activity (approximately the limit of detection of the enzyme assay) or $<$0.4% predicted

residual GALT activity based on the average of activities predicted for their two *GALT* alleles.

Statistical Analyses

We performed all statistical analyses in R (https://www.r-project.org/). Because there are no good estimates for the relevant population prevalence of the GI symptoms we report, we used the reported symptoms in our controls as a guide for calculating the statistical power of our study. Reported symptoms ranged from a prevalence of 1.6% (nausea) to 6.3% (heartburn) in our controls. With our sample size, we had 80% power to detect an increase in cases of 5.2–7.8%.

To determine if there were significant differences in population structure or outcomes between related and unrelated control groups, we used chi-square tests, *t*-tests, and Fisher's exact tests, as appropriate. For case/control comparisons we performed logistic regression using generalized estimating equations (GEE) (Liang and Zeger 1986) to account for within-family correlations. With "frequent" (symptom experienced more than once a month) or "infrequent" (symptom experienced once a month or less) GI symptom as the outcome, our full models included "case" or "control" diagnosis as the predictor of interest and age, gender, probiotic use, and antibiotic use as covariates. Covariates were tested individually for association with outcome and retained in our reduced model if their *p*-value was ≤ 0.1. To adjust for multiple testing of various GI symptoms, we used permutation procedures that randomly shuffled each subject's set of GI symptoms within the study. To perform permutations while maintaining the existing familial structure in the dataset, we performed separate shuffling of unrelated subjects (unrelated cases and unrelated controls) and related subjects (related cases and controls). For related subjects, we assigned each individual's set of GI symptoms randomly among subjects from the same family. Symptoms significantly associated or close to associated with diagnosis ($p \leq 0.1$) were subjected to 10,000 such permutations of outcome to account for multiple testing.

For case-only diet and residual activity analyses we used Fisher's exact tests because all cases were unrelated (independent observations) and at least one cell in each comparison included fewer than five individuals.

Results

Study Population Characteristics

In total, 499 people responded to our GI health survey. However, we restricted analyses to respondents between ages 1 and 55 because of differences in distribution of cases and controls outside this range. Additionally, because >90% of our cases self-reported as white and non-Hispanic, we restricted our analyses to this demographic. We ultimately analyzed GI health survey results from 183 children and adults with classic galactosemia (cases) and 190 children and adults without classic galactosemia (controls). These 190 controls included 75 volunteers who were related to cases in the study, and 115 unrelated volunteers. There were only 4 reports of frequent vomiting in our entire cohort (evenly split between cases and controls), so we excluded this outcome from our analysis.

Notably, GI outcomes were not significantly different between the related and unrelated control groups for abdominal pain, constipation, heartburn, or nausea (Fisher's exact test $p = 1$, $p = 1$, $p = 1$, and $p = 0.3$, respectively). This is important because it suggests there were no strong "household" effects impacting the outcomes studied here. However, 13 unrelated controls reported severe diarrhea, compared to 0 reports in the related control group (Fisher's exact test $p = 0.002$). Privacy issues prevented us from re-contacting these 13 individuals for clarification, and because they did not clearly differ from other controls with regard to other parameters assessed, we did not exclude them from the study but instead did not test diarrhea as an outcome in subsequent analyses.

Unrelated controls were significantly older than related controls (24 ± 14 years old compared to 18 ± 14 years old; *t*-test $p = 0.003$), and overall this combined control group was significantly older than the case group (22 ± 14 years old compared to 16 ± 12 years old; $p = 1E\text{-}05$ based on *t*-test). We therefore tested age as a potential covariate in all case/control analyses. We likewise tested gender as a potential covariate because of differences in gender distribution between related and unrelated controls (63 and 42% female, respectively; chi-square $p = 0.008$). However, the gender distributions of cases and combined controls were not significantly different (55 and 50% female, respectively). Table S1 shows a summary of the numbers of cases and controls used in all comparisons.

Individuals with Classic Galactosemia Experience Some GI Symptoms More Frequently Than Controls

Our final GEE models comparing frequency of GI symptoms between cases and controls included: probiotic usage for abdominal pain and constipation, age for heartburn, and antibiotic usage for nausea (Table S2 provides a summary of full and reduced models). Of note, both antibiotic and probiotic usage were similar between cases and controls, so this was not a confounding variable (Table S3). Gender did not approach significant association

Table 1 Odds ratios from logistic regression using generalized estimating equations (GEE) to calculate odds of cases experiencing frequent symptoms compared to controls, with 95% confidence intervals, *p*-values (^ indicates after 10,000 permutations), and the number of observations included in model

Symptom	Odds ratio for cases	95% CI	*p*-value	*N*
Abdominal pain	2.1	0.8, 5.4	0.1^	360
Constipation*	4.5	1.8, 11.5	0.0008^	362
Heartburn	1.2	0.5, 2.9	0.8	356
Nausea*	4.2	1.2, 15.5	0.03^	356

Asterisk (*) indicates outcome was significantly higher among cases

with any outcome ($p > 0.1$ for all analyses) and therefore was not included in any of our reduced models.

Using case/control status as a binary predictor in our GEE framework, we were able to calculate adjusted odds ratios for experience of frequent symptoms controlled for relevant covariates (Table 1). Comparing unadjusted prevalence numbers we found that a diagnosis of classic galactosemia was significantly associated with a 4.5-fold increase in frequent constipation (95% CI 1.8–11.5, permuted $p = 0.0008$) and a 4.2-fold increase in frequent nausea (95% CI 1.6–18.7, permuted $p = 0.03$) (Fig. S1). Differences in abdominal pain (2.1-fold, 95% CI 0.8–5.4) and heartburn (1.2-fold, 95% CI 0.5–2.9) were not significant.

Residual GALT Activity and GI Health

For the case-only residual GALT activity question, we had *GALT* genotype information for 153 of the 183 cases who completed our GI health survey, 29 of whom had *GALT* alleles either not yet tested or not appropriate for study in our yeast system (Fridovich-Keil and Jinks-Robertson 1993; Riehman et al. 2001). Of the 124 cases for whom we could predict GALT activity, 27 had ≥0.4% predicted residual GALT activity and 97 had <0.4%. *GALT* genotypes and predicted activities for this study are summarized in Table S4.

While cases with predicted residual GALT activity ≥0.4% reported one-fifth the frequent constipation reported by cases with lower predicted activity (Table 2, odds ratios, upper rows and Fig. S2A, unadjusted prevalence), this difference was not statistically significant (95% CI 0.005–1.6, $p = 0.2$). There was no evidence of a difference in frequency of nausea between the two groups ($p = 1$).

Dietary Restriction in Infancy and GI Health

We received completed parent-response diet surveys with historical galactose restriction data for 114 of the 183 cases who also completed our GI health survey. The diet survey asked respondents to indicate categories of food restricted in infancy to avoid galactose. Options included: (1) milk and other high galactose dairy products, (2) legumes, (3) some fruits, (4) some vegetables, and (5) other. Milk and other dairy products were universally restricted among cases in infancy, and most families also restricted legumes which have long been considered a significant source of galactose (Acosta and Gross 1995). A smaller proportion of families also restricted some fruits/vegetables, or other foods believed to contain potentially concerning levels of galactose. Because of this distribution, we defined diets restricting only milk/dairy or milk/dairy plus legumes as "moderate" and diets restricting these plus any additional food groups (e.g., some fruits and vegetables) as "strict."

Nausea was not significantly different between "moderate" and "strict" dietary groups (Table 2, odds ratios, lower rows and Fig. S2B, unadjusted prevalence). We noted a 3.9-fold increase in odds for frequent constipation in the "strict" group but this result was not significant (95% CI 0.8–38.3, $p = 0.1$). Importantly, our findings were not confounded by the effect of residual GALT activity, because similar proportions of cases in the "moderate" and "strict" dietary groups had ≥0.4% predicted residual activity (19 and 22%, respectively, Table S5).

Discussion

The main goal of this study was to test whether there was a link between classic galactosemia and specific GI symptoms among a relatively large cohort of volunteers. Our results demonstrated that cases indeed reported significantly more frequent constipation and nausea than controls. Specifically, we found that individuals with classic galactosemia in our study were 4.5 times more likely to report frequent constipation and 4.2 times more likely to report frequent nausea compared to controls. It is important to note that while these increases were significant, the absolute prevalence of each symptom in our CG study group was fairly low at 11 and 5%, respectively. Therefore, while individuals with classic galactosemia do experience these GI problems more frequently than controls, these symptoms are not universal.

As a first step toward identifying possible genetic and environmental modifiers of GI health outcomes in classic galactosemia we addressed two obvious possibilities: predicted residual GALT activity and diet in infancy. We found suggestive trends for residual GALT activity: cases with ≥0.4% predicted residual GALT activity reported less frequent constipation than individuals with <0.4% predicted residual GALT activity (Table 2). We saw no evidence of a difference in frequency of nausea. However,

Table 2 Results of Fisher's exact tests for association of <0.4% predicted residual GALT activity (upper set of rows) or strict diet (lower set of rows) with frequent experience of GI symptoms

Symptom	Odds ratio for ≥0.4%	95% CI	*p*-value	*N*
Association of ≥0.4% predicted residual GALT activity with frequent experience of GI symptoms				
Constipation	0.2	0.005, 1.6	0.2	122
Nausea	0.8	0.02, 7.4	1	118
Symptom	Odds ratio for strict diet	95% CI	*p*-value	*N*
Association of strict dietary galactose restriction in infancy with frequent experience of GI symptoms				
Constipation	3.9	0.8, 38.3	0.1	112
Nausea	1.2	0.2, 8.4	1	109

a larger study is needed to confirm or refute the significance of these results.

Considering dietary galactose restriction in infancy, we noted a nearly fourfold increase in reported frequent constipation among cases on strict compared to moderate galactose restriction in infancy. This difference was not statistically significant, but our sample size may not have been adequately powered to detect a difference. We saw no notable difference in the frequency of nausea between the two diet groups.

We did not have concurrent GI health and general nutritional information for our study cohort. It is therefore possible that cases on more restrictive diets in infancy also followed more restrictive diets later in life, potentially leading to lower fiber intake due to a reduction in fruit and/or vegetable consumption. A larger study, with data gathered concurrently for diet and GI symptoms, will be needed to test this possibility. We also did not have information concerning a number of other factors that might have potentially influenced the GI outcomes we measured here, including type of milk substitute consumed, if any, presence or absence of calcium supplementation, psychosocial distress or psychiatric comorbidity, alcohol ingestion, obesity, or use of medications not covered by our survey.

Because classic galactosemia is a rare disorder with limited treatment options, individuals experiencing complications may be more likely to participate in research than those not experiencing complications, resulting in ascertainment bias. However, our observation that less than 12% of cases reported "frequent" experience for each GI symptom helps counter the concern that only those with frequent GI problems were motivated to participate in this study.

One other study limitation is the retrospective nature of our diet survey. Because classic galactosemia is a rare condition (1/50,000 live births), it took many years to assemble our study cohort, at all times welcoming cases of any age to join. While recall bias is therefore potentially a concern, there was no practical way to conduct this study otherwise. Of note, we have anecdotally found that parents of children with classic galactosemia tend to remember incredible detail of their child's early diet, perhaps because they worried about it so much.

Another potential limitation is our control group. We originally wanted to use siblings of cases to control for shared environment and genetics. However, we worried that as the parents raising a child with classic galactosemia might be so focused on the considerable health needs of their affected child they might under-report possible health concerns for their non-CG child. A comparison of related and unrelated control groups demonstrated no significant differences in reported frequency of GI symptoms between the two groups (with the exception of frequent vomiting in a small number of unrelated controls as a clear outlier). Additionally, performing GEE analysis of binary outcome data allowed us to account for within-family correlations that could have biased our results.

Importantly, our findings open up new avenues of investigation into pathophysiology of CG and possibilities for therapeutic intervention. One potential explanation for increased GI problems in CG is that defective glycosylation due to perturbation in UDP sugar substrate pools might impact the mucosal layer of the gut, compromising gut barrier function and potentially commensal bacterial population structure (reviewed in Bergstrom and Xia 2013). A "leaky" gut, microbiome dysbiosis, or both, could help explain increased GI problems as well as some of the other complications commonly seen in CG.

Importantly, diet also has a significant impact on establishment of the gut microbiome (Albenberg and Wu 2014; David et al. 2014), and the diet of infants and children with CG is fundamentally altered because of restriction of galactose-containing foods. Deficiency of the probiotic effect of milk and other dairy products alone could result in differences in the gut microbiome between cases and controls. Perhaps the most appealing aspect of testing this hypothesis is that it could offer opportunities for

therapeutic intervention such as dietary supplementation with appropriate probiotics.

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Compliance with Ethics Guidelines

Competing Interests

None of the authors have any competing interests to declare.

Human Subjects Ethics Approval

This study was conducted with approval of the Emory University Institutional Review Board (Emory IRB Protocol # 00024933, PI: JL Fridovich-Keil) and all study volunteers completed appropriate informed consent prior to participation.

Conflict of Interest

Kelly A. Shaw declares that she has no conflict of interest.

Jennifer G. Mulle declares that she has no conflict of interest.

Michael P. Epstein declares that he has no conflict of interest.

Judith L. Fridovich-Keil declares that she has no conflict of interest.

Author Contributions

Kelly A. Shaw helped to design the study, collected much of the data, conducted most of the statistical analyses, wrote the original draft of the manuscript, and contributed to editing the final manuscript.

Jennifer G. Mulle contributed to the study design, statistical analyses, and manuscript editing.

Michael P. Epstein contributed to the study design, statistical analyses, and manuscript editing.

Judith L. Fridovich-Keil oversaw the project and contributed to the study design and preparation and editing of the final manuscript.

References

Acosta PB, Gross KC (1995) Hidden sources of galactose in the environment. Eur J Pediatr 154:S87–S92

Albenberg L, Wu G (2014) Diet and the intestinal microbiome: associations, functions, and implications for health and disease. Gastroenterology 146:1564–1572

Bergstrom K, Xia L (2013) Mucin-type O-glycans and their roles in intestinal homeostasis. Glycobiology 23:1026–1037

Berry G (2014) Classic galactosemia and clinical variant galactosemia. In: Pagon R, Adam M, Ardinger H, Bird T, Dolan C, Fong C, Smith R, Stephens K (eds) GeneReviews®. University of Washington, Seattle

Calderon F, Phansalkar A, Crockett D, Miller M, Mao R (2007) Mutation database for the galactose-1-phosphate uridyltransferase (<I>GALT</I>) gene. Hum Mutat 28:939–943

David LA, Maurice CF, Carmody RN et al (2014) Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559–563

Fridovich-Keil JL, Jinks-Robertson S (1993) A yeast expression system for human galactose-1-phosphate uridylyltransferase. Proc Natl Acad Sci U S A 90:398–402

Fridovich-Keil JL, Gubbels CS, Spencer JB, Sanders RD, Land JA, Rubio-Gozalbo E (2011) Ovarian function in girls and women with GALT-deficiency galactosemia. J Inherit Metab Dis 34:357–366

Gleason L, Rasberry M, VanCalcar S (2010) Understanding galactosemia: a diet guide (W. C. Biochemical Genetics Program, University of Wisconsin-Madison, ed.). Abbot Nutrition and Abbot Laboratories, Columbus, Ohio

Kaufman F, Kogut MD, Donnell GN, Koch H, Goebelsmann U (1979) Ovarian failure in galactosaemia. Lancet 2:737–738

Kaufman FR, McBride-Chang C, Manis FR, Wolff JA, Nelson MD (1995) Cognitive functioning, neurologic status and brain imaging in classical galactosemia. Eur J Pediatr 154:S2–S5

Liang KY, Zeger SL (1986) Longitudinal data-analysis using generalized linear-models. Biometrika 73:13–22

Pyhtila BM, Shaw KA, Neumann SE, Fridovich-Keil JL (2015) Newborn screening for galactosemia in the United States: looking back, looking around, and looking ahead. JIMD Rep 15:79–93

Riehman K, Crews C, Fridovich-Keil JL (2001) Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase. J Biol Chem 276:10634–10640

Ryan EL, Lynch ME, Taddeo E, Gleason TJ, Epstein MP, Fridovich-Keil JL (2013) Cryptic residual GALT activity is a potential modifier of scholastic outcome in school age children with classic galactosemia. J Inherit Metab Dis 36:1049–1061

Spencer JB, Badik JR, Ryan EL et al (2013) Modifiers of ovarian function in girls and women with classic galactosemia. J Clin Endocrinol Metab 98:E1257–E1265

Tyfield L, Reichardt J, Fridovich-Keil J et al (1999) Classical galactosemia and mutations at the galactose-1-phosphate uridyl transferase (GALT) gene. Hum Mutat 13:417–430

van Calcar SC, Bernstein LE, Rohr FJ, Scaman CH, Yannicelli S, Berry GT (2014) A re-evaluation of life-long severe galactose restriction for the nutrition management of classic galactosemia. Mol Genet Metab 112:191–197

Waggoner DD, Buist NR, Donnell GN (1990) Long-term prognosis in galactosaemia: results of a survey of 350 cases. J Inherit Metab Dis 13:802–818

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RESEARCH REPORT

Management of Life-Threatening Tracheal Stenosis and Tracheomalacia in Patients with Mucopolysaccharidoses

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Abstract Several different lysosomal storage diseases, mainly mucopolysaccharidosis (MPS) type I, II, and VI, are complicated by severe obstruction of the upper airways, tracheobronchial malacia, and/or stenosis of the lower airways. Although enzyme replacement therapies (ERTs) are available, the impact of these on tracheobronchial alterations has not been reported. By extending the life expectancy of MPS patients with ERTs, airway problems may become more prevalent at advanced ages. These airway abnormalities can result in severe, potentially fatal, difficulties during anesthetic procedures. Usually, upper airway obstruction is treated by tracheostomy. However, with lower airway malacia and/or stenosis, there are no procedures available to date to address these difficulties. We report the first cases using a new technique of tracheal stenting in patients with MPS type VI (Maroteaux–Lamy syndrome) and type II (Hunter syndrome) who had almost complete tracheal occlusion and total airway collapse. An updated literature review is also reported.

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Introduction

Mucopolysaccharidoses (MPS) are a heterogeneous group of different lysosomal storage disorders, resulting from the abnormal degradation of glycosaminoglycans (GAGs) (Muenzer 2004). The crude cumulative incidence is approximately 1 in 25,000 newborns (Baehner et al. 2005; Nelson 1997). The main accumulated storage products include GAGs containing heparan, dermatan, keratan, and chondroitin sulfates (Neufeld and Muenzer 2001). The wide clinical spectrum for MPS is related to the particular GAG products involved, the degree of enzyme deficiency, and the associated amount of accumulated storage products.

In patients with MPS types I, II, and VI (OMIM #607014, #309900, and #253200, respectively; each including dermatan sulfate as the storage product) and MPS types IV A and B (OMIM #253000 and #253010, respectively; including chondroitin and/or keratan sulfates as storage products) (Muenzer 2004), treating physicians have raised considerable attention concerning the development of airway obstructions (Shapiro et al. 1985; Semenza and Pyeritz 1988), which may lead to fatal complications in emergency cases or during planned ear, nose, and throat procedures, including death during intubation (Yoskovitch et al. 1998; Muhlebach et al. 2011; Muhlebach et al. 2013; Walker et al. 2013). Additionally, patients affected by MPS face a substantial number of surgical procedures (and therefore intubations) during childhood and adolescence (Young and Harper 1979; Arn et al. 2012).

A high postsurgical mortality rate due to respiratory and cardiac disease has been reported in MPS patients, with

higher mortality among those with a more severe phenotype (Young and Harper 1979; Arn et al. 2012). Among 196 deceased patients with MPS type I, 32 had surgery within 1 month of death, including 20 who had surgery within 10 days of death (Arn et al. 2012).

Several reports have been published concerning the difficulties in airway management among MPS patients (Adachi and Chole 1990; Nicolson et al. 1992; Gaitini et al. 1998; Walker et al. 2003; Ard et al. 2005). Aside from the difficulties in airway management, the use of neuromuscular blocking agents, the prolongation of anesthesia and/or aggressive ventilator support will contribute to the deterioration of these findings.

Airway obstructions may be localized in any of the physiological airways, from the nose to the peripheral bronchiae (Steven Sims and Kempiners 2007). While the upper airways are usually obstructed by large amounts of storage material, the more common complications seen in the trachea and bronchiae are tracheomalacia and bronchomalacia, which sometimes lead to complete major airway collapse (Morehead and Parsons 1993). This malacia may occur in combination with bronchial and tracheal stenosis due to large storage material "tumors" in the mucosa of airways and surrounding tissues, leading to a compression of the airway (Morehead and Parsons 1993). Usually, these stenoses occur around adolescence and are progressive, and may lead to almost complete obstruction of a bronchus or the trachea. Because of upper airway obstruction, a substantial number of MPS patients are treated with tracheotomy (Jeong et al. 2006). But, tracheostomy itself may cause significant problems to the patients, when the auto-positive end-expiratory pressure (PEEP) function of the glottis is reduced or excluded, and an airway collapse caused by the malacia will become apparent. It is reported that the most common cause of death in Hunter syndrome (MPS type II, at least in the attenuated phenotype) is impairment of cardiorespiratory function, which may aggravate the neurological deterioration, and these patients do not usually survive beyond adolescence (Brama et al. 1986; Gaitini et al. 1998; Gross and Lemmens 2010). For MPS types I, II, and VI, enzyme replacement therapy (ERT) has been available for several years. ERT with recombinant human *N*-acetylgalactosamine 4-sulfatase has been shown to halt and partially reverse the decline in pulmonary function in patients with MPS type VI [Harmatz et al. 2010]. However, to date, there are no reports available regarding the impact of ERT on airway obstruction or malacia. In Hurler syndrome, even after early bone marrow transplantation and donor engraftment, tracheal stenosis and tracheomalacia may occur in the long term and complicate the course of the patient (Valayannopoulos et al. 2010). It is noteworthy that the reported Hurler patient developed 12 years after successful engraftment, without signs of graft-versus-host disease and with leukocyte enzyme activity of 50% of normal, severe pulmonary hypertension and tracheal and bronchial obstructions and malacia, which resolved after additional ERT with weekly infusions of 100 U/kg of α-L-ironidase.

It is therefore conceivable that by extending the life expectancy of the patients with ERT, tracheobronchial airway obstruction or malacia may become more prominent at advanced ages.

No conclusive management guidelines exist regarding the treatment of upper airway obstructions by tracheotomy to date or for the treatment of tracheomalacia in combination with severe tracheal stenosis in patients with MPS. Many patients therefore remain untreated, mandatory surgical procedures are withheld, and they ultimately die.

Presentation of Case 1

We report a 20-year-old female patient with severe MPS type VI (Maroteaux–Lamy syndrome). At the age of 17 years, the patient received a tracheotomy during a neurosurgical procedure because of complicated intubation and the inability to wean the patient off ventilation because of upper airway obstruction. At the same time, the patient started ERT. Besides her severe clinical phenotype, the patient's situation was complicated by moderate mitral and aortic valve stenosis, and moderate mitral valve regurgitation. Since the age of 17 years, the patient developed progressive respiratory impairment with the need for home ventilation overnight. During an upper airway infection, the patient developed severe respiratory failure with sepsis and required continuous 24-h ventilation. During a 3-month period of intensive care, the patient's respiratory function deteriorated further with the need for assisted spontaneous ventilation with a PEEP of 18 mmHg and a pressure support of 10 mmHg. Multiple attempts failed to wean the patient off ventilation.

Bronchoscopy revealed subtotal tracheal stenosis (beyond which the bronchoscope could not pass) and tracheomalacia. A three-dimensional computed tomography scan showed a large tumorous mass infiltrating and impressing the lower trachea proximal to the bronchial bifurcation and impressing the origin of the left main bronchus (Fig. 1a, b). Under a PEEP of 18 mmHg, the inner lumen of the tracheal stenosis was <3 mm. By reduction of PEEP to <10 mmHg, the trachea collapsed and showed a total occlusion of the airway. It was demonstrated during bronchoscopy that the long-term high PEEP ventilation

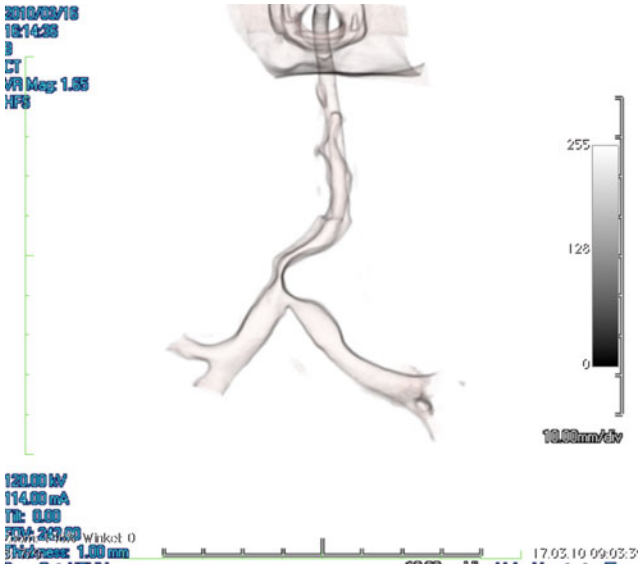

Fig. 1 3D computerized tomography scans showing severe tracheal stenosis with tracheo-bronchomalacia of the left main bronchus during hold inspiration with a positive end-expiratory pressure (PEEP) of 18 mmHg. Note severe obstruction of the trachea and bifurcation, and the post-stenotic bronchomalacia. The patient was ventilated with intubation past the tracheostoma but with its end remaining proximal to the tracheal stenosis

may have aggravated the tracheomalacia above the stenosis by un-physiological high pressure ventilation. All assessments were done under mild sedation without deep anesthesia, and during bronchoscopy additional local anesthesia. These findings were judged as being untreatable by surgery.

After informed consent from the patient and her parents, it was decided to attempt a palliative stent implantation into the tracheal stenosis. The procedure was performed under fluoroscopic and bronchoscopic control. After passing a guide wire across the stenosis into the left main bronchus, a sizing of the internal diameter of the stenosis was performed with an inflatable balloon (Fig. 2a). Because the stenosis was in close proximity to the tracheal bifurcation, two guide wires were advanced through the stenosis into both the right and the left main bronchi. One 22-mm long, uncovered bare platinum/iridium stent (CP8Z22 Stent, NuMED Inc., Hopkinton, NY) was mounted on two balloon catheters (20-mm long and 6-mm diameter when fully inflated) simultaneously. This ensemble of stent and two balloons was advanced over both guide wires across the tracheal stenosis and placed into the bifurcation (Fig. 2b). Both balloon catheters were simultaneously inflated to their nominal diameter. Thereafter, each balloon catheter was exchanged to a 12-mm balloon catheter and alternately inflated to allow airflow to the left and right main bronchi (Fig. 2c). Immediately after stent implantation, PEEP could be reduced to 8 mmHg. No tracheal rupture, stent migration, or dysphagia occurred.

Three weeks after the first stent implantation, a second stent was placed at the proximal end of the initial previously implanted stent and dilated to 12 mm. The patient recovered well and could be weaned off after 24-h ventilation. Bronchoscopy at discharge showed an open trachea (Fig. 3a) and a free origin to the left main bronchus (Fig. 3b). At 18 months after the second procedure, the patient needed re-dilatation of the implanted stents to 12-mm diameter because of granulomatous tissue formation. Two years after stenting of the trachea, the patient showed no signs of respiratory failure or requirement for single-sided ventilation.

Presentation of Case 2

A 23-year-old severely affected patient with Hunter syndrome without major neurological involvement on ERT since 2008 required an operation because of the failure of his implanted port system. The introduction of anesthesia was complicated by pharyngeal obstructions and the stiffness of the cranio-cervical junction. After successful oro-tracheal intubation and operation, the patient could not be weaned from ventilation anymore, requiring a PEEP of 15 mmHg and a pressure support of 10 mmHg. Even after tracheostomy, ventilation could not be reduced and the patient could not be weaned from the ventilator. Tracheography under fluoroscopic control and bronchoscopy, all procedures were performed under mild intravenous sedation

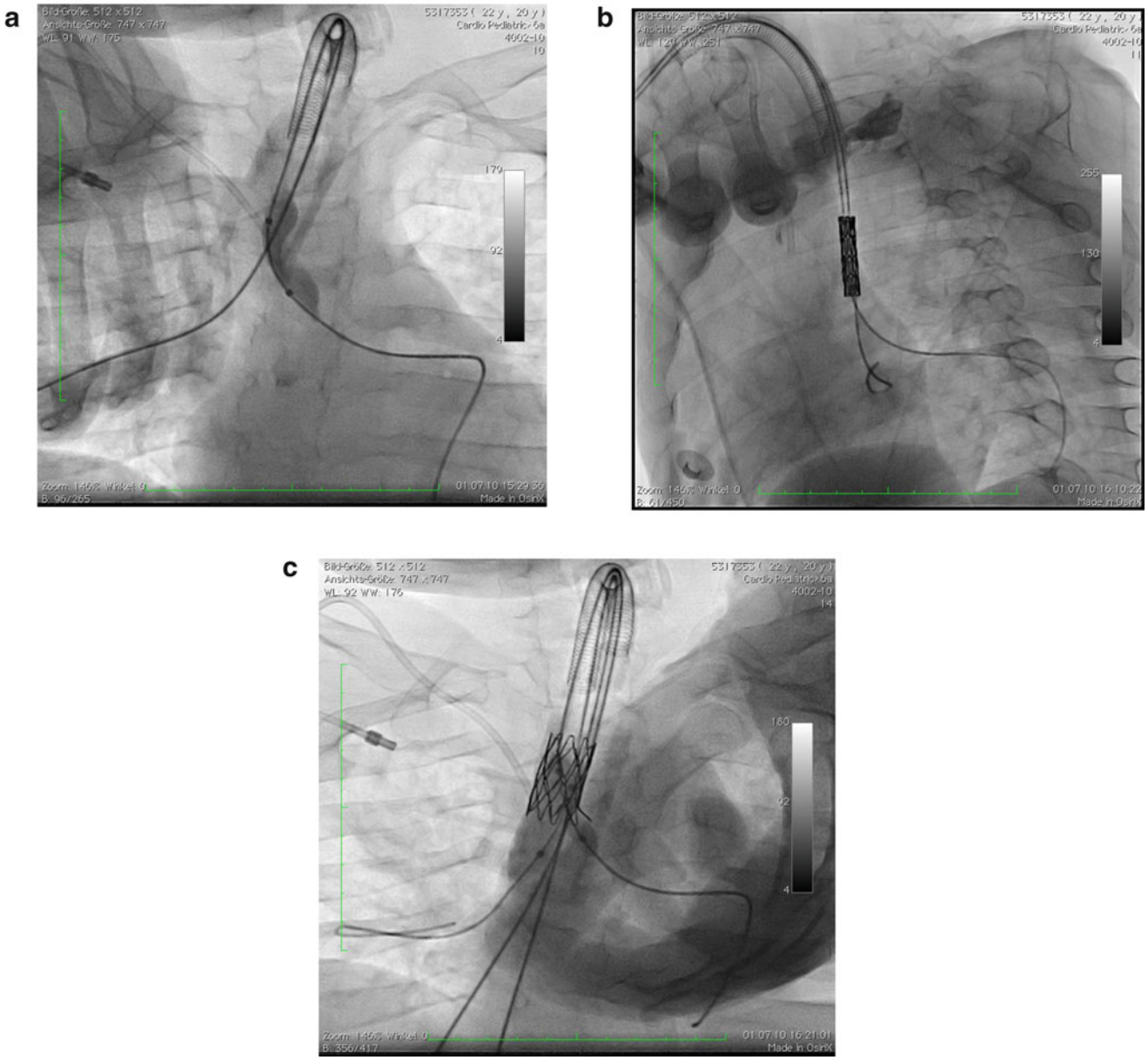

Fig. 2 Balloon sizing of the true tracheal stenosis after placing a guide wire into the left lower bronchus. Note the severe narrowing of the trachea at the bifurcation (**a**). After placing guide wires into the right and left main bronchus the advanced ensemble of a stent mounted on two balloons immediately before inflation (**b**). Fully deployed stent with two balloons inflated: one balloon in the right main bronchus (12-mm diameter) and the other balloon in the left main bronchus (6-mm diameter). Note the stent opening to the left main bronchus to avoid tumorous obstruction of the main left bronchus (**c**)

with midazolam with doses between 0.02 and 0.05 mg/kg body weight, revealed a total tracheal collapse, once the PEEP was reduced below 8 mmHg. The tracheal collapse affected the whole trachea. Therefore, the above mentioned approach was used to stent the trachea from the bifurcation upwards to the tracheostoma. Here the trachea was stented to an internal diameter of 14 mm. After successful implantation of two CP stents over a total length of 48 mm the distal tip of the tracheal cannula was advanced 5 mm within the proximal end of the stent (Fig. 4). Immediately after stent deployment the ventilation could be reduced to a spontaneous mode with a PEEP of 5 mmHg and a pressure support of 10 mmHg. During follow-up, the patient could be weaned from ventilation. Aside from

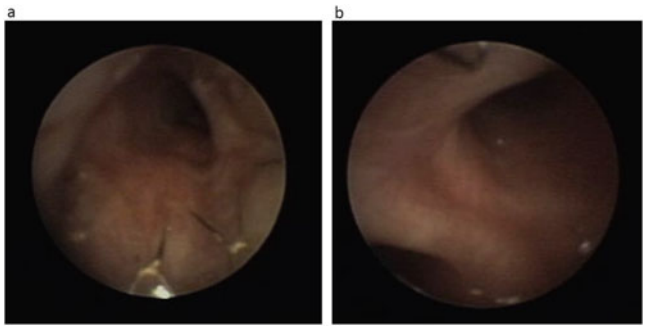

Fig. 3 Bronchoscopy before discharge of the patient. The tracheal airway is almost free of stenosis, and the stent is in place with mild granulation tissue (**a**). Origin of the left main bronchus at 2 o'clock and distal stent ending at 12 o'clock. Note that the entrance of the left main bronchus is only minimally impressed (**b**)

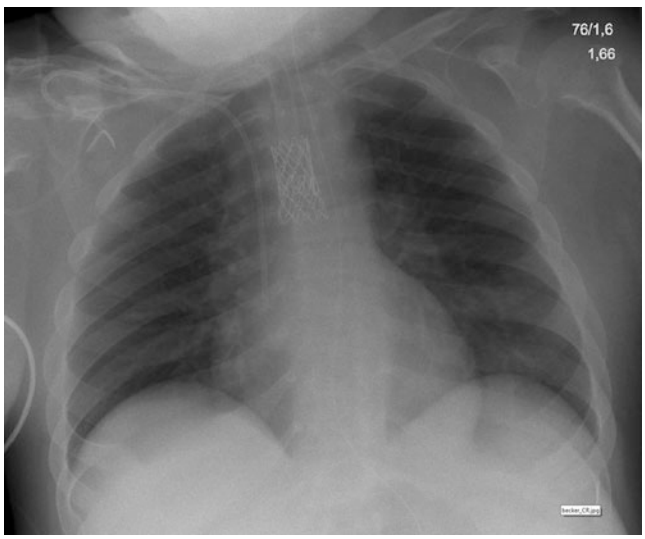

Fig. 4 Chest radiogram of the second patient with complete tracheomalacia and after stenting of the trachea. Of note, the tracheal cannula reaches in the upper orifice of the stent. Patient is spontaneously breathing

tracheostomy, the patient has not required mechanical ventilation and has had just one upper airway infection in 22 months of follow-up.

Discussion

Tracheobronchial obstructions are infrequent in pediatric or adolescent subjects unaffected by MPS, but when they are seen, they are associated with significant morbidity and mortality (Anton-Pacheco et al. 2008). Airway obstruction is usually caused by malignant diseases in adults and occurs more frequently after reconstructive surgery in children (Nicolai 2008). Nevertheless, malacia and stenosis, either congenital or acquired, are the most frequent airway anomalies encountered (Davitt et al. 2002). Symptomatic airway stenosis is usually treated surgically with good results so intraluminal airway stenting remains a therapeutic approach for only very selected cases (Filler et al. 1998; Nicolai et al. 2001; Nicolai 2008), and may be accompanied by potential long-term complications, like chronic pulmonary infections, re-obstruction, and migration of the implanted stent into surrounding structures.

These cases are the first reports of palliative permanent tracheal stenting in a patient with severe Maroteaux–Lamy (MPS type VI) and Hunter (MPS type II) syndrome. Furthermore, the presented technique of stent implantation, using a double-wire double-balloon technique, has not been previously described. It offers the possibility of tracheal stenting in the immediate proximity to the tracheobronchial bifurcation with preservation of airflow to both the right and left main bronchi.

Following a review of the literature, airway stenting has only been reported in two MPS patients, although there are several reports of bronchio-tracheal stenting among non-malignant cases, for which indications were mainly tracheo- or bronchomalacia, but rarely stenosis (Furman et al. 1999). One patient with MPS type II (Hunter syndrome) had peripheral bronchial stenosis (Davitt et al. 2002) and another patient with MPS type IV (Morquio syndrome) had bronchial malacia (Anton-Pacheco et al. 2008). Unfortunately, follow-up data were not presented in either patient (Davitt et al. 2002; Anton-Pacheco et al. 2008).

Other therapeutic options for the treatment of tracheal stenosis in MPS patients might include the use of CO_2 or Nd-Yak laser surgery for either upper or lower airway stenosis (Lin et al. 2000; Mierzwinski et al. 2006). However, laser surgery might fail with a combination of stenosis and malacia. Bronchio-tracheal stenosis with tracheomalacia has been reported for MPS types I, II, and VI, while tracheomalacia might occur predominantly in MPS type IV (Shapiro et al. 1985; Semenza and Pyeritz 1988). It seems that from all MPS patients, those with abnormal dermatan sulfate storage are at risk for developing tracheobronchial complications. It has been shown that in non-MPS patients approximately 14.8% of the dry weight of tracheal material consists of GAGs, which usually bind to hyaluronan (Roberts and Paré 1991, Rains et al. 1992). During aging, around 30% of the chondroitin sulfates are being replaced by keratan sulfate, which has less water binding capacity, and is therefore responsible for the decrease of elasticity of the airways during aging (Rains 1992). Nevertheless, except within the cartilage, dermatan sulfate accounts for 20–40% of the weight of the tracheal muscles, the adventitia, and the intercartilage tissue, while heparan sulfate plays a neglectible role (Binette et al. 1994). Therefore it can be suggested that in these tracheal structures a high turnover of dermatan sulfates takes place and in diseases with a disturbed dermatan sulfate metabolism a missing dermatan sulfate brake down will lead to a substantial storage of material within these structures.

Even after bone marrow transplant, a close follow-up of these patients is mandatory, and once those patients are showing signs of deterioration of pulmonary function, or the development of trachea bronchial complications or pulmonary hypertension, increasing the dose of ERT or initiating enzyme replacement should be considered. Furthermore, the use of neuromuscular blocking agents, prolonged anesthesia and/or aggressive ventilator support should be limited or avoided, because it may contribute to the deterioration of these trachea bronchial complications.

Nevertheless, stent implantation offers a palliative *ultima ratio* approach to treat bronchio-tracheal stenoses and/or with malacia and maintain open airways in these patients permanently.

Synopsis

We report the first cases and their follow-up of tracheal stenting using a new technique in patients with MPS type VI and II who had almost complete tracheal occlusion and total airway collapse.

Conflict of Interest

Christoph Kampmann declares that he has no conflict of interest.

Christiane M. Wiethoff declares that she has no conflict of interest.

Ralf G. Huth declares that he has no conflict of interest.

Eugen Mengel declares that he has no conflicts of interest.

Gundula Staatz declares that she has no conflicts of interest.

Michael Beck declares no conflicts of interest.

Stefan Gehring declares no conflicts of interest.

Torsten Mewes declares that he has no conflicts of interest.

Tariq Abu-Tair declares that he has no conflicts of interest.

Christoph Kampmann has received honoraria for lectures and compensation for travel and accommodation from Biomarin and Shire.

Eugen Mengel has received honoraria for lectures and compensation for travel and accommodation from Biomarin and Shire and an unrestricted grant from Genzyme.

Michael Beck has received honoraria for lectures and compensation for travel and accommodation and unrestricted grants from Shire, Genzyme, Actelion, and Biomarin.

All authors declare that they have no competing interests.

Informed Consent

Treated and reported patients were in end-stage disease with an acute life-threatening condition; it was the absolute wish of the parents and guardians to offer the patients a treatment strategy. Therefore, the basis of this report is on intention to treat.

Details of the Contribution of Individual Authors

CK, TW, RGH, and CMW have conducted the interventions,

CK and CMW have written the manuscript,

GS and TA-T delivered 3D scanning,

MB, EM, and SG taken care of the patients before and during procedures,

CK, CMW, and TA-T were planning and reporting the work described in this article, and

MB and EM made important contributions to the manuscript.

References

Adachi K, Chole RA (1990) Management of tracheal lesions in Hurler syndrome. Arch Otolaryngol Head Neck Surg 116:1205–1207

Anton-Pacheco JL, Cabezali D, Tejedor R et al (2008) The role of airway stenting in pediatric tracheobronchial obstruction. Eur J Cardiothorac Surg 33:1069–1075

Ard JL Jr, Bekker A, Frempong-Boadu AK (2005) Anesthesia for an adult with mucopolysaccharidosis I. J Clin Anesth 17:624–626

Arn P, Whitley C, Wraith JE et al (2012) High rate of postoperative mortality in patients with mucopolysaccharidosis I: findings from the MPS I Registry. J Pediatr Surg 47:477–484

Baehner F, Schmiedeskamp C, Krummenauer F et al (2005) Cumulative incidence rates of the mucopolysaccharidoses in Germany. J Inherit Metab Dis 28:1011–1017

Binette JP, Burgi W, Ohishi H, Grundboeck-Jusko J, Burki R, Maekawa Y, Tschopp FA, Kimura A, Schmid K (1994) The glycosaminoglycan composition of human tracheas and the changes observed during aging and disease. Clin Chem Acta 225:179–185

Brama I, Gay I, Feinmesser R, Springer C (1986) Upper airway obstruction in Hunter syndrome. Int J Pediatr Otorhinolaryngol 11:229–235

Davitt SM, Hatrick A, Sabharwal T, Pearce A, Gleeson M, Adam A (2002) Tracheobronchial stent insertions in the management of major airway obstruction in a patient with Hunter syndrome (type-II mucopolysaccharidosis). Eur Radiol 12:458–462

Filler RM, Forte V, Chait P (1998) Tracheobronchial stenting for the treatment of airway obstruction. J Pediatr Surg 33:304–311

Furman RH, Backer CL, Dunham ME, Donaldson J, Mavroudis C, Holinger LD (1999) The use of balloon-expandable metallic stents in the treatment of pediatric tracheomalacia and bronchomalacia. Arch Otolaryngol Head Neck Surg 125:203–207

Gaitini L, Fradis M, Vaida S et al (1998) Failure to control the airway in a patient with Hunter's syndrome. J Laryngol Otol 112:380–382

Gross ER, Lemmens HJ (2010) Hunter syndrome in an adult: beware of tracheal stenosis. Anesth Analg 110:642–643

Harmatz P, Yu Z-F, Giugliani R et al (2010) Enzyme replacement therapy for mucopolysaccharidosis VI: evaluation of long-term pulmonary function in patients treated with recombinant human N-acetylgalactosamine 4-sulfatase. J Inherit Metab Dis 33:51–60

Jeong HS, Cho DY, Ahn KM, Jin DK (2006) Complications of tracheotomy in patients with mucopolysaccharidoses type II (Hunter syndrome). Int J Pediatr Otorhinolaryngol 70:1765–1769

Lin CM, Hsu JC, Liu HP, Li HY, Tan PP (2000) Anesthesia of CO_2 laser surgery in a patient with Hunter syndrome: case report. Chang Gung Med J 23:614–618

Mierzwinski J, Zwierz A, Jaworski A et al (2006) The use of Nd-Yag laser in the treatment of tracheal tumor in 14 years old child with mucopolysaccharidosis type II. Otolaryngol Pol 60:603–606

Morehead JM, Parsons DS (1993) Tracheobronchomalacia in Hunter's syndrome. Int J Pediatr Otorhinolaryngol 26:255–261

Muenzer J (2004) The mucopolysaccharidoses: a heterogenous group of disorders with variable pediatric presentations. J Pediatr 144 (5 Suppl):S27–S34

Muhlebach MS, Wooten W, Muenzer J (2011) Respiratory manifestations in mucopolysaccharidoses. Paediatr Respir Rev 12:133–138

Muhlebach MS, Shaffer CB, Georges L, Abode K, Muenzer J (2013) Bronchoscopy and airway management in patients with mucopolysaccharidoses (MPS). Pediatr Pulmonol 48:601–607

Nelson J (1997) Incidence of the mucopolysaccharidoses in Northern Ireland. Hum Genet 101:355–358

Neufeld EF, Muenzer J (2001) The mucopolysaccharidoses. In: Scriver CR, Beaudet AL, Sly WS (eds) The metabolic and molecular basis of inherited disease. McGraw Hill, New York, pp 3421–3452

Nicolai T (2008) Airway stents in children. Pediatr Pulmonol 43:330–344

Nicolai T, Huber RM, Reiter K, Merkenschlager A, Hautmann H, Mantel K (2001) Metal airway stent implantation in children: follow-up of seven children. Pediatr Pulmonol 31:289–296

Nicolson SC, Black AE, Kraras CM (1992) Management of a difficult airway in a patient with Hurler-Scheie syndrome during cardiac surgery. Anesth Analg 75:830–832

Rains JK, Bert L, Roberts CR, Paré PD (1992) Mechanical properties of human tracheal cartilage. J Appl Physiol 72(1):219–225

Roberts CR, Paré PD (1991) Composition changes in human tracheal cartilage in growth and aging, including changes in proteoglycan structure. Am J Physiol 261:L92–L101

Semenza GL, Pyeritz RE (1988) Respiratory complications of mucopolysaccharide storage disorders. Medicine (Baltimore) 67:209–219

Shapiro J, Strome M, Crocker AC (1985) Airway obstruction and sleep apnea in Hurler and Hunter syndromes. Ann Otol Rhinol Laryngol 94:458–461

Steven Sims H, Kempiners JJ (2007) Special airway concerns in patients with mucopolysaccharidoses. Respir Med 101:1779–1782

Valayannopoulos V, de Blic J, Mahlaoui N et al (2010) Laronidase for cardiopulmonary disease in Hurler syndrome 12 years after bone marrow transplantation. Pediatrics 126:e1242–e1247

Walker PP, Rose E, Williams JG (2003) Upper airways abnormalities and tracheal problems in Morquio's disease. Thorax 58:458–459

Walker R, Belani KG, Braunlin EA et al (2013) Anaesthesia and airway management in mucopolysaccharidosis. J Inherit Metab Dis 36:211–219

Yoskovitch A, Tewfik TL, Brouillette RT, Schloss MD, Der Kaloustian VM (1998) Acute airway obstruction in Hunter syndrome. Int J Pediatr Otorhinolaryngol 44:273–278

Young ID, Harper PS (1979) Long-term complications in Hunter's syndrome. Clin Genet 16:125–132

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RESEARCH REPORT

Brain White Matter Integrity Mediates the Relationship Between Phenylalanine Control and Executive Abilities in Children with Phenylketonuria

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Abstract We tested the hypothesis that brain white matter integrity mediates the relationship between phenylalanine (Phe) control and executive abilities in children with phenylketonuria (PKU; $N = 36$). To do so, we examined mean diffusivity (MD) from diffusion tensor imaging (DTI) in two white matter brain regions (posterior parietal–occipital, PPO; centrum semiovale, CSO) and lifetime phenylalanine (Phe) exposure; the executive abilities examined included verbal strategic processing, nonverbal strategic processing, and working memory. Mediation modeling showed that MD in the PPO and CSO mediated the relationship between Phe exposure and nonverbal strategic processing, MD in the CSO mediated the relationship between Phe exposure and verbal strategic processing, and MD in the PPO mediated the relationship between Phe exposure and working memory. These exploratory findings demonstrate the importance of using sophisticated modeling procedures to understand the interplay among metabolic control, neural factors, and functional outcomes in individuals with PKU.

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Introduction

Phenylketonuria (PKU; 261600) is an inherited metabolic disorder associated with a deficiency in or absence of the phenylalanine hydroxylase enzyme (EC 1.14.16.1). As a consequence, the amino acid phenylalanine (Phe) is improperly metabolized, which leads to higher than normal Phe levels (De Groot et al. 2010). Although serious cognitive sequelae are generally avoided through early detection and dietary treatment to limit Phe intake (Mitchell et al. 2011; Paine 1957), individuals with early and continuously treated PKU often have lower than expected intellectual abilities (Waisbren et al. 2007), as well as impairments in processing speed (Janos et al. 2012) and executive abilities (Christ et al. 2010; DeRoche and Welsh 2008).

For decades it has been hypothesized that PKU-related cognitive impairment is associated with dopamine deficiency (De Groot et al. 2010), because elevations in Phe disrupt the neurochemical cascade by which Phe is converted to tyrosine, a precursor of dopamine, and other catecholaminergic neurotransmitters (Scriver 2007). Compromised white matter integrity in the brain, however, is another possible mechanism underlying PKU-related cognitive impairment and represents the focus of the current study. Recent research using diffusion tensor imaging (DTI) indicates that white matter compromise is widespread throughout the brain in individuals with early and continuously treated PKU (Antenor-Dorsey et al. 2013; Hood et al. 2014a). More specifically, although fractional anisotropy

(FA; reflecting the degree of water diffusion asymmetry) is relatively normal, mean diffusivity (MD; reflecting degree of displacement of water molecules) is significantly decreased (Antenor-Dorsey et al. 2013; Scarabino et al. 2009; White et al. 2010, 2013).

In terms of relationships between white matter integrity, cognition, and Phe control in individuals with PKU, a number of studies have shown that higher Phe is associated with both decreases in MD (e.g., Vermathen et al. 2007; Hood et al. 2014a) and poorer cognition (e.g., Christ et al. 2010; Weglage et al. 2013). Our knowledge of relationships between DTI findings and cognition is quite limited, but the three relevant studies to date point to associations between MD across a range of brain regions and IQ (Peng et al. 2004, 2013; Antenor-Dorsey et al. 2013). There is, however, no research in which the interplay among white matter integrity, cognition, and Phe control has been modeled within a single study. The purpose of this exploratory study was to address this substantial gap in our knowledge of PKU. Specifically, we tested the hypothesis that white matter integrity mediates the relationship between Phe control and executive abilities.

Material and Methods

Participants

Children with PKU ($n = 36$; 17 males, 19 females) were diagnosed soon after birth and received early dietary management to limit Phe intake. Phe levels were obtained from heparinized plasma done via MS/MS in children fasted for a minimum 2.5–3 h before the blood draw. Lifetime Phe levels, with gaps of no more than 2 years prior to neuroimaging and cognitive evaluation, were available for all children and ranged from 0 to 2742 μmol/L (sample; $M = 371.1$, SD = 282.5, males; $M = 420.3$, SD = 303.9, females; $M = 352.6$, SD = 271.9). Age ranged from 6 to 18 years (sample; $M = 12.2$, SD = 3.8, males; $M = 12.3$, SD = 3.7, females; $M = 12.2$, SD = 4.1), education ranged from 0 to 13 years ($M = 6.4$, SD = 3.8), and IQ ranged from 75 to 122 (sample; $M = 102.1$, SD = 10.9, males; $M = 75$, SD = 117, females; $M = 86$, SD = 122). No child had a reported history of major medical, psychiatric, or learning disorder unrelated to PKU, and no child was treated with sapropterin dihydrochloride at the time of study.

Procedures

Approval to conduct this study was obtained from institutional review boards for the protection of human subjects at Washington University in St. Louis (WU) and Oregon Health & Science University (OHSU). All participants and/or guardians provided written informed consent prior to initiation of study procedures. Referring metabolic clinics provided blood Phe levels over the lifetime based on medical records. Executive and neuroimaging procedures were administered during a single session lasting approximately 4 h. Some data reported here were used in previous reports (e.g., Hood et al. 2014a, b) but not in relation to mediation modeling.

Index of Phe Control

Mean Phe exposure over the lifetime was computed based on all available Phe levels prior to evaluation. The number of lifetime blood Phe levels for individual children who participated in our study ranged from 86 to 466 ($M = 215.0$, SD = 98.0). The rationale for examining this index is detailed elsewhere (Hood et al. 2014a). Briefly, mean Phe exposure was computed to take into account the duration (i.e., years) and accumulative effects of exposure to elevations in Phe, because older children with PKU have experienced more prolonged exposure to elevated Phe than younger children. As the first step in calculating mean exposure, we obtained the mean and standard deviation of lifetime Phe and age across the entire sample of children with PKU. Z scores for lifetime Phe and age were then computed for each child based on the means and standard deviations of the sample. Mean exposure for each child was then calculated by summing the z scores for lifetime Phe and age. This method of calculation for mean exposure ($M = 0$, SD = 1.8, range = −2.5–4.5) results in scores that approximate a normal distribution, with higher scores indicating greater exposure.

Executive Abilities

The Matrix Reasoning subtest from the Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler 1999) was used to measure nonverbal strategic processing. During this task, children viewed series of incomplete matrices and selected which of five solutions best completed each matrix. The number of correct completions was recorded. A verbal wordlist learning task was used to measure verbal strategic processing. Children listened as a list of 18 words was read aloud then orally recalled as many words as possible in any order over five learning trials. The list comprised six words from each of three semantic categories (e.g., furniture, food, and parts of the body). A ratio reflecting the number of words reported serially in semantic clusters to the total number of words recalled over the five trials was recorded. An n-back task with two conditions, location and letter, was used to measure working memory.

Children observed 1 of 8 letters (C, F, H, J, N, P, Q, and S) appearing alone at 1 of 8 locations along an imaginary circle on a computer monitor. In the location condition, children pressed a button when any letter appeared in the same location as two trials previously; in the letter condition, children pressed a button when the letter presented was identical to the letter presented two trials earlier. Otherwise, children withheld responses. The mean number of correct nonresponses averaged across location and letter conditions was recorded.

Standard scores ($M = 100$, SD = 15) from each task were used in mediation analyses. Matrix Reasoning standard scores in children with PKU ($M = 99.3$, SD = 11.1, range = 74.5–116.5) were based on age-referenced normative data that accompanied the subtest. Normative data were not available for the experimental wordlist learning and n-back tasks. However, to provide a similar interpretative context, we computed age-referenced standard scores for each task for children with PKU (wordlist learning: $M = 99.4$, SD = 8.2, range = 86.2–116.5; n-back task: $M = 100.6$, SD = 7.2, range = 89.0–113.1) based on data previously collected in our laboratory from a group of 80 healthy control children who ranged in age from 7 to 18 years ($M = 12.4$, SD = 3.2).

White Matter Integrity

Children were scanned with a 3.0T Siemens Trio at OHSU and with a 1.5T Siemens Sonata at WU. DTI was acquired using an echo planar imaging (EPI) sequence (TR = 9,000 ms, TE = 84 ms (OHSU) and 78 ms (WU), 2.5 mm (OHSU) and 3.0 mm (WU) isotropic voxels, conventional hexahedral (6 direction) encoding with diffusion sensitization of *b*-values = 0 and 1,000 s/mm^2). Four complete DTI datasets were acquired for each participant, with a total imaging time of approximately 1 h. The first image-processing step was registration of all images. We defined the spatial relationships between all images in terms of affine transforms. T2W image registration was accomplished using vector gradient measure maximization. The first acquired, unsensitized ($b = 0$ s/mm^2; I0) DTI volume was registered to the T2W image; stretch and shear were enabled (12 parameter affine transform) to partially compensate for EPI distortion. The remaining DTI images were then registered to the unsensitized DTI volume. The diffusion tensor and its three eigenvalues were calculated using log-linear regression in each voxel for each ROI (Shimony et al. 1999). Using standard methods, the DTI parameters were computed from the eigenvalues.

To minimize false-positives, we focused on two white matter brain regions: posterior parietal–occipital (PPO) and centrum semiovale (CSO) (see Fig. 1). These regions were selected based on a well-established DTI atlas (Oishi et al. 2008), and placement of ROIs was compared on each participant's FA map and TW2 images simultaneously. ROIs were shifted by a few voxels as necessary by a trained neuroradiology technician to better conform to each individual's native anatomy. Finalized ROIs were then applied to each subject's mean diffusivity, axial diffusivity, and radial diffusivity parametric maps and sampled using Analyze 8.0 software similar to the methods of Shimony et al. 1999. Raters have established interrater correlation coefficients above 0.90 for mean diffusivity values for all ROIs.

We have previously shown that MD in these ROIs was significantly lower in individuals with PKU than in age-matched controls (White et al. 2013) and that MD in these ROIs was related to a range of indices of Phe control (Hood et al. 2014b). In addition, visually observable white matter abnormalities often occur in the PPO and CSO in individuals with PKU (Citton et al. 2012). For mediation analyses, standard scores were generated for MD based on data from a subset ($N = 62$; not all children completed neuroimaging) of the healthy control children whose data were used to generate executive abilities standard scores.

Data Analyses

As a starting point in our analyses, we conducted Pearson correlations to determine the simple bivariate relationships between mean Phe exposure over the lifetime, MD in the PPO and CSO, and executive abilities. Statistical rigor was increased by considering findings significant only if $p < 0.05$ and effect sizes were either medium or large (Cohen 1988).

For mediation analyses, we used a bootstrapping approach, which is a non-parametric resampling procedure for the assessment of indirect effects (Preacher and Hayes 2004, 2008). This method used an ordinary least squares regression-based path analytic framework for estimating direct and indirect effects. Mediation analyses indicate whether the total effect (weight *c*) of an independent variable (IV; mean Phe exposure) on a dependent variable (DV; nonverbal strategic processing, verbal strategic processing, or working memory) comprises a direct effect (weight *c*') of an IV on a DV and an indirect effect (weight $a \times b$) of an IV on a DV through a predicted mediator (MD). Weight *a* denotes the effect of an IV on a mediator, whereas weight *b* denotes the effect of a mediator on a DV. From our original dataset of 36 cases, random sampling with replacement generated a bootstrap sample of 36 cases. Repeating the process 5,000 times provided the basis for the bootstrap estimates. Means and standard errors of the 5,000 samples were then calculated.

Current recommendations indicate that inferences should not be based on the significance of paths *a* and *b*; instead, inferences should be an explicit quantification of the

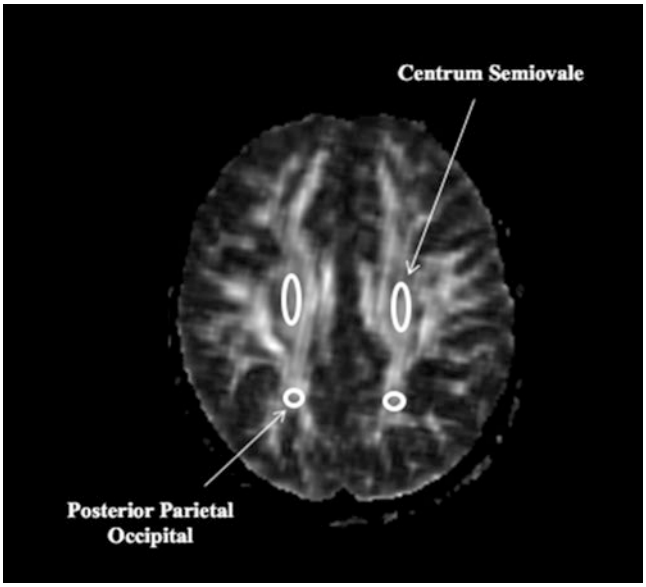

Fig. 1 ROI placement

Table 1 Correlations between MD, Phe exposure, and executive abilities

Variable	PPO	CSO
Nonverbal strategic processing	0.34*	0.35*
Verbal strategic processing	0.22	0.41*
Working memory	0.36*	0.19
Mean Phe exposure	−0.65*	−0.41*

Note: * = $p < 0.05$ with medium or large effect sizes

indirect effect, and significant indirect effects can occur in the absence of significant total or direct effects (Preacher and Hayes 2004, 2008). Given our small sample size, we focused on Kappa-squared (κ^2) to measure effect size because it is standardized and insensitive to sample size. Kappa-squared represents the proportion of the total possible effect in the sample and it can be interpreted analogous to R^2, with a kappa-squared of 0.01, 0.09, and 0.25 representing small, medium, and large effects, respectively. In addition, due to small sample size and the exploratory nature of the current study, there was no correction for multiple statistical comparisons. To increase statistical rigor, indirect effects were considered significant only if zero was not within the 95% confidence interval (CI), $p < 0.05$ two tailed, and effect size was medium or large. Given that three aspects of executive abilities and two ROIs were examined, a total of six mediation analyses were conducted.

We would also note that exploratory mediation analyses were conducted to determine whether subcomponents of MD, including axial diffusivity (AD) and radial diffusivity (RD), mediated the relationship between Phe exposure and executive abilities. Because results from these analyses were not explanatory beyond those conducted using MD, they are not discussed further.

Results

Correlation Analyses

Pearson correlations indicated that MD in the PPO was significantly related to nonverbal strategic processing and working memory, whereas MD in the CSO was significantly related to nonverbal and verbal strategic processing. MD in both the PPO and CSO was significantly related to mean Phe exposure. Executive abilities were not significantly related to mean Phe exposure, but because significant indirect effects may be present in the absence of total or direct relationships, we next conducted mediation analyses (Preacher and Hayes 2004; Rucker et al. 2011) (Table 1).

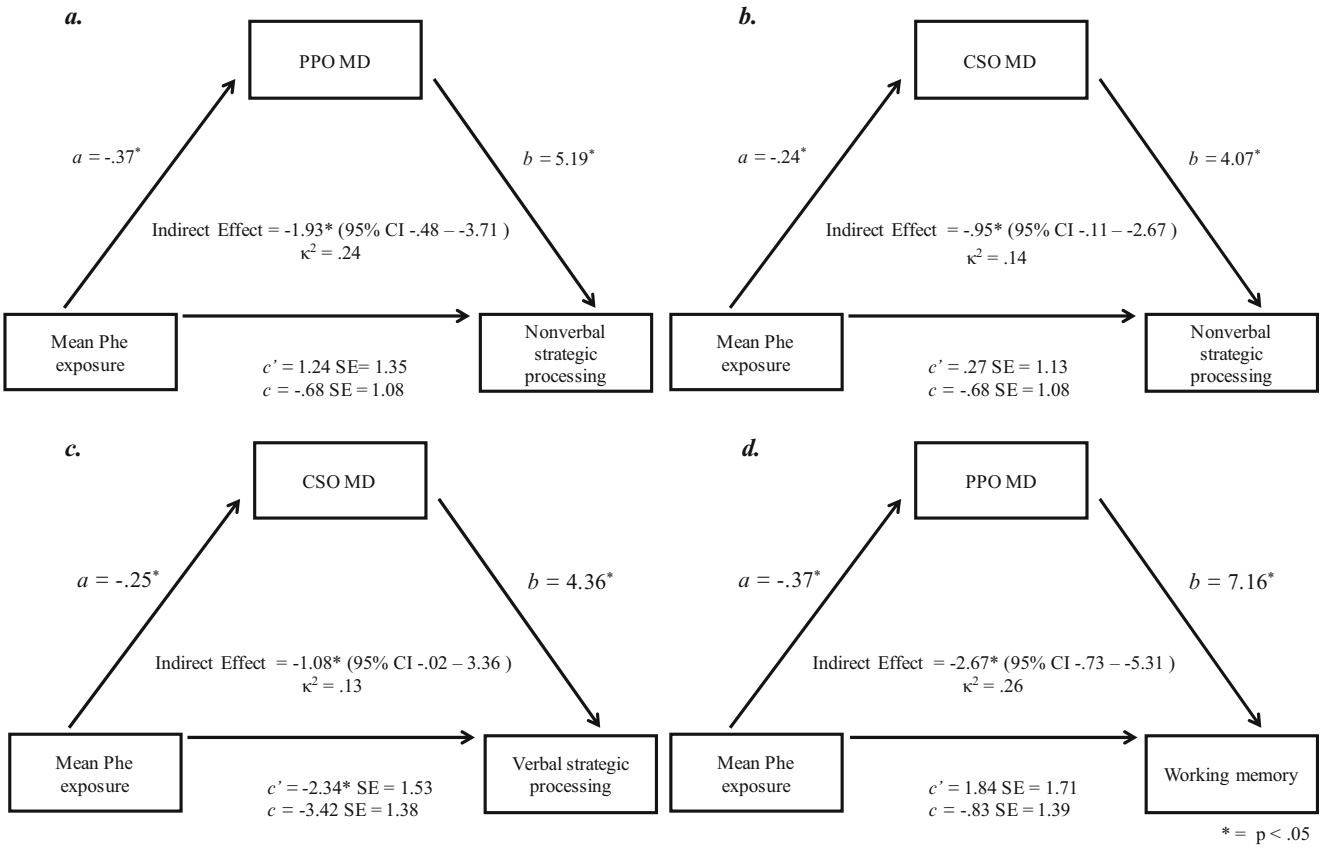

Fig. 2 PPO and CSO as mediators of the relationships between mean Phe exposure and executive abilities

Mediation Analyses

Of the six mediation analyses conducted, four yielded the statistically significant results of interest that are discussed here. As shown in Fig. 1, neither the total (weight *c*) nor direct (weight *c'*) effects of mean Phe exposure on nonverbal strategic processing were significant in analyses including either PPO or CSO as mediators (panels a and b, respectively).

Of greater interest, the indirect effects of mean Phe exposure on nonverbal strategic processing through both the PPO (panel a; 95% CI entirely below zero, $\kappa^2 = 0.24$, medium effect) and CSO (panel b; 95% CI entirely below zero, $\kappa^2 = 0.14$, medium effect) were statistically significant. In terms of verbal strategic processing (panel c), with CSO as the mediator, the total effect (weight *c*) of mean Phe exposure on verbal strategic processing was not significant, although the direct effect (weight *c'*) was significant. More importantly, the indirect effect of mean Phe exposure on verbal strategic processing through the CSO was statistically significant (95% CI entirely above zero, $\kappa^2 = 0.13$, medium effect). Turning to working memory (panel d), with PPO as the mediator, neither the total (weight *c*) nor direct (weight *c'*) effects of mean Phe exposure on working memory were significant. The indirect effect of mean Phe exposure on working memory through PPO, however, was statistically significant (95% CI below zero, $\kappa^2 = 0.26$, large effect) (Fig. 2).

Discussion

White matter compromise in individuals with PKU has been related to poorer Phe control (Anderson et al. 2007; Das et al. 2013; Hood et al. 2014a), and this relationship has been shown more consistently when examined across the lifetime rather than at discrete points (Viau et al. 2011). More rarely, significant relationships have been shown between white matter integrity and cognition in individuals with PKU (Peng et al. 2004; Antenor-Dorsey et al. 2013). This is somewhat surprising, as compromised white matter integrity has been related to poorer cognition in many other neurological disorders (Chiaravalloti and DeLuca 2008).

There are a number of possible reasons why direct relationships between white matter integrity and cognition have rarely been found in studies of PKU. First, cognition has most often been examined in relation to observable white matter abnormalities using structural MRI, which

makes it difficult to identify subtle relationships (Pietz et al. 1996; Weglage et al. 2013). In contrast, DTI permits detection of subtle disruptions in microstructural white matter integrity that are not detectable on structural images. Second, in previous studies, cognition has often been investigated using only IQ (Rupp et al. 2001). Such global measures, although useful, are less specific and do not address relationships with executive abilities, which are often impaired in individuals with PKU (Anderson et al. 2007; Christ et al. 2006). Finally, other studies have assessed relationships between cognition and white matter compromise in adults rather than children. It is possible that white matter compromise particularly affects cognition during earlier development.

In our exploratory study, mediation was used to assess the indirect effects of microstructural white matter integrity in PPO and CSO brain regions on the relationship between Phe exposure over the lifetime and executive abilities. Although both Phe and MD have been related to cognitive outcomes, such direct effects are not necessarily predictive of indirect effects. In fact, in our study, only one of the four significant mediation models showed a significant direct effect of Phe on cognition. Overall, results from our study are the first to show that white matter integrity mediates the relationship between Phe and executive abilities and suggest that white matter integrity may be a sensitive marker of cognitive dysfunction. Our results also suggest that to fully understand the complex interplay between metabolic control, white matter integrity, and executive abilities in children with PKU, all of these domains should be analyzed in conjunction.

In addition to the strengths of our study, such as the use of mediation and DTI, there are limitations that should be acknowledged. For example, our exploratory study had a small sample size, which could have resulted in decreased power to detect additional significant effects, and so requires replication in a larger sample. In addition, because the study was cross-sectional, causality was implied rather than determined. Future longitudinal research will be helpful in determining whether white matter integrity in childhood predicts executive abilities later in life and whether improvements in white matter integrity and in turn executive abilities can be obtained through lower lifetime Phe exposures.

Despite these limitations, our study provides unique information about neural processes that may affect cognition in children with PKU. Mediation analyses are rare in PKU research and have not previously been conducted in relation to executive abilities. We suggest that these types of analyses are crucial if we are to understand the complex relationships between metabolic control, neural factors, and functional outcomes in individuals with PKU.

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Compliance with Ethics Guidelines

Conflict of Interest

Anna Hood, Jerrel Rutlin, and Joshua Shimony declare that they have no conflict of interest. Desiree White and Dorothy Grange have received research grants from BioMarin Pharmaceutical Inc. and have served as consultants for BioMarin Pharmaceutical Inc.

Informed Consent

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

Contribution of Authors

Desiree White designed the study, wrote the protocol, trained research staff, supervised data collection, interpreted data, and co-wrote the manuscript. Anna Hood conducted literature review, analyzed, and interpreted data, and co-wrote the manuscript. Jerrel Rutlin analyzed and interpreted data and provided input on the writing of the paper. Joshua Shimony contributed to study design and provided statistical analysis, neuroimaging consultation, and input on the writing of the paper. Dorothy Grange contributed to study design, participant recruitment, and provided input on the

writing of the paper. All authors contributed to and have approved the final manuscript.

References

Anderson PJ, Wood SJ, Francis DE, Coleman L, Anderson V, Boneh A (2007) Are neuropsychological impairments in children with early-treated phenylketonuria (PKU) related to white matter abnormalities or elevated phenylalanine levels? Dev Neuropsychol 32(2):645–668

Antenor-Dorsey JAV, Hershey T, Rutlin J, Shimony JS, McKinstry RC, Grange DK, White DA (2013) White matter integrity and executive abilities in individuals with phenylketonuria. Mol Genet Metab 109(2):125–131

Chiaravalloti ND, DeLuca J (2008) Cognitive impairment in multiple sclerosis. Lancet Neurol 7(12):1139–1151

Christ SE, Steiner RD, Grange DK, Abrams RA, White DA (2006) Inhibitory control in children with phenylketonuria. Dev Neuropsychol 30(3):845–864

Christ SE, Huijbregts SCJ, de Sonneville LMJ, White DA (2010) Executive function in early-treated phenylketonuria: profile and underlying mechanisms. Mol Genet Metab 99:S22–S32

Citton V, Burlina A, Baracchini C, Gallucci M, Catalucci A, Dal Pos S, Burlina A, Manara R (2012) Apparent diffusion coefficient restriction in the white matter: going beyond acute brain territorial ischemia. Insights Imaging 3(2):155–164

Cohen J (1988) Statistical power analysis for the behavioral sciences. Lawrence Erlbaum, Hillsdale

Das AM, Goedecke K, Meyer U, Kanzelmeyer N, Koch S, Illsinger S et al (2013) Dietary habits and metabolic control in adolescents and young adults with phenylketonuria: self-imposed protein restriction may be harmful. In: JIMD reports-case and research reports, vol 13. Springer, Heidelberg, pp 149–158

De Groot MJ, Hoeksma M, Blau N, Reijngoud DJ, Van Spronsen FJ (2010) Pathogenesis of cognitive dysfunction in phenylketonuria: review of hypotheses. Mol Genet Metab 99:S86–S89

DeRoche K, Welsh M (2008) Twenty-five years of research on neurocognitive outcomes in early-treated phenylketonuria: intelligence and executive function. Dev Neuropsychol 33(4):474–504

Hood A, Antenor-Dorsey JAV, Rutlin J, Hershey T, Shimony JS, McKinstry RC et al (2014) Prolonged exposure to high and variable phenylalanine levels over the lifetime predicts brain white matter integrity in children with phenylketonuria. Mol Genet Metab 114(1):19–24

Hood A, Grange DK, Christ SE, Steiner R, White DA (2014b) Variability in phenylalanine control predicts IQ and executive abilities in children with phenylketonuria. Mol Genet Metab 111 (4):445–451

Janos AL, Grange DK, Steiner RD, White DA (2012) Processing speed and executive abilities in children with phenylketonuria. Neuropsychology 26(6):735

Mitchell JJ, Trakadis YJ, Scriver CR (2011) Phenylalanine hydroxylase deficiency. Genet Med 13(8):697–707

Oishi K, Zilles K, Amunts K, Faria A, Jiang H, Li X et al (2008) Human brain white matter atlas: identification and assignment of common anatomical structures in superficial white matter. Neuroimage 43(3):447–457

Paine RS (1957) The variability in manifestations of untreated patients with phenylketonuria (phenylpyruvic aciduria). Pediatrics 20 (2):290–302

Peng SS-F, Tseng W-YI, Chien Y-H, Hwu W-L, Liu H-M (2004) Diffusion tensor images in children with early-treated, chronic, malignant phenylketonuric: correlation with intelligence assessment. Am J Neuroradiol 25(9):1569–1574

Peng H, Peck D, White DA, Christ SE (2013) Tract-based evaluation of white matter damage in individuals with early-treated phenylketonuria. J Inherit Metab Dis 37(2):237–243

Pietz J, Kreis R, Schmidt H, Meyding-Lamade UK, Rupp A, Boesch C (1996) Phenylketonuria: findings at MR imaging and localized in vivo H-1 MR spectroscopy of the brain in patients with early treatment. Radiology 201(2):413–420

Preacher KJ, Hayes AF (2004) SPSS and SAS procedures for estimating indirect effects in simple mediation models. Behav Res Methods Instrum Comput 36(4):717–731

Preacher KJ, Hayes AF (2008) Asymptotic and resampling strategies for assessing and comparing indirect effects in multiple mediator models. Behav Res Methods 40(3):879–891

Rucker DD, Preacher KJ, Tormala ZL, Petty RE (2011) Mediation analysis in social psychology: current practices and new recommendations. Soc Personal Psychol Compass 5 (6):359–371

Rupp A, Kreis R, Zschocke J, Slotboom J, Boesch C (2001) Variability of blood–brain ratios of phenylalanine in typical patients with phenylketonuria. J Cereb Blood Flow Metab 21 (3):276–284

Scarabino T, Popolizio T, Tosetti M, Montanaro D, Giannatempo GM, Terlizzi R et al (2009) Phenylketonuria: white-matter changes assessed by 3.0-T magnetic resonance (MR) imaging, MR spectroscopy and MR diffusion. Radiol Med 114(3):461–474

Scriver CR (2007) The PAH gene, phenylketonuria, and a paradigm shift. Hum Mutat 28(9):831–845

Shimony JS, McKinstry RC, Akbudak E, Aronovitz JA, Snyder AZ, Lori NF et al (1999) Quantitative diffusion-tensor anisotropy brain MR imaging: normative human data and anatomic analysis. Radiology 212(3):770–784

Vermathen P, Robert-Tissot L, Pietz J, Lutz T, Boesch C, Kreis R (2007) Characterization of white matter alterations in phenylketonuria by magnetic resonance relaxometry and diffusion tensor imaging. Magn Reson Med 58(6):1145–1156

Viau KS, Wengreen HJ, Ernst SL, Cantor NL, Furtado LV, Longo N (2011) Correlation of age-specific phenylalanine levels with intellectual outcome in patients with phenylketonuria. J Inherit Metab Dis 34(4):963–971

Waisbren SE, Noel K, Fahrbach K, Cella C, Frame D, Dorenbaum A, Levy H (2007) Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis. Mol Genet Metab 92(1–2):63–70

Wechsler D (1999) Wechsler abbreviated scale of intelligence (WASI). The Psychological Corporation, San Antonio

Weglage J, Fromm J, van Teeffelen-Heithoff A, Möller HE, Koletzko B, Marquardt T et al (2013) Neurocognitive functioning in adults with phenylketonuria: results of a long term study. Mol Genet Metab 110:S44–S48

White DA, Connor LT, Nardos B, Shimony JS, Archer R, Snyder AZ et al (2010) Age-related decline in the microstructural integrity of white matter in children with early- and continuously-treated PKU: A DTI study of the corpus callosum. Mol Genet Metab 99: S41–S46

White DA, Antenor-Dorsey JAV, Grange DK, Hershey T, Rutlin J, Shimony JS et al (2013) White matter integrity and executive abilities following treatment with tetrahydrobiopterin (BH4) in individuals with phenylketonuria. Mol Genet Metab 110 (3):213–217

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CASE REPORT

The Challenges of a Successful Pregnancy in a Patient with Adult Refsum's Disease due to Phytanoyl-CoA Hydroxylase Deficiency

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Abstract We describe the management and outcomes of pregnancy in a 27-year-old woman with infantile-onset Adult Refsum's disease (ARD). She presented in infancy but was diagnosed with ARD at the age of 10 on basis of phytanic acidaemia and later confirmed to have the phytanoyl-CoA hydroxylase (*(PHYH)* c.164delT, p. L55fsX12) mutation. Despite repeated plasmapheresis sessions and strict dietary surveillance for 20 years, her phytanic acid levels persistently stayed above the ideal target level of 100 μmol/L but remained below 400 μmol/L. Initially the pregnancy was uncomplicated but in the third trimester of pregnancy the patient was admitted to the hospital with fluctuating hypertension, sinus tachycardia and breathlessness. The patient was compliant with diet during pregnancy and her phytanic levels were remained well controlled: 177 and 188 μmol/L in the first and second trimester, respectively. Peri-partum management required a coordinated team approach including a high-calorie and restricted diet to reduce the risk of acute metabolic decompensation. During the induced labour she required 10% dextrose infusions.

Post-partum it took the mother a long time to recover from childbirth – her appetite was poor due to post-natal depression and her body weight decreased rapidly by 11 kg within 3 weeks after childbirth, resulting in a spike in phytanic acid to 366 μmol/L. Measures were taken to minimise the risk of acute neurological decompensation. The infant was unaffected and has made normal developmental progress in the subsequent 2 years.

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Refsum's Disease Syndrome

Classical or Adult Refsum's disease (ARD; OMIM 266510) is a rare autosomal recessive condition. About 250 cases have been identified and the frequency of the condition in the population is thought to be 1 in 1,000,000. The ARD syndrome is associated with raised levels of phytanic acid due to defective peroxisomal alpha-oxidation of this dietary-derived isoprenoid fatty acid. About 90% of cases are caused by mutations in phytanoyl-CoA hydroxylase (*PHYH;* EC 1.14.11.18) with phenocopies caused by mutations in peroxin-7 (van den Brink et al. 2003) and alpha-methyl-alpha-acyl CoA racemase (AMACR; EC 5.1.99.4) (Lloyd et al. 2008).

ARD usually presents in adolescence with impaired night vision and retinitis pigmentosa though anosmia and

osteological features are present from birth (Wierzbicki et al. 2002). Most patients develop retinitis pigmentosa, demyelinating polyneuropathy, cerebellar ataxia and elevated CSF protein by the age of 20 (Skjeldal et al. 1987). Other clinical features of ARD include sensorineural hearing loss, cataracts, cardiac arrhythmias and ichthyosis (Burns et al. 2003). The retinal condition is slowly progressive. Neuropathic symptoms may appear intermittently and are associated with acute rises in phytanic levels that occur during intercurrent illness, anorexia, deliberate weight loss or starvation prior to surgery (Burns et al. 2003; Wierzbicki et al. 2003). Dietary restriction lowers plasma phytanic acid levels by 50–100%, leading to significant improvement in neurological function (Baldwin et al. 2010). Acute or long-term plasmapheresis is also used in the management of ARD (Kohlschutter et al. 2012; Zolotov et al. 2012). Although visual loss, anosmia and deafness are irreversible, their progression may slow with dietary therapy (Baldwin et al. 2010). Pregnancy is a stress-inducing state characterised by fat mobilisation. Pregnancy-related anorexia or weight loss potentially could lead to decompensation of phytanic acid levels. This is the first case report of infantile-onset ARD who had successful pregnancy.

Case

The mother was born at full term with no obvious abnormalities to consanguineous parents. At week six post-partum the neonate presented with horizontal nystagmus. As a child she had poor eyesight and limited night vision. Her vision gradually deteriorated over time from 6/36 to 6/24 at the age of 10 years and she was registered as partially sighted. Fundoscopy showed a waxy pallid disk and very early macular atrophic scarring in keeping with 'salt and pepper' retinitis pigmentosa of ARD. Sensorineural hearing loss was diagnosed at age 9. She had slight developmental delay at school and coped using short sentences but managed to start a course in fashion design. She suffered from chronic constipation from the neonatal period. She had multiple admissions with abdominal pain and heartburn. At the age of 11 years she was diagnosed with 'psoriasis' (probably ichthyosis) affecting her scalp and eyelids. She had eczema on her hands, feet and legs that responded well to topical therapy. On examination at the age of 10 she was noted to have short fingers and toes and incurved fifth fingers. Hand and foot X-rays confirmed symmetrical shortening of fourth and fifth metacarpals and short fourth and fifth toes due to short fourth metatarsals. The proximal phalangeal epiphyses of each hallux were cone-shaped.

At 11 years of age, the local ophthalmologist noted visual impairment, horizontal nystagmus and osteological abnormalities, and referred her to the regional genetics department. A diagnosis of ARD was made. Her initial phytanic acid level was 670 μmol/L (<15 μmol/L). Her very-long chain fatty acids (VLCFA) were within normal reference ranges: C22 = 72 (15–112), C24 = 51.9 (14–80), C26 = 0.99 (0.5–4.0), C24:C22 = 0.72 (0.35–1.10), C26:C22 = 0.008 (0.005–0.030). Pristanate was suppressed. Skin fibroblast culture showed a reduced rate of phytanic acid alpha-oxidation (25–31; normal range 57–90 pmol/h/mg). Peroxisomal enzyme activities for beta-oxidation and dihydroxyacetone phosphate acyltransferase (DHAP-AT; EC 2.3.1.42) activity (6.76; normal 3.5–7.5 μmol/h/g protein) were normal. Genetic sequencing later confirmed homozygosity for phytanoyl-CoA hydroxylase (*PHYH)* c.164delT, p.L55fsX12 mutation (Jansen et al. 2000). Her younger brother was also affected. He commenced a strict dietary regime and did not acquire visual and hearing impairments.

She was managed with a low phytanic acid diet and multivitamins (Brown et al. 1993; Baldwin et al. 2010, 2016). Her phytanic acid could be brought down temporarily with plasmapheresis sessions but varied between 300 and 600 μmol/L between sessions (Fig. 1). She adhered well to her diet reasonably but on one occasion her phytanic acid spiked to 860 μmol/L as a result of acute weight loss. Standardised portion meals were introduced to help her maintain body weight and prevent rapid weight loss.

Pregnancy

At the age of 27 she presented to the regional Adult Inherited Metabolic Disorders Clinic in the 6th week of pregnancy. She was reviewed monthly in person by a Metabolic Clinician or by telephone by a Metabolic Dietician. She also had regular appointments with the local Obstetrics Team. In the first trimester she was admitted locally with epigastric pain and lower abdominal pain. Foetal ultrasound scans showed a normal foetal development. She was advised to avoid any dairy spreads, cheese, fat olive, vegetable oil, tomatoes, walnuts and peanuts (Baldwin et al. 2010). Omega-3 fatty acids, iron and vitamin supplements were introduced throughout the pregnancy (Baldwin et al. 2016). The aim was to maintain adherence to the restrictive diet and a phytanic acid below 100 μmol/L (our local guidelines) and optimise her calorie intake to minimise weight gain during pregnancy. Her phytanic acid levels remained stable during pregnancy (Fig. 1).

At 5 months, she had gained 7 kg and weighed 82.6 kg. During pregnancy she developed some common complications including carpal tunnel syndrome in 6th month. In the third trimester she presented with sinus tachycardia of mean

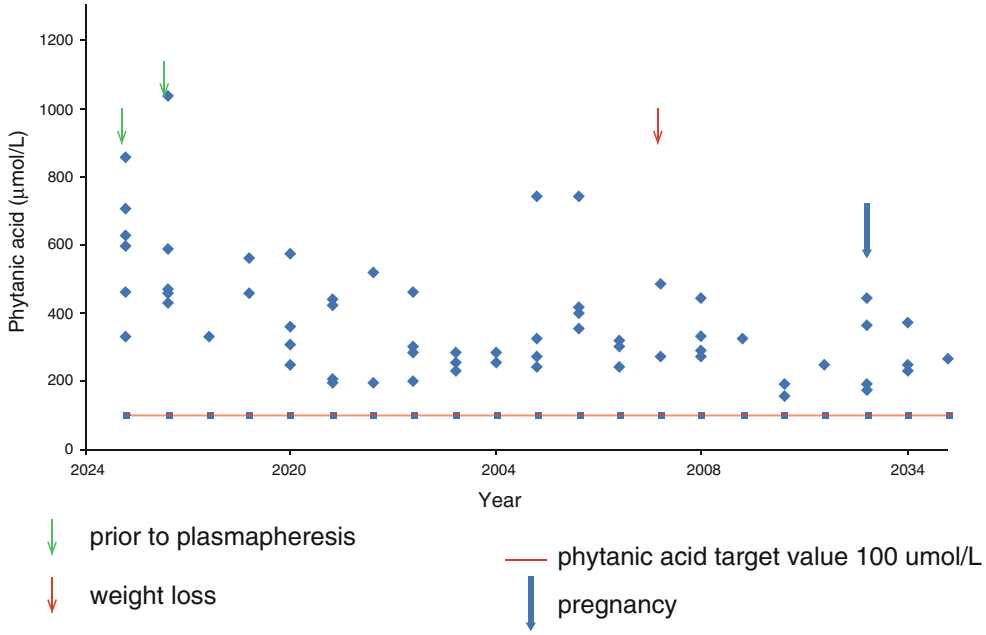

Fig. 1 Fluctuations in phytanic acid over 20 years

120/min on 24-hour ECG and shortness of breath. Investigations for pulmonary embolism and echocardiogram were normal. No cause for her sinus tachycardia was found but she was advised to start labetalol. She had breathlessness, fluctuating hypertension, dry skin and pruritus until the end of her pregnancy which improved post-partum. The dryness of the skin and pruritus were thought to be exacerbated by high phytanic acid levels (DiGiovanna and Robinson-Bostom 2003). She treated her dry skin with hydroxyzine and emollient as a supplement of soap. At 36 weeks her blood pressure was 156/93 mmHg and fluctuating. Her height of 155 cm and weight 92.2 kg gave a body mass index of 38.38 kg/m^2. Her clinical examination was normal. The patient was compliant with diet during pregnancy. Her phytanic acid levels were 177 µmol/L and 188 µmol/L in the first and second trimester, respectively. They increased to 443 µmol/L at 36 weeks.

During the induced labour, 100 g/day Polycal® (384 kcal) and 30mls tds Calogen® (420 kcal) were provided to reduce the risk of acute metabolic decompensation. After the delivery of a healthy girl at term (whose phytanic acid at the age of 6 weeks was not raised), it took the mother a long time to recover from her childbirth – her appetite was poor due to post-natal depression (1,417 kcal daily intake, including 40 g protein). She was advised to continue isotonic drinks and eating small portions when she could. She decided not to breastfeed. Artificial formula feeding was used by the infant from birth.

She was advised not to lose more than 0.5 kg per week post-partum but her weight decreased rapidly by 11 kg within 3 weeks resulting in a spike of phytanic acid to 366 µmol/L. The patient was prescribed Fresubin® (3 cartons daily; 90 kcal) as a source of additional calories and was advised to monitor her body weight on a daily basis. She continued experiencing episodes of fatigue and general weakness. She had problems with her episiotomy for many months causing dyspareunia that required a refashioning of episiotomy and treatment with antibiotics (gentamycin). Physical examination and ultrasound scan of her pelvis did not show any abnormal findings. Post-natally her blood pressure was raised and she was treated with diuretics. Echocardiography was normal. She developed headaches and dizziness and tried a calcium channel blocker, diuretics, amitriptyline and tricyclic anti-depressants but could not tolerate any. She had persistent episodes of nausea, vomiting and epigastric abdominal pain radiating to her back associated with raised amylase (429; reference range $<$110 IU/L). Cholecystitis and pancreatitis were considered but never confirmed. A gastroscopy showed a 3 cm hernia. She underwent detailed specialist investigations and her symptoms were eventually attributed to irritable bowel syndrome.

She has mild learning difficulties and receives support from her husband and family. She is engaging effectively with healthcare services and tries to be compliant with diet. Twelve months post-delivery, her BMI is stable at 33.6 kg/m^2. She has remained on strict diet with 1,500 kcal intake including 78 g protein but is still struggling on a daily basis with fatigue, shortness of breath and variable bowel movements. The child is healthy and developing well.

Discussion

We present a case of infantile-onset ARD syndrome in a female patient, who despite significantly raised levels of phytanic acid, has had a successful pregnancy. One other case description of infantile-onset ARD syndrome exists but describes a more severe clinical but similar biochemical phenotype in a male child (Herbert and Clayton 1994). Her delayed diagnosis is not unusual in cases of ARD where the delay in diagnosis is typically 11 years (Claridge et al. 1992; Ruther 2005). This is the first case report describing the birth of a healthy baby despite very high phytanic acid levels in the mother. In our patient the only significant defect was in alpha-oxidation of phytanic acid. Pristanate was suppressed as is typical in ARD rather than elevated as in severe peroxisomal biogenesis disorders (Poulos et al. 1988). Despite a phytanic acid-restricted diet for many years and repeated plasmapheresis, she continued to have an elevated plasma phytanic acid in the typical range for many treated patients (Baldwin et al. 2010). The effect of elevated maternal phytanic acid on maternal or foetal outcomes in pregnancy has never been described. It has been postulated that phytanic acid does not cross the placenta (Zomer et al. 2000) but clinical data is lacking. In this case it seems that elevated plasma phytanic acid that was not detrimental to the mother or foetus.

Vomiting, nausea and anorexia in pregnancy may exacerbate the underlying condition (Langendonk et al. 2012). Decreases in glucose, insulin and alanine and increases in ketone body production occur in a fasting state, and occur faster than in a non-pregnant state (Butte 2000). In ARD starvation induces a rapid rise in phytanic acid levels with long-term sequelae in some patients (Wierzbicki et al. 2003). In our patient nausea and vomiting led to decrease in meal frequency and food intake, but this had only a small effect on plasma phytanic acid levels. Ideally phytanic acid levels need to be monitored monthly in pregnancy. The patient maintained good contact with IMD services and was very compliant with diet. As a result, her phytanic acid levels were better than prior to the pregnancy and repeated monitoring was not thought necessary.

It has been shown that enhanced catabolism and increased phytanic acid levels may exacerbate retinitis pigmentosa and reduce visual fields in the third trimester of pregnancy as described in an unpublished case report of a woman with ARD (Leroy BP, unpublished observations). No deterioration in visual fields was seen in our patient. Pruritus is a feature of pregnancy. The incorporation of phytanic acid into cholesterol esters reduces the amount of free cholesterol in the blood and may lead to a relative deficiency of linoleate in many organs, including skin. This may contribute to ichthyosis which is also seen in essential fatty acid deficiency (Laurell et al. 1972).

During labour and delivery the requirements for energy increase. In principle it is important that the woman keeps her caloric intake adequately maintained during labour so that she does not go into catabolic stress at the time of delivery. Therefore she should be encouraged to have oral foods and drinks at frequent intervals during the earlier stages of labour when she is able to tolerate this. In our case, labour was induced and not prolonged and the woman was managed on intravenous 10% dextrose (2 mL/kg/h). After she delivered and was able to eat and drink there was no need for the dextrose infusion. Breastfeeding will increase energy demands on a mother (Langendonk et al. 2012) but our patient did not breastfeed so this was not an issue.

There is little data on drug interactions with the alpha-oxidation pathway. Ibuprofen is generally avoided because it is metabolised by AMACR and may interfere with the metabolism of phytanic acid (Lloyd et al. 2008). Most medications are safe as long as they do not contain animal fat but even if these contain phytanic acid then the additional exposure is likely to be small. The only exception is in omega-3 fatty acid preparations. Partially purified preparations derived from fish oils (e.g. MaxEPA) may contain significant quantities of phytanic acid though ultra-pure formulations derived from plant sources (e.g. Omacor) do not (Wierzbicki AS, unpublished data). Gelatine and lactose are safe as are lidocaine, diamorphine, phenylephrine, ondansetron, co-amoxiclav, suxamethonium, paracetamol, codeine and morphine (Stepien KM, unpublished data). All those medications were considered prior to childbirth and confirmed with the pharmacist to likely be safe. There is no evidence that levonorgestrel (the active medication in a mirena coil) will affect phytanic acid levels so this method of contraception was proposed to the patient after pregnancy.

The risk of metabolic decompensation is increased in pregnancy in a patient with ARD. Dietary advice prior to the pregnancy should include detailed assessment to ensure adequate caloric intake and this should be reviewed and adjusted during the pregnancy. In conclusion, a multidisciplinary team approach is required to manage patients with inherited metabolic disorders in pregnancy.

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Synopsis

The challenges in the management of pregnancy in a patient with Adult Refsum's disease.

Compliance with Ethics Guidelines

Contributions

All authors performed the literature search. KS wrote the first draft of the manuscript and reviewed the literature, with AW, BTPT, HRW and CJH involved in revisions. All authors approved the final article.

Guarantor

CJH is the guarantor.

Conflict of Interest

All authors declare no conflict of interest for this publication.

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Patient's Consent

Informed consent was obtained from the patient.

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

References

Baldwin EJ, Gibberd FB, Harley C, Sidey MC, Feher MD, Wierzbicki AS (2010) The effectiveness of long-term dietary therapy in the treatment of adult Refsum disease. J Neurol Neurosurg Psychiatry 81:954–957

Baldwin EJ, Harrington DJ, Sampson B, Feher MD, Wierzbicki AS (2016) Safety of long-term restrictive diets for peroxisomal disorders: vitamin and trace element status of patients treated for Adult Refsum Disease. Int J Clin Pract 70:229–235

Brown PJ, Mei G, Gibberd FB et al (1993) Diet and Refsum's disease. The determination of phytanic acid and phytol in certain foods and application of this knowledge to the choice of suitable convenience foods for patients with Refsum's disease. J Hum Nutr Diet 6:295–305

Burns TM, Ryan MM, Darras B, Jones HR Jr (2003) Current therapeutic strategies for patients with polyneuropathies secondary to inherited metabolic disorders. Mayo Clin Proc 78:858–868

Butte NF (2000) Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus. Am J Clin Nutr 71:1256S–1261S

Claridge KG, Gibberd FB, Sidey MC (1992) Refsum disease: the presentation and ophthalmic aspects of Refsum disease in a series of 23 patients. Eye (Lond) 6(Pt 4):371–375

DiGiovanna JJ, Robinson-Bostom L (2003) Ichthyosis: etiology, diagnosis, and management. Am J Clin Dermatol 4:81–95

Herbert MA, Clayton PT (1994) Phytanic acid alpha-oxidase deficiency (Refsum disease) presenting in infancy. J Inherit Metab Dis 17:211–214

Jansen GA, Hogenhout EM, Ferdinandusse S et al (2000) Human phytanoyl-CoA hydroxylase: resolution of the gene structure and the molecular basis of Refsum's disease. Hum Mol Genet 9:1195–1200

Kohlschutter A, Santer R, Lukacs Z, Altenburg C, Kemper MJ, Ruther K (2012) A child with night blindness: preventing serious symptoms of Refsum disease. J Child Neurol 27:654–656

Langendonk JG, Roos JC, Angus L et al (2012) A series of pregnancies in women with inherited metabolic disease. J Inherit Metab Dis 35:419–424

Laurell S, Nilsen R, Norden A (1972) Incorporation of phytanic and linoleic acid into plasma lipids in Refsum's disease. Clin Chim Acta 36:169–174

Lloyd MD, Darley DJ, Wierzbicki AS, Threadgill MD (2008) Alpha-Methylacyl-CoA racemase – an 'obscure' metabolic enzyme takes centre stage. FEBS J 275:1089–1102

Poulos A, Sharp P, Fellenberg AJ, Johnson DW (1988) Accumulation of pristanic acid (2, 6, 10, 14 tetramethylpentadecanoic acid) in the plasma of patients with generalised peroxisomal dysfunction. Eur J Pediatr 147:143–147

Ruther K (2005) Adult Refsum disease. A tapetoretinal degeneration accessible for therapy. Ophthalmologe 102:772–777

Skjeldal OH, Stokke O, Refsum S, Norseth J, Petit H (1987) Clinical and biochemical heterogeneity in conditions with phytanic acid accumulations. J Neurol Sci 77:87–96

van den Brink DM, Brites P, Haasjes J et al (2003) Identification of PEX7 as the second gene involved in Refsum disease. Am J Hum Genet 72:471–477

Wierzbicki AS, Lloyd MD, Schofield CJ, Feher MD, Gibberd FB (2002) Refsum's disease: a peroxisomal disorder affecting phytanic acid alpha-oxidation. J Neurochem 80:727–735

Wierzbicki AS, Mayne PD, Lloyd MD et al (2003) Metabolism of phytanic acid and 3-methyl-adipic acid excretion in patients with adult Refsum disease. J Lipid Res 44:1481–1488

Zolotov D, Wagner S, Kalb K, Bunia J, Heibges A, Klingel R (2012) Long-term strategies for the treatment of Refsum's disease using therapeutic apheresis. J Clin Apher 27:99–105

Zomer AW, Jansen GA, van Der BB et al (2000) Phytanoyl-CoA hydroxylase activity is induced by phytanic acid. Eur J Biochem 267:4063–4067

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CASE REPORT

Novel Homozygous Missense Mutation in *SPG20* Gene Results in Troyer Syndrome Associated with Mitochondrial Cytochrome *c* Oxidase Deficiency

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Abstract Troyer syndrome is an autosomal recessive form of hereditary spastic paraplegia (HSP) caused by deleterious mutations in the *SPG20* gene. Although the disease is associated with a loss of function mechanism of spartin, the protein encoded by *SPG20*, the precise pathogenesis is yet to be elucidated. Recent data indicated an important role for spartin in both mitochondrial maintenance and function. Here we report a child presenting with progressive spastic paraparesis, generalized muscle weakness, dysarthria, impaired growth, and severe isolated decrease in muscle cytochrome *c* oxidase (COX) activity. Whole exome sequencing identified the homozygous c.988A>G variant in *SPG20* gene (p.Met330Val) resulting in almost complete loss of spartin in skeletal muscle. Further analyses demonstrated significant tissue specific reduction of COX 4, a nuclear encoded subunit of COX, in muscle suggesting a role for spartin in proper mitochondrial respiratory chain function mediated by COX activity. Our findings need to be verified in other Troyer syndrome patients in order to classify it as a form of HSP caused by mitochondrial dysfunction.

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Introduction

Hereditary spastic paraplegia (HSP) comprises a highly heterogeneous group of neurogenetic disorders with multiple identified causative genes involved in various cellular pathophysiologic pathways. HSP is further classified as pure or complicated depending on accompanying clinical (mainly neurologic) features. In general complicated HSP is mainly inherited as autosomal recessive and is associated with variable manifestations such as intellectual disability, peripheral neuropathy, optic atrophy, and others (Hensiek et al. 2015). Several HSP forms may be caused by mutations in genes involved in mitochondrial function. Few recent examples include *FARS2* (Yang et al. 2016), *C12ORF65* (Shimazaki et al. 2012), *SPG7* (Shanmughapriya et al. 2015), *HSPD1* (Bross et al. 2008), *IBA57* (Lossos et al. 2015), and others.

Troyer syndrome (MIM # 275900) is an autosomal recessive complicated HSP characterized in addition by distal amyotrophy, dysarthria, cerebellar signs, developmental delay, and short stature caused by mutations in the *SPG20* gene leading to loss of the encoded spartin protein (Patel et al. 2002). Since the initial identification of the founder c.1110delA mutation in the Amish population only two other mutations have been reported, both predicted deleterious and associated with the Troyer phenotype (Manzini et al. 2010; Tawamie et al. 2015; Alazami et al. 2015; Butler et al. 2016). Herein, we report a patient with a

novel homozygous mutation in *SPG20* gene with concomitantly reduced mitochondrial cytochrome *c* oxidase (COX) activity, suggesting a link between spartin and the mitochondrial respiratory chain.

Patients and Methods

Case Report

The patient is the first child of healthy Arab Muslim parents who are first degree cousins once removed. She has twin male siblings with confirmed glycogen storage disease type 1a. The patient was born vaginally at term with no further complications. Her pregnancy was unremarkable except for intrauterine growth retardation initially documented at the third trimester. Her birth weight was 2,270 g and the Apgar score was normal. Growth retardation was a problem since infancy as weight and height remained below the third centile. Development was mildly delayed as she started to walk at 1 year and 9 months and to gain her first words at 2 years. At the age of 5 years she was referred for neurological evaluation due to global developmental delay dominated by hypotonia and gross motor impairment. At that time she already displayed dysarthric speech. A year later she was diagnosed with attention deficit disorder and treatment with methylphenidate was started.

Disease course was progressive with gait disturbance being the major problem manifested by significant clumsiness while walking, recurrent falls, and instability associated with further worsening of dysarthria. At the age of 8 years intention tremor occurred in both hands. This was relatively mild at rest but exacerbated during exercise or anxiety. Physical examination at that time showed weight and height below the third centile, elongated face with drooling, and reduced facial movements compatible with pseudobulbar palsy. She had generalized muscle wasting reflected by pectus carinatum deformity and clear atrophy of her four limbs as well as distal amyotrophy. Her neurological examination revealed mildly delayed cognitive skills, positive Gower sign consistent with proximal muscle weakness, generalized tendon hyperreflexia predominantly in the lower limbs associated with bilateral ankle clonus and positive Babinski sign. In addition upper extremities dysmetria was also noted. Brain MR imaging at this age was normal.

The patient had an extensive metabolic investigation that included among others blood count, serum creatine phosphokinase, liver transaminases, renal functions, thyroid hormones, repeated lactate and ammonia in plasma, amino acids in plasma and urine, acylcarnitine profile, organic acids in urine, total plasma homocysteine, serum transferrin isoelectric focusing, and serum very long chain fatty acids all of which were normal. Serum fibroblast growth factor 21 (FGF21), a novel biomarker for mitochondrial diseases (Suomalainen et al. 2011), was mildly elevated to 455 pg/ml (normal range < 200 pg/ml). Chromosomal microarray assay did not reveal pathological copy number variations.

Methods

Biochemical Assay of OXPHOS

Enzymatic activities of the five respiratory chain complexes, rotenone sensitive NADH CoQ reductase (Complex I), succinate cytochrome *c* reductase (complex II + III), succinate dehydrogenase (complex II) COX (complex IV), and Mg^{2+}ATPase (complex V) were determined in isolated muscle mitochondria from the patient. We used the same methodology as the one employed in a previous study (Saada et al. 2004). The activities were normalized to citrate synthase and compared to normal control means.

Whole Exome Sequencing

Exonic sequences from DNA sample of the patient were enriched with the SureSelect Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA, USA). Sequences (100-bp paired-end) were generated on a HiSeq2000 (Illumina, San Diego, CA, USA). Read alignment and variant calling were performed with DNAnexus (Palo Alto, CA, USA) using default parameters with the human genome assembly hg19 (GRCh37) as reference.

Western Blot Analysis of Muscle and Fibroblasts

Proteins from muscle and fibroblast homogenates were treated with sample solubilization buffer and separated by SDS-polyacrylamide gel electrophoresis on a 12% gel. Proteins were transferred to PVDF membranes and probed with the indicated antibodies using the Enhanced Chemiluminescence Western blotting method as according to the manufacturer's instruction (Biological Industries, HaEmek, Israel). The primary antibodies used were mouse-anti-B-tubulin-loading control (Santa Cruz Biotechnology), mouse-anti-COX 1, 2, 4 subunits (Molecular Probes), and rabbit anti-SPG20 (Proteintech). The secondary antibodies were HRP-conjugated anti-mouse and anti-rabbit (Jackson).

Results

Muscle biopsy from the right quadriceps was obtained at the age of 9 years. Light microscopy, immunohistochemical staining including COX, and electron microscopy were largely normal (supplementary Fig. S1). Enzymatic activities

Table 1 Enzymatic activities of mitochondrial respiratory chain complexes

	Muscle mitochondria		Fibroblast homogenate	
Assay[a]	Patient	Controls ± SD (range) $n = 50$	Patient	Control mean $n = 4$
CI	106 [98%]	98 ± 46 (46–215)	nd	nd
CI + III	159 [65%]	305 ± 148 (104–717)	nd	nd
CII	141 [116%]	124 ± 57 (52–230)	nd	nd
CII + III	75 [105%]	80 ± 40 (41–172)	nd	nd
CIV	209 [25%]	1,061 ± 47 (265–2,896)	61 [115%]	72 ± 22 (62–135)
CS	980	1,132 ± 438 (438–2,350)	42	45 ± 12 (30–66)

nd not determined
[a] nmol/min/mg (%) percentage residual activity normalized to citrate synthase (CS) activity compared to controls

of the five respiratory chain complexes determined in muscle and mitochondria assays disclosed significantly reduced COX (respiratory chain complex IV) activity in muscle mitochondria, 209 nmol/min/mg (controls 1,061 ± 47 nmol/min/mg) which is 25% of control mean when normalized to citrate synthase (CS). The activities of complexes I–III and V were within normal limits. However, COX activity was normal in fibroblast homogenate (Table 1). ATP production in fibroblasts was tested normal both on glucose and galactose.

Molecular genetic investigations for the patient and her family were initiated after obtaining the relevant written informed consents and local ethical review board approval. Given the complicated HSP phenotype and the presumed nuclear encoded mitochondrial impairment we elected to proceed with whole exome sequencing (WES) under a rare allele, recessively inherited hypothesis. WES of the patient sample yielded 42.5 million mapped reads with a mean coverage of X56. Following alignment and variant calling, we performed a series of filtering steps. These included removing variants called less than X8, were off-target, synonymous, heterozygous, or had MAF > 0.5% at ExAC (Exome Aggregation Consortium, Cambridge, MA [URL: http://exac.broadinstitute.org]). We also removed variants with MAF > 4% at the Hadassah in-house database (~800 ethnic matched exome analyses) or variants which were predicted benign (Mutation Taster, http://mutationtaster.org/). Only three variants survived this filtering (supplementary Table S1). Of these three variants only *SPG20* is associated with HSP phenotype whereas the other two variants are associated with completely unrelated phenotypes and non-recessive inheritance.

WES underscored a homozygous c.988A>G in the *SPG20* gene, predicting the substitution of highly evolutionary conserved methionine at position 330 with valine (p.Met330Val) as the only relevant HSP-targeted gene. This previously unreported variant was predicted to be disease causing by Mutation Taster (http://mutationtaster.org), Polyphen 2 (http://genetics.bwh.harvard.edu/pph2), and SIFT (http://siftdna.org). Both parents were heterozygous for this variant and it segregated perfectly in the family.

In order to further assess the pathogenicity of this c.988A>G variant, Western blot analysis was performed on homogenate muscle tissue and fibroblasts from the patient using commercial monoclonal antibodies. There was an almost complete reduction in steady state SPG20 protein level in patient's muscle and decrease but to a lesser degree in patient's fibroblasts (Fig. 1a).

Discussion

Our patient presented with clinical features consistent with Troyer syndrome. This phenotype is usually attributed to deleterious mutations in *SPG20* gene leading to loss of expression of the encoded protein termed spartin (Bakowska et al. 2008). In contrast with previously reported mutations that were all caused by frameshift mutations resulting in early truncation and absent protein (Patel et al. 2002; Manzini et al. 2010; Alazami et al. 2015; Butler et al. 2016) the missense mutation identified in our patient was not predicted to disrupt the protein. This mutation was not located in either of the two conserved domains of the spartin, i.e., the microtubule interacting and trafficking motif and the plant-related senescence domain but it is located in an evolutionary highly conserved residue (supplementary Fig. S2) rendering an unstable protein as suggested by the absence of spartin demonstrated by Western blot analysis. In order to assess for absence of mRNA resulting in lack of protein, RNA was isolated from control and patient's fibroblasts, reverse transcribed and the cDNA was subjected to PCR using the following primers: Forward: GCT CAG AGG GAT CAG CAT TT and Reverse: CCT CCT TTA CTT CCT TCG TCT. No difference in the length and abundance was noted (data not shown). Notably, spartin was almost completely

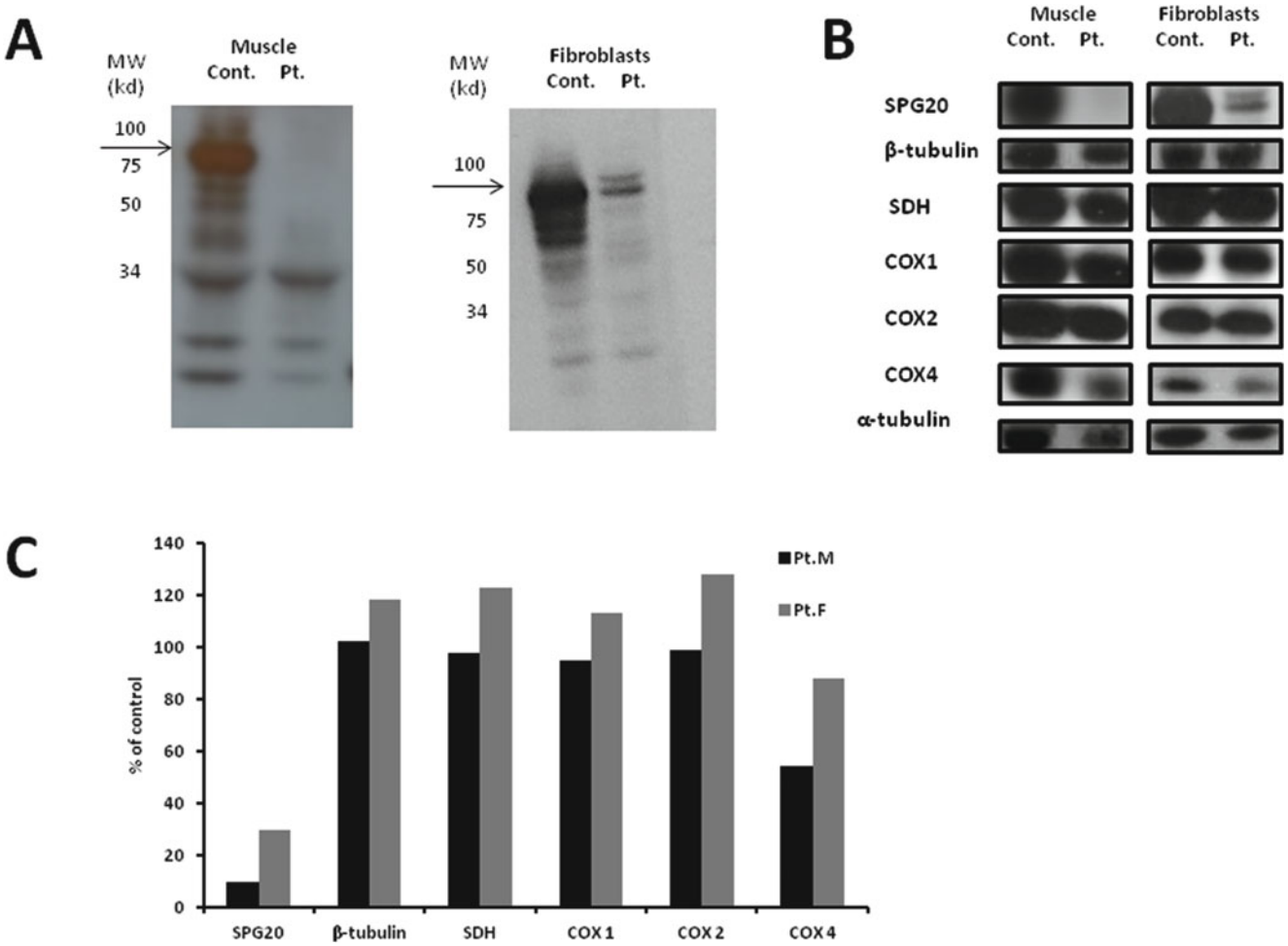

Fig. 1 Western blot of spartin and COX subunits in muscle and fibroblasts. (**a**) Control and patient's muscle (*right*) and fibroblasts (*left*) were exposed to anti-spartin antibodies and detected with Enhanced Chemiluminescence. The location of molecular weight markers is marked and the spartin protein (*arrow*) is located as expected at 85 kd. (**b**) Western blot in muscle (*right*) and fibroblasts (*left*) specific antibodies after exposure to Enhanced Chemiluminescence. (**c**) Quantification relative to control (Image J software). Pt. M = patient's muscle, Pt. F = patient's fibroblasts

depleted in muscle compared with some residual protein in fibroblasts thus inferring tissue variability (Fig. 1b, c).

Spartin is a multifunctional protein widely expressed in human tissues and is localized to several cellular organelles and compartments including endosomes, lipid droplets, and mitochondria (Bakowska et al. 2007; Eastman et al. 2009; Lu et al. 2006). The precise role of spartin in mitochondrial function is yet to be elucidated but elegant studies by Joshi et al. illustrated that spartin binds to mitochondrial cardiolipin via its plant-related senescence domain where it associates with mitochondrial outer membrane playing a crucial role in mitochondrial Ca^{+2} influx which is essential in the maintenance of mitochondrial membrane potential (Joshi and Bakowska 2011). Furthermore, recent evidence obtained from studies on *C. elegans* SPG20 orthologue showed a protective effect of spartin on mitochondrial oxidation stress thereby allowing more effective mitochondrial ATP production in wild type worms compared with animals harboring a null allele (Truong et al. 2015).

A major clinical feature observed in our patient was abnormal skeletal muscle function manifested by generalized muscle wasting, facial weakness, and Gower sign suggesting proximal muscle weakness as well as clear evidence of distal muscle weakness. These manifestations prompted us to perform a muscle biopsy which revealed severe isolated reduction in COX activity relative to CS. The seemingly unremarkable COX stain is due the qualitative rather than quantitative nature of the histochemical stain. Importantly, the decreased COX activity was in correlation with the almost undetectable spartin protein levels in muscle while intact COX activity in fibroblasts where spartin was clearly present albeit in lower amount. Moreover, the nuclear encoded subunit of COX, COX 4, was significantly reduced in muscle but only mildly

decreased in fibroblasts (Fig. 1b, c). Given that spartin associates with cardiolipin located mainly in the mitochondrial outer membrane (Joshi and Bakowska 2011) its effect on the inner mitochondrial membrane COX enzyme is not intuitively explained. We therefore speculate a link between spartin and mitochondrial oxidative phosphorylation mediated by COX activity via COX4 although the exact mechanism remains to be elucidated. This may explain the tissue specificity seen in our patient where high energy producing tissues such as skeletal muscles are more severely affected following the lack of spartin expression. However, similar findings should be verified in another patient with Troyer syndrome in order to confirm pathogenesis caused by mitochondrial dysfunction. Of note, tissue specific COX assembly defects were previously reported with variable levels of the enzymatic subcomplexes (Stiburek et al. 2005) but the profile seen in our patient is yet uncommon. Noteworthy, another form of HSP caused by mutations in the *SPG7* gene encoding paraplegin, a mitochondrial metallopeptidase result in a secondary assembly defect of mitochondrial complex I of the respiratory chain of affected patients (Atorino et al. 2003).

In conclusion Troyer syndrome is a complex disease with variable clinical features caused by lack of spartin in various tissues including skeletal muscle, central nervous system, growth plates of bones, and adipose tissue. Although the pathogenesis of the disease is not completely understood, growing evidence implies that mitochondrial dysfunction plays a major role. Our study supports this assumption and adds more evidence to disease pathogenesis that needs to be further investigated in future research.

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Authors' Contributions

Dr. Spiegel leads the composition and evaluation of the manuscript; designed and conceptualized the study, and interpreted the data. Dr. Spiegel 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.

Dr. Soiferman evaluated the manuscript for content and analyzed and interpreted the biochemical studies as well as Western blot analyses.

Dr. Shaag evaluated the manuscript for content and analyzed and interpreted the genetic data.

Prof. Shalev evaluated the manuscript for content and analyzed and interpreted the clinical data.

Prof. Elpeleg evaluated the manuscript for content, including medical writing for content, analyzed and interpreted the genetic data in particular the exome sequencing.

Prof. Saada leads the composition and evaluation of the manuscript, designed and conceptualized the study, and analyzed and interpreted the biochemical data as well as the Western blot analysis.

Conflict of Interests

Dr Spiegel, Dr. Soiferman, Dr. Shaag, Prof. Shalev, Prof. Elpeleg and Prof. Saada all declare that they have no conflict of interests.

Ethics and Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained accordingly as approved by the local ethical review board.

References

Alazami AM, Patel N, Shamseldin HE, Anazi S, Al-Dosari MS, Alzahrani F (2015) Accelerating novel candidate gene discovery in neurogenetic disorders via whole-exome sequencing of prescreened multiplex consanguineous families. Cell Rep 10: 148–161

Atorino L, Silvestri L, Koppen M et al (2003) Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia. J Cell Biol 163:777–787

Bakowska JC, Jupille H, Fatheddin P, Puertollano R, Blackstone C (2007) Troyer syndrome protein spartin is mono-ubiquitinated and functions in EGF receptor trafficking. Mol Biol Cell 18: 1683–1692

Bakowska JC, Wang H, Xin B, Sumner CJ, Blackstone C (2008) Lack of spartin protein in Troyer syndrome: a loss-of-function disease mechanism? Arch Neurol 65:520–524

Bross P, Naundrup S, Hansen J et al (2008) The Hsp60-(p.V98I) mutation associated with hereditary spastic paraplegia SPG13 compromises chaperonin function both in vitro and in vivo. J Biol Chem 283:15694–15700

Butler S, Helbig KL, Alcaraz W, Seaver LH, Hsieh DT, Rohena L (2016) Three cases of Troyer syndrome in two families of Filipino descent. Am J Med Genet A 170(7):1780–1785

Eastman SW, Yassaee M, Bieniasz PD (2009) A role for ubiquitin ligases and Spartin/SPG20 in lipid droplet turnover. J Cell Biol 184:881–894

Hensiek A, Kirker S, Reid E (2015) Diagnosis, investigation and management of hereditary spastic paraplegias in the era of next-generation sequencing. J Neurol 262:1601–1612

Joshi DC, Bakowska JC (2011) SPG20 protein spartin associates with cardiolipin via its plant-related senescence domain and regulates mitochondrial Ca^{2+} homeostasis. PLoS One 6(4), e19290

Lossos A, Stümpfig C, Stevanin G et al (2015) Fe/S protein assembly gene IBA57 mutation causes hereditary spastic paraplegia. Neurology 84:659–667

Lu J, Rashid F, Byrne PC (2006) The hereditary spastic paraplegia protein spartin localises to mitochondria. J Neurochem 98: 1908–1919

Manzini MC, Rajab A, Maynard TM et al (2010) Developmental and degenerative features in a complicated spastic paraplegia. Ann Neurol 67:516–525

Patel H, Cross H, Proukakis C et al (2002) SPG20 is mutated in Troyer syndrome, an hereditary spastic paraplegia. Nat Genet 31:347–348

Saada A, Bar-Meir M, Belaiche C, Miller C, Elpeleg O (2004) Evaluation of enzymatic assays and compounds affecting ATP production in mitochondrial respiratory chain complex I deficiency. Anal Biochem 335:66–72

Shanmughapriya S, Rajan S, Hoffman NE et al (2015) SPG7 is an essential and conserved component of the mitochondrial permeability transition pore. Mol Cell 60:47–62

Shimazaki H, Takiyama Y, Ishiura H et al (2012) A homozygous mutation of *C12orf65* causes spastic paraplegia with optic atrophy and neuropathy (SPG55). J Med Genet 49:777–784

Stiburek L, Vesela K, Hansikova H et al (2005) Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1. Biochem J 392:625–632

Suomalainen A, Elo JM, Pietiläinen KH et al (2011) FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a diagnostic study. Lancet Neurol 10:806–818

Tawamie H, Wohlleber E, Uebe S, Schmäl C, Nöthen MM, Abou Jamra R (2015) Recurrent null mutation in SPG20 leads to Troyer syndrome. Mol Cell Probes 29:315–318

Truong T, Karlinski ZA, O'Hara C, Cabe M, Kim H, Bakowska JC (2015) Oxidative stress in *Caenorhabditis elegans*: protective effects of spartin. PLoS One 10(6), e0130455

Yang Y, Liu W, Fang Z et al (2016) A newly identified missense mutation in *FARS 2* causes autosomal recessive spastic paraplegia. Hum Mutat 37:165–169

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RESEARCH REPORT

Lethal Neonatal LTBL Associated with Biallelic *EARS2* Variants: Case Report and Review of the Reported Neuroradiological Features

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Abstract Mitochondrial translation defects are important causes of early onset mitochondrial disease. Although the biochemical (combined respiratory chain deficiency) signature and neuroimaging are usually distinctive, they are not diagnostic as the genetic origin of mitochondrial translation defects is heterogeneous. We report a female child, born at term to non-consanguineous parents, who exhibited global hypotonia, failure to thrive, persistent and progressive hyperlactacidaemia with lactic acidosis, liver dysfunction and encephalopathy and died at the age of 5 months. Brain MRI revealed hypogenesis of the *corpus callosum*, T2 signal abnormalities in the medulla oblongata, pons, midbrain, thalami, cerebellar white matter, and a lactate peak on MRS. Muscle histochemistry showed cytochrome *c* oxidase (COX)-deficient and ragged-red fibres, while muscle biochemical studies showed decreased activities of mitochondrial respiratory chain complexes I and IV. Whole exome sequencing (WES) identified biallelic *EARS2* (NM_001083614) variants, a previously reported start-loss (c.1>G, p.Met1?) variant and a novel missense (c.184A>T, p.Ile62Phe) variant. Patient fibroblasts and muscle homogenate displayed markedly decreased EARS2 protein levels, although decreased steady-state levels of complex I (NDUFB8) and complex IV (MT-CO1 and MT-CO2) subunits were only observed in muscle. Pathogenic variants in *EARS2*, encoding mitochondrial glutamyl-tRNA synthetase (mtGluR), are associated with Leukoencephalopathy involving the Thalamus and Brainstem with high Lactate (LTBL), a mitochondrial disorder characterised by a

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distinctive brain MRI pattern and a biphasic clinical course. We further outline the unique phenotypic spectrum of LTBL and review the neuroradiological features reported in all patients documented in the literature.

Introduction

In recent years, next-generation sequencing (NGS) technologies have allowed identification of many novel disease genes, particularly in those human conditions that are characterised by clinical and genetic heterogeneity. This is true of mitochondrial disorders, which are clinically, biochemically and genetically heterogeneous; respectively, characterised by multisystemic features and mitochondrial respiratory chain complex activity deficiency. Defects of mitochondrial protein translation, which is closely coordinated between both mitochondrial DNA and the nuclear genome, are associated with multiple respiratory chain deficiency (Calvo et al. 2012; Lightowlers et al. 2015).

The mitochondrial amino-acyl tRNA synthetases (mt-aaRSs) are a group of nuclear-encoded enzymes that catalyse the specific attachment and conjugation of each amino acid to their corresponding cognate tRNA; hence, mt-aaRSs are critical for mitochondrial protein synthesis (Diodato et al. 2014). Mutations of mt-aaRSs genes are important causes of autosomal recessive mitochondrial translation disorders associated with multiple mitochondrial respiratory chain complex deficiencies. These disorders often manifest during childhood with typically multi-organ phenotypes. A genotype–phenotype correlation has been ascertained for several mt-aaRS genes with some tissue specificity, probably due to cell-type specific differences in response to mutations (Diodato et al. 2014; Biancheri et al. 2015; Taylor et al. 2014).

Several mt-aaRS defects unveil discrete and specific clinical and neuroradiological phenotypes including *DARS2* (OMIM#611105) (Scheper et al. 2007); *RARS2* (OMIM#611523) (Edvardson et al. 2007); *FARS2* (OMIM#614946) (Elo et al. 2012); *EARS2* (OMIM#614924) (Steenweg et al. 2012) and *AARS2* (OMIM#615889) (Dallabona et al. 2014). Although the biochemical signatures and neuroimaging of combined respiratory chain deficiency can be distinctive, it remains difficult to infer the genetic origin from often heterogeneous phenotypes. Mutations of *EARS2*, encoding the mitochondrial glutamyl-tRNA synthetase (mtGluR), cause Leukoencephalopathy involving the Thalamus and Brainstem with high Lactate (LTBL), also known as Combined Oxidative Phosphorylation Deficiency 12 (COXPD12) (Steenweg et al. 2012). This mitochondrial disorder is characterised by a distinctive brain magnetic resonance (MR) pattern and a biphasic clinical course; either severe onset with partial clinical and radiological recovery/stagnation or a variable clinical course ranging from mild or intermediate to severe (Steenweg et al. 2012; Biancheri et al. 2015).

We describe the clinical, biochemical and pathological features, alongside the *post-mortem* diagnostic workup of a patient with a severe and fatal form of LTBL. We also review the literature for reported patients with pathogenic *EARS2* variants and highlight the clinical and neuroradiological spectrum of this disorder.

Patient Report and Methods

Patient Report

The proband was a female child, born at term to non-consanguineous parents of Portuguese ancestry. Her birth weight, length and occipitofrontal circumference were normal and she was an otherwise normal baby. At 21 weeks gestation, mild ventriculomegaly was observed by ultrasound and confirmed by foetal brain MR at 22 weeks. A second foetal MR that was performed at 24 weeks was normal.

She was first admitted at day 7 of life due to severe feeding problems, failure to thrive and global hypotonia. Subsequent admissions due to decompensations showed signs of undernutrition and she developed encephalopathy. There was also persistent and progressive lactic acidosis and liver dysfunction. No ketonuria was detected and ammonia was normal. She had no sign of respiratory distress and cardiac evaluation was normal. Newborn screening was also normal. Brain MRI at 2 months revealed symmetrical lesions in brainstem, cerebellum and thalami, suggestive of a mitochondrial disorder (Fig. 1). Delayed myelination was noted (matched with term newborn). MR spectroscopy showed elevated lactate peak (short TE). Analysis of mitochondrial respiratory chain complexes in muscle and liver revealed decreased activities of complex I (19%) and complex IV (11%) only in muscle compared to controls. Histopathology analysis of the muscle revealed cytochrome *c* oxidase (COX)-deficient and ragged-red fibres (Fig. 2a). Liver histology disclosed distention of sinusoids with mix steatosis peri-centrilobular and hemosiderosis. The girl developed multi-organ failure, presenting skin infiltrate and hepatomegaly, and progressive depression of consciousness, went into coma around 4 months old and died at 5 months old. She was the third pregnancy of the couple and second child born. The previous pregnancy was interrupted due to a brain malformation; *corpus callosum* agenesis was described, but no medical records were available.

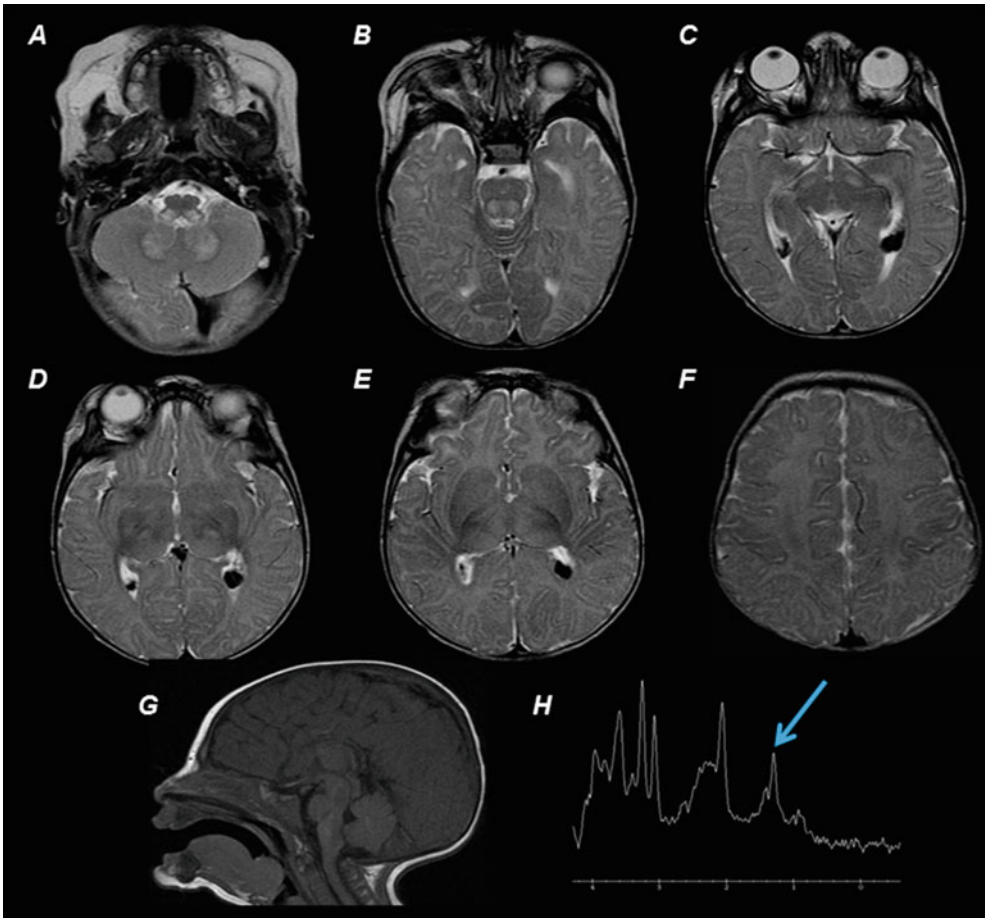

Fig. 1 Brain MRI at 2 months old revealed bilateral and symmetrical abnormal high signal on T2-weighted images in the posterior part of the medulla oblongata and cerebellar white matter and dentate nucleus (**a**), posterior part of the pons (**b**), cerebral peduncles (**c**), subthalamus and thalamus (**d**, **e**); Delayed myelination in pericentral regions (**f**); Hypogenesis of the *corpus callosum* was noted (**g**); Short TE MR spectroscopy demonstrated elevated lactate peak (*arrow*) (**h**)

Genetic Studies

Whole mitochondrial genome sequencing of blood, muscle and liver DNA was performed to exclude pathogenic point mutations. Mitochondrial DNA depletion and rearrangements were excluded from blood, muscle and liver using established diagnostic quantitative real-time PCR and long-range PCR assays, respectively. Pathogenic *SURF1* and *POLG1* variants and larger rearrangements were excluded by Sanger sequencing and Multiplex Ligation Probe Amplification (MLPA) according to standard protocols.

Whole Exome Sequencing and Analysis

Exome capture was attained using the Nextera Rapid Exome Capture (37 Mb) kit, sequenced using the Illumina HiSeq 2000 system in 100 base pair reads and aligned to the human reference genome (UCSC hg19). Variants with a minor allele frequency of >0.01 (1%) from in-house and external exome variant databases were excluded. Recessive (homozygous or compound heterozygous) exonic or splice-site variants of nuclear genes encoding mitochondrial-targeted proteins were prioritised. Polyphen 2, SIFT and Align GVGD were used to predict effect on protein function. Identified recessive variants were validated and segregation studies were performed by Sanger sequencing (Taylor et al. 2014).

Biochemical and Histopathological Studies

Relevant plasma biochemical measurements were liver function tests (AST, ALT, γGT and LDH), creatine kinase, lactate and amino acids. Measurements of amino acids and organic acids in urine were also performed.

Diagnostic skin, muscle and liver biopsies were performed; samples were frozen and processed for diagnostic histology and histochemistry according to standard procedures. Mitochondrial OXPHOS activities (complexes I–V) were determined in muscle and liver (normalised to citrate synthase activity) as described (Grazina 2012).

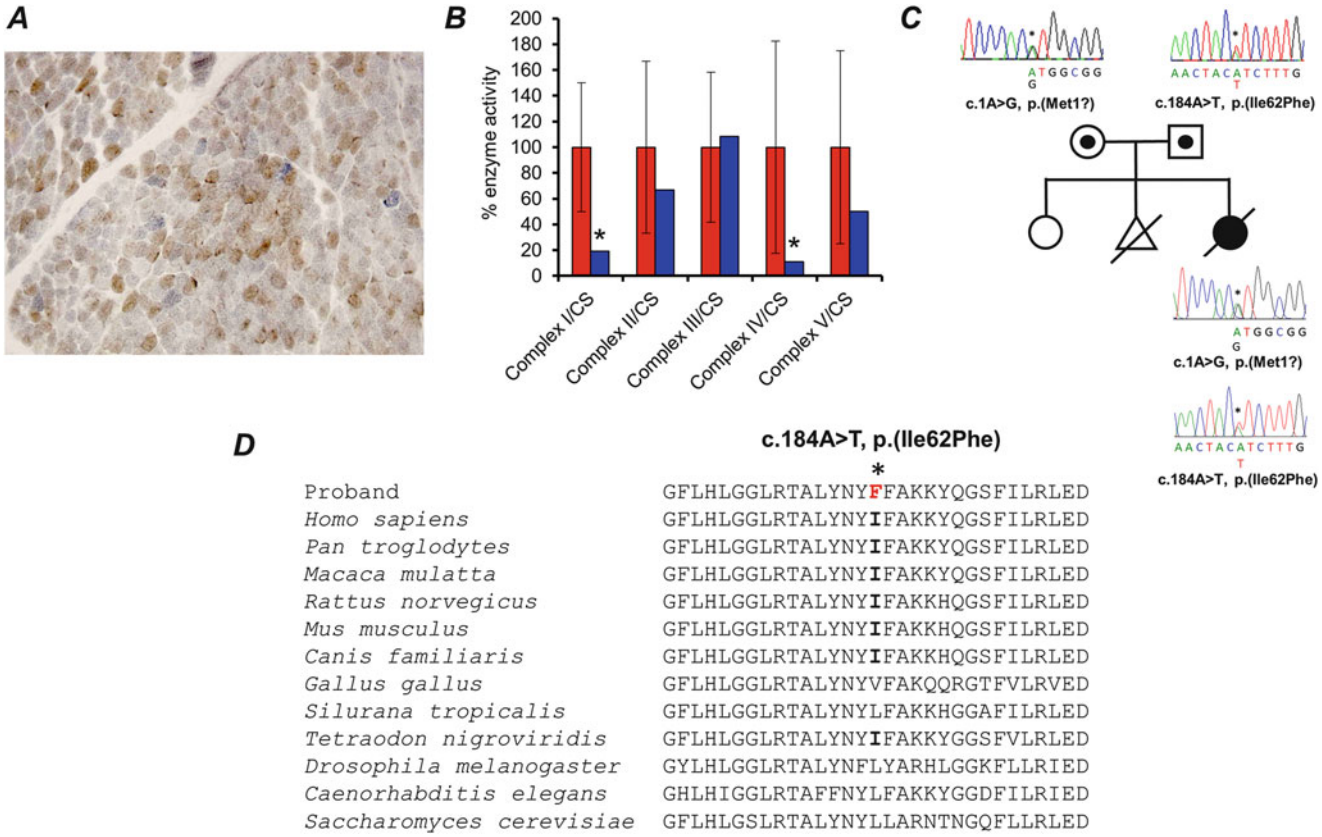

Fig. 2 Muscle histochemical and biochemical studies, segregation analysis and p.Ile62 amino acid sequence conservation. (**a**) Sequential COX-SDH histochemical reaction demonstrating a mosaic pattern of COX-deficiency in patient muscle. (**b**) Biochemical activity of mitochondrial respiratory chain complexes I (nmol NADH oxidised·min^{-1}·unit citrate synthase^{-1}), II (nmol DCPIP reduced·min^{-1}·unit citrate synthase^{-1}), III, IV ($\times 10^{-3}$ K s^{-1}·unit citrate synthase^{-1}) and V (nmol NADH oxidised·min^{-1}·unit citrate synthase^{-1}), as a percentage of residual controls. (**c**) Family pedigree showing confirmation of parental carrier status for the p.Met1? (maternal) and p.Ile62Phe (paternal) *EARS2* variants. (**d**) Multiple sequence alignment of p.Ile62 residue across species

Cell Culture

Cultured fibroblasts from the proband and two controls were grown in minimum essential medium (MEM) (Gibco) with 10% foetal bovine serum (FBS) (Gibco), 1× MEM vitamins (Sigma), 1× non-essential amino acids (Sigma), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma), 1 mM sodium pyruvate solution (Sigma), 0.05 mg/ml uridine and 2 mM L-glutamine (Sigma).

Western Blotting

Fibroblast cell lysates (50 μg) or muscle lysates (25 μg) were subjected to 12% SDS-PAGE and transferred to PVDF membranes (Immobilon P). Membranes were incubated overnight at 4°C with primary antibodies specific to EARS2 (HPA043633, Sigma), SDHA (ab14715, Abcam), ATP5B (ab14730, Abcam), UQCRC2 (ab14745, Abcam), MT-CO1 (ab14705, Abcam), MT-CO2 (ab110258, Abcam), NDUFB8 (ab110242, Abcam), β-actin (A5316, Sigma) or α-tubulin (ab7291, Abcam), followed by HRP-conjugated secondary antibodies (DakoCytomation) for 1 h at room temperature and were visualised using ECL-prime (GE Healthcare) and BioRad ChemiDoc MP with Image Lab software according to manufacturer's guidelines.

Results

Diagnostic Mitochondrial Investigations

In agreement with the muscle histopathology, which revealed mitochondrial abnormalities, assessment of mitochondrial respiratory chain complex activities in skeletal muscle demonstrated a combined deficiency involving both complex I and complex IV (Fig. 2b). Assessment of enzyme activities in liver was normal (data not shown). Whole mitochondrial genome sequencing failed to identify likely pathogenic point mutations, while diagnostic quantitative real-time PCR and long-range PCR assays excluded mitochondrial DNA depletion and large-scale mitochondrial DNA rearrangements, respectively. Targeted screening

of *SURF1* and *POLG1* failed to detect likely pathogenic variants.

Whole Exome Sequencing

Prioritisation of rare recessive variants in nuclear genes encoding mitochondrial-targeted proteins identified by whole exome sequencing (WES) revealed two heterozygous variants in *EARS2* (NM_001083614); c.1>G, p.Met1? and c.184A>T, p.Ile62Phe. Segregation analysis confirmed parental carrier status of the identified variants (Fig. 2c). The start-loss p.Met1? variant was described previously in a single *EARS2* patient of Portuguese ancestry (Steenweg et al. 2012). The p.Ile62Phe missense variant was novel and unrepresented in exome variant databases including Exome Aggregation Consortium (ExAC), NHLBI Exome Variant Server (ESP) and the 1000 Genomes Project. This missense change affects a moderately conserved residue within an evolutionary conserved region of the protein (Fig. 2d).

Western Blot Analysis

Patient fibroblasts showed no decrease in steady-state protein levels of respiratory chain complex subunits (Fig. 3a). In contrast, there was a marked loss of NDUFB8 (complex I), MT-CO1 and MT-CO2 (complex IV) protein levels in patient muscle homogenate (Fig. 3b). Steady-state levels of EARS2 were markedly decreased in both patient fibroblasts and muscle homogenate compared to controls, demonstrating a functional consequence of the *EARS2* mutations.

Discussion

We report the clinical, biochemical and brain image signature of a patient with a severe mitochondrial disease, disclosed to be LTBL. Genetic characterisation identified two heterozygous *EARS2* variants; a maternally inherited start-loss p.Met1? variant and a paternally inherited novel missense p.Ile62Phe change.

The bilateral and symmetrical lesions that predominantly affected grey matter structures and brainstem plus the elevated lactate observed at 2 months of age were suggestive of Leigh syndrome. Muscle studies demonstrated COX-deficient and ragged-red fibres, while complex I and IV respiratory chain activities were significantly reduced. Although liver respiratory chain activity was normal, there was evident liver dysfunction. Liver involvement was also described in six additional *EARS2* patients, which included one or more of neonatal transient icterus, hepatomegaly, fatty liver and steatosis (Steenweg et al. 2012; Talim et al. 2013; Taylor et al. 2014; Pronicka et al. 2016). Post-mortem examination of the liver from the patient reported by Talim et al. (2013) also found COX-deficiency.

Patient fibroblast and muscle samples displayed a marked decrease in EARS2 protein levels (Fig. 3). This was expected due to the start-loss variant, while the missense may affect EARS2 stability. Decreased EARS2 has also been previously demonstrated in fibroblasts with compound heterozygous missense *EARS2* variants (Danhauser et al. 2016). We demonstrated decreased abundance of complex I and IV subunits in the muscle, which

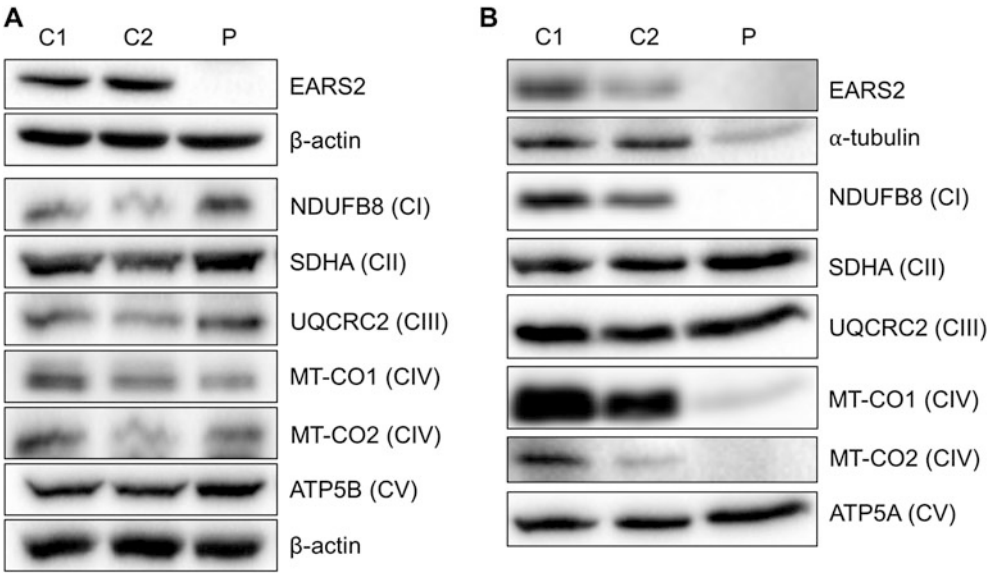

Fig. 3 Western blot analysis of patient fibroblasts and muscle homogenate. Western blots of lysates from patient fibroblasts (**a**) and skeletal muscle (**b**) from the proband (P) and two controls (C), Antibodies against EARS2, NDUFB8 (CI), SDHA (CII), UQCRC2 (CIII), MT-CO1 (CIV), MT-CO2 (CIV), and ATP5B (CV) were used, with β-actin and α-tubulin as loading controls

corresponded with impaired complex I and IV activities by direct enzymatic assay (Fig. 2b). In contrast, the abundance of respiratory chain complex subunits was normal in patient fibroblasts (Fig. 3a), which corresponds to the tissue-specific consequences of *EARS2* variants.

To date, 26 patients are reported with LTBL (12 by Steenweg et al. (2012), one by Talim et al. (2013), two by Taylor et al. (2014), one by Biancheri et al. (2015), one by Kohda et al. (2016), one by Kevelam et al. (2016), one by Danhauser et al. (2016), one by Taskin et al. (2016), one by Güngör et al. (2016), two by Şahin et al. (2016) and two by Pronicka et al. (2016)), with our patient being the 26th reported patient. The phenotype of our patient fits the severe group of LTBL patients, mostly due to signs of perinatal presentation of the disease and rapid decline of her clinical status. The relative immature brain associated with a slight delay in myelination limited the evaluation of possible involvement of the cerebral white matter in our patient. Nevertheless, no obvious signal abnormality of the supratentorial white matter was found, namely the spared periventricular rim described in older patients (Steenweg et al. 2012; Biancheri et al. 2015; Kevelam et al. 2016; Güngör et al. 2016; Taskin et al. 2016; Şahin et al. 2016).

Cerebral lesions appeared less expressive than reported previously, perhaps because at 2 months of age it could be too early to observe white matter abnormalities such as leukoencephalopathy. Still, the remaining lesions were typical of LTBL, as the brainstem and cerebellar lesions were present at that early age. Hypogenesis of the *corpus callosum* was also noted, as has been consistently described (Steenweg et al. 2012; Biancheri et al. 2015; Kevelam et al. 2016; Güngör et al. 2016). The collective findings of symmetrical signal changes of the deep white matter (usually sparing the periventricular rim), thalami and brainstem together with increased lactate on MRS is the hallmark of LTBL. In patients described with either mild (Biancheri et al. 2015; Güngör et al. 2016; Taskin et al. 2016; Şahin et al. 2016) or severe disease (Steenweg et al. 2012; Kevelam et al. 2016), the clinical presentation appeared to correlate with the severity of neuroimaging at each stage of this biphasic disease. However, lesions of the main structures involved were not different between patients in each group of severity, except for the significant improvement without new lesions of brain MR/MRS associated with the mild-intermediate forms. In severe situations, a progressive atrophy of the affected structures has been detected (Biancheri et al. 2015). Also, as observed by Steenweg et al. (2012), patients in the severe group had early onset neurodevelopment compromise and persistent hyperlactacidaemia. Due to the rapid and fatal disclosure, neuroimaging alone was insufficient to predict the outcome of our patient, but she had signs of neurodevelopment compromise such as hypotonia. She had a fatal course that was similar to patients reported by Talim et al. (2013) and Danhauser et al. (2016). Unfortunately none of these reports had brain MR reports and images available for comparison, except for *corpus callosum* dysgenesis and agenesis, described for each patient on brain MR and brain sonogram, respectively. The two patients reported by Taylor et al. (2014) (patients 16 and 17) also had a fatal outcome with a severe neurometabolic disorder, with neonatal or very early onset presentation of LTBL.

Of the severe cases, three had compound heterozygous variants and two had a homozygous variant. In total, only five patients have been reported with homozygous pathogenic *EARS2* variants, presenting forms of LTBL with different clinical outcomes. One patient each from Talim et al. (2013) and Taylor et al. (2014) had a homozygous c.193A>G (p.Lys65Glu) missense change affecting a well conserved residue, and had a severe and fatal form of the disease. The patient presented by Biancheri et al. (2015) with a homozygous c.902G>C (p.Gly301Ala) missense change was described as having an intermediate form of LTBL. The patient from Kevelam et al. (2016) had a homozygous c.454_456del (p.Lys152del) in-frame deletion of a highly conserved residue, which presented as a severe form of disease, and was alive at the time of publication (aged 18 years). The fifth patient from Taskin et al. (2016) had a homozygous c.322C>T (p.Arg108Trp) missense change and presented a mild phenotype. Compound heterozygous changes were identified in three additional patients as part of large-scale WES studies but without specific details of phenotypes for comparison (Kohda et al. 2016; Pronicka et al. 2016). Regarding the two heterozygous variants in our patient, the start-loss p.Met1? variant is predicted to abolish the initiation translation site and has only been described previously in a single patient of Portuguese ancestry (Steenweg et al. 2012), suggesting a possible founder effect in this population. The missense p.Ile62Phe variant affects a moderately conserved amino acid within a conserved region of EARS2, which we postulate causes the tertiary structure of the protein to become unstable. For the patient here reported, as leukoencephalopathy was not clear on the only MR performed, the diagnosis could therefore be missed without WES analysis.

Hence, from the current 26 patients reported (including this report), almost half (11 patients) presented with a severe form of LTBL with early onset and various *EARS2* pathogenic variants (patients 1, 8, 9, 10 and 11 from Steenweg et al. (2012); Talim et al. (2013); patients 16 and 17 from Taylor et al. (2014); Kevelam et al. (2016); Danhauser et al. (2016), and the current report). Five died soon after the first clinical event (our patient, Talim et al. 2013; Taylor et al. 2014; Danhauser et al. 2016) and six patients had severe neurodevelopmental compromise, although alive at the time of the respective publication,

the oldest being 18 years old (Steenweg et al. 2012; Kevelam et al. 2016). Further studies will be needed to better understand which, if any, specific neuroimaging features would correlate with the severity and course of the disease. We were unable to establish a particular genotype–phenotype correlation among these patients.

A syndromic phenotype has been carefully delineated for LTBL as described by Steenweg et al. (2012), which is consistent with all subsequent patients reported, including our patient. Though the clinical and bio-histochemical characteristics are not specific, when combined with brain MR/MRS pattern there can be enough specificity to implement a targeted *EARS2* gene analysis in similar cases. Supplementary Table S1 summarises and compares patients reported so far with brain MR available.

For our patient, the application of WES was fundamental in reaching a definitive genetic diagnosis, but the functional studies were essential to validate the significance of the novel variant. At the time of presentation, the clinical diagnosis of LTBL had only recently been described in the literature, although we did suspect a nuclear-driven, Mendelian mitochondrial disorder (autosomal recessive inheritance) as the likely cause in our family. The establishment of the molecular diagnosis is always important for accurate genetic counselling for families and should allow the option for prenatal diagnosis or pre-implantation diagnosis for future pregnancies.

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Take-Home Message (Synopsis)

This report reinforces the phenotypic spectrum of pathogenic *EARS2* variants by reporting a severe clinical manifestation and highlights diagnostic methods to recognise biochemical and biological consequences of the genetic error.

Compliance with Ethics Guidelines

Conflict of Interest

Renata Oliveira, Ewen W. Sommerville, Kyle Thompson, Joana Nunes, Angela Pyle, Manuela Grazina, Patrick F. Chinnery, Luísa Diogo, Paula Garcia and Robert W. Taylor declare that they have no conflict of interest.

Informed Consent

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

Authors' Contributions

Renata Oliveira, Joana Nunes, Manuela Grazina, Luísa Diogo, Paula Garcia and Robert W. Taylor acquired the clinical data and performed genetic, biochemical and histochemical investigations. Ewen W. Sommerville, Angela Pyle and Patrick F. Chinnery generated and analysed WES data. Ewen W. Sommerville and Kyle Thompson undertook the western blot studies. Renata Oliveira, Ewen W. Sommerville and Robert W. Taylor drafted the manuscript; all authors contributed to critical revision of the manuscript.

References

Biancheri R, Lamantea E, Severino M et al (2015) Expanding the clinical and magnetic resonance spectrum of leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL) in a patient harboring a novel *EARS2* mutation. JIMD Rep 23:85–89

Calvo SE, Compton AG, Hershman SG et al (2012) Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. Sci Transl Med 118:118ra10

Dallabona C, Diodato D, Kevelam SH et al (2014) Novel (ovario) leukodystrophy related to *AARS2* mutations. Neurology 82 (23):2063–2071

Danhauser K, Haack TB, Alhaddad B et al (2016) *EARS2* mutations cause fatal neonatal lactic acidosis, recurrent hypoglycemia and agenesis of *corpus callosum*. Metab Brain Dis 31(3):717–721

Diodato D, Ghezzi D, Tiranti V (2014) The mitochondrial aminoacyl tRNA synthetases: genes and syndromes. Int J Cell Biol 2014:787956

Edvardson S, Shaag A, Kolesnikova O et al (2007) Deleterious mutation in the mitochondrial arginyl-transfer RNA synthetase gene is associated with pontocerebellar hypoplasia. Am J Hum Genet 81(4):857–862

Elo JM, Yadavalli SS, Euro L et al (2012) Mitochondrial phenylalanyl-tRNA synthetase mutations underlie fatal infantile Alpers encephalopathy. Hum Mol Genet 21(20):4521–4529

Grazina MM (2012) Mitochondrial respiratory chain: biochemical analysis and criterion for deficiency in diagnosis. Methods Mol Biol 837:73–91

Güngör O, Özkaya AK, Şahin Y et al (2016) A compound heterozygous *EARS2* mutation associated with mild leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL). Brain Dev. pii: S0387-7604(16)30030-4

Kevelam SH, Klouwer FCC, Fock JM et al (2016) Absent thalami caused by a homozygous *EARS2* mutation: expanding disease spectrum of LTBL. Neuropediatrics 47(1):64–67

Kohda M, Tokuzawa Y, Kishita Y et al (2016) A comprehensive genomic analysis reveals the genetic landscape of mitochondrial respiratory chain complex deficiencies. PLoS Genet 12(1), e1005679

Lightowlers RN, Taylor RW, Turnbull DM (2015) Mutations causing mitochondrial disease: what is new and what challenges remain? Science 349(6255):1494–1499

Pronicka E, Piekutowska-Abramczuk D, Ciara E et al (2016) New perspective in diagnostics of mitochondrial disorders: two years' experience with whole-exome sequencing at a national paediatric centre. J Transl Med 14(1):174

Şahin S, Cansu A, Kalay E et al (2016) Leukoencephalopathy with thalamus and brainstem involvement and high lactate caused by novel mutations in the *EARS2* gene in two siblings. J Neurol Sci 365:54–58

Scheper GC, van der Klok T, van Andel RJ et al (2007) Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. Nat Genet 39(4):534–539

Steenweg ME, Ghezzi D, Haack T et al (2012) Leukoencephalopathy with thalamus and brainstem involvement and high lactate 'LTBL' caused by *EARS2* mutations. Brain 135(Pt 5):1387–1394

Talim B, Pyle A, Griffin H et al (2013) Multisystem fatal infantile disease caused by a novel homozygous *EARS2* mutation. Brain 136:1–3

Taskin BD, Karalok ZS, Gurkas E et al (2016) Early-onset mild type leukoencephalopathy caused by a homozygous *EARS2* mutation. J Child Neurol 31(7):1–4

Taylor RW, Pyle A, Griffin H et al (2014) Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. JAMA 312(1):68–77

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CASE REPORT

Leukoencephalopathy due to Complex II Deficiency and Bi-Allelic *SDHB* Mutations: Further Cases and Implications for Genetic Counselling

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Abstract Isolated complex II deficiency is a rare cause of mitochondrial disease and bi-allelic mutations in *SDHB* have been identified in only a few patients with complex II deficiency and a progressive neurological phenotype with onset in infancy. On the other hand, heterozygous *SDHB* mutations are a well-known cause of familial paraganglioma/pheochromocytoma and renal cell cancer. Here, we describe two additional patients with respiratory chain deficiency due to bi-allelic *SDHB* mutations. The patients' clinical, neuroradiological, and biochemical phenotype is discussed according to current knowledge on complex II and SDHB deficiency and is well in line with previously described cases, thus confirming the specific neuroradiological presentation of complex II deficiency that recently has emerged. The patients' genotype revealed one novel *SDHB* mutation, and one *SDHB* mutation, which previously has been described in heterozygous form in patients with familial paraganglioma/pheochromocytoma and/or renal cell cancer. This is only the second example in the literature where one specific *SDHx* mutation is associated with both recessive mitochondrial disease in one patient and familial paraganglioma/pheochromocytoma in others. Due to uncertainties regarding penetrance of different heterozygous *SDHB* mutations, we argue that all heterozygous *SDHB* mutation carriers identified in relation to *SDHB*-related leukoencephalopathy should be referred to relevant surveillance programs for paraganglioma/pheochromocytoma and renal cell cancer. The diagnosis of complex II deficiency due to *SDHB* mutations therefore raises implications for genetic counselling that go beyond the recurrence risk in the family according to an autosomal recessive inheritance.

Introduction

Mitochondrial respiratory chain diseases are inborn errors of metabolism that are caused by defective oxidative phosphorylation. Oxidative phosphorylation is the primary mechanism of ATP production in eukaryotic cells, utilizing the five transmembrane complexes (complex I–V), which constitute the respiratory chain (RC) in the inner mitochondrial membrane. The RC consists of at least 90 protein subunits encoded by nuclear and mitochondrial genes (DiMauro et al. 2013). Clinically, deficiency of the RC with onset in childhood is associated with a variable spectrum of manifestations, including Leigh syndrome, mitochondrial encephalomyopathy with multisystem involvement, and leukoencephalopathy (Goldstein et al. 2013). Brain imaging with magnetic resonance (MRI) may show affection of basal ganglia, cerebellum, brain stem, white matter, and/or cortical structures. Different patterns of MRI abnormalities can support the diagnostic process (Bricout et al. 2014; Helman et al. 2015). Even though involvement of the basal ganglia is a frequent and well-known feature of mitochondrial disease, white matter involvement has been increasingly recognized in more recent patient descriptions (Wong 2012; Morato et al. 2014).

The four subunits of complex II, which is also termed succinate dehydrogenase (SDH), are exclusively encoded by the nuclear genome. Complex II has a dual role in mitochondrial metabolism: it functions in the citric acid cycle as well as in the RC, transferring electrons to ubiquinone (Jain-Ghai et al. 2013). Two subunits, SDHA and SDHB, have a catalytic function, while SDHC and SDHD are considered anchoring proteins. In addition, a number of proteins, including SDHAF1, SDHAF2 (SDH5), SDHAF3, and SDHAF4, are required for correct assembly and functioning of the protein complex (Jain-Ghai et al. 2013). Overall, complex II deficiency is a rare cause of RC deficiency and was primarily identified in cases with bi-allelic *SDHA* and *SDHAF1* mutations, while heterozygous mutations of *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* were found to cause familial paraganglioma/pheochromocytoma (Ghezzi et al. 2009; Rutter et al. 2010; Jain-Ghai et al. 2013). Familial paraganglioma/pheochromocytoma is a hereditary tumor susceptibility disorder with development of paraganglioma and pheochromocytoma with high penetrance, and with an increased risk for malignancy and renal cell carcinoma in the case of *SDHB* mutations (Ricketts et al. 2010). Complex II-related RC deficiencies show a wide clinical spectrum including presentations with isolated myopathy/cardiomyopathy as well as severe multisystem disorders with neurological manifestations. Recently, the first patients with complex II deficiency due to bi-allelic mutations in *SDHB* and *SDHD* were described and patients presented with progressive mitochondrial encephalomyopathy (Alston et al. 2012; Jackson et al. 2014; Helman et al. 2015).

Here, we present two additional patients with RC deficiency due to *SDHB* mutations. The patients' phenotype including their leukoencephalopathic MRI pathology is described and compared to the phenotypic spectrum associated with complex II deficiency. The implications of identifying complex II deficiency for genetic counseling are discussed in the context of current knowledge regarding familial paraganglioma/pheochromocytoma.

Subjects and Methods

Patient 1 Patient 1 was the first common female child of consanguineous parents of Lebanese descent. The mother's sister deceased at the age of 18 months due to an undiagnosed progressive neurological disease with onset at the age of 10 months; the pedigree is presented in Fig. 1. There was no family history of cancer or paraganglioma/pheochromocytoma. The patient was born by cesarean section in gestational week 35 because of intrauterine growth retardation; birth weight was 1,663 g (−34% SGA), length 41 cm (−2 SD), head circumference 30 cm (−1.1 SD), and Apgar score normal. The pregnancy had been complicated by preeclampsia. The neonatal course was uncomplicated. The patient's development during the first 12 months was slightly delayed with truncal hypotonia and instable sitting position, but the patient crawled at 8 months and pulled herself into a standing position at 10 months of age. From the age of 12 months, a progressive loss of these previously acquired abilities was noted and the deterioration was more pronounced during infections. At 15 months of age, she had pronounced truncal hypotonia, increased muscle tone and pathologically increased deep tendon reflexes in upper and lower extremities, extensor plantar response, and intermittent vertical nystagmus. Weight, length, and head circumference had developed along −1.8, −1.4 SD, and −1.6 SD, respectively. Initial laboratory analysis showed normal blood counts and routine biochemistry parameters. Blood gas analysis showed compensated metabolic acidosis with a lactate level of 3.5 mmol/L (ref < 2.1 mmol/L). Lactate, glucose, and protein concentration, as well as cell count, and amino acid profile in cerebrospinal fluid were normal. Urine metabolic screening showed a normal lactate concentration, but increased levels of ketone bodies and Krebs cycle metabolites, especially succinate. All additional metabolic

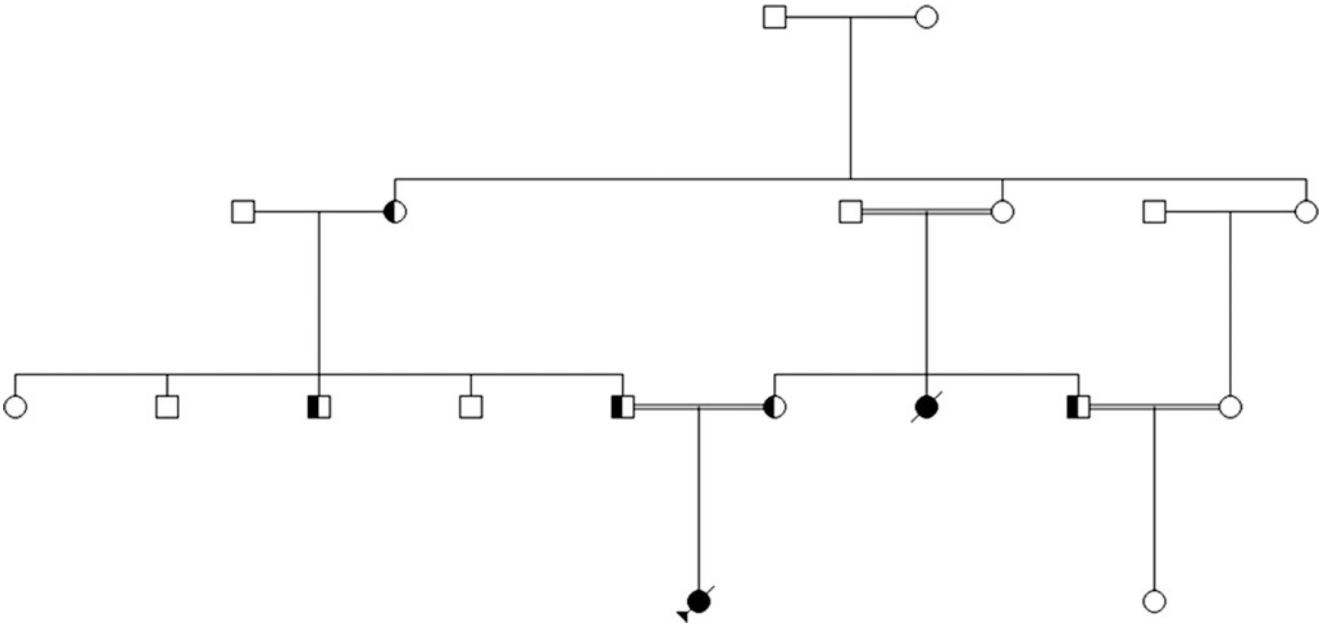

Fig. 1 Pedigree of the family of patient 1 (*arrow*). Affected family members are shown with *filled symbols*, and heterozygotes for the p.Leu257Val variant are shown with *half-filled symbols*

examinations and array CGH analysis were without abnormalities. Eye examination revealed slight atrophy of the optic nerves bilaterally, reduced vision (6/40), and horizontal nystagmus with elements of opsoclonus. Cerebral MRI at 16 months of age was pathological and is presented in Fig. 2a–h. A muscle biopsy was performed at the age of 18 months. In the further course, the patient's neurological deterioration continued, and she became dependent on gavage feeding. Her truncal hypotonia with an opisthotonic neck position and her spasticity increased. At the age of 25 months, the patient deceased in the course of a severe respiratory infection causing multi-organ failure.

Patient 2 Patient 2 was the third male child of healthy and unrelated parents of Turkish and Swedish descent (mother and father, respectively). There was no family history of cancer or paraganglioma/pheochromocytoma. Pregnancy was complicated by preeclampsia. The patient was born by elective cesarean section in gestational week 35 because of intrauterine growth retardation. Birth weight was 1,200 g (< -3 SD), length was 38.5 cm (< -3 SD), and head circumference was 30 cm (-2 SD). Apgar values were normal. The neonatal course was uncomplicated. The early psychomotor development was normal, but from age 6 months a progressive loss of previously acquired skills was noted with episodic deterioration during infections. At 9 months of age, he had pneumonia with respiratory failure necessitating assisted ventilation and lost the ability to babble, grasp, turn around, and needed tube feeding. Neuropediatric examination showed complete head lag with pronounced truncal hypotonia, increased muscle tone in upper and lower extremities as well as increased tendon reflexes. Weight, length, and head circumference had developed along -3.5, -3 SD, and -1.5 SD, respectively. Initial laboratory analysis showed increased blood lactate levels up to 9 mmol/L (ref < 1.8), serum levels of ALAT and ASAT up to 40 (ref < 0.6) and 30 μkat/L (ref < 0.8), respectively, creatine kinase level to 4,970 U/L (ref < 150), and the INR level up to 2.4. A cardiomyopathy was found with severe dilatation and hypertrophy of the septum and posterior wall of the left ventricle. Upon treatment with enalapril, metoprolol, and furosemide, the biochemical parameters normalized except for lactate, which remained slightly increased. Cerebral MRI and MR spectroscopy at 11 months of age showed leukoencephalopathy, detailed in Fig. 2i–o. Cerebrospinal fluid analysis showed a normal cell count and glucose level, while the lactate level was 3.0 mmol/L (ref < 1.7). Urine organic acid screening showed slightly increased lactate, but no succinate. Eye examination revealed strabismus and anisometropia with abnormal pursuit and saccades. EEG was normal. A muscle biopsy was performed at the age of 11 months (Fig. 3a). The patient was put on treatment with riboflavin and coenzyme Q10. In the further course, the patient's condition was stable with some improvement until the age of 1 year when he had a severe relapse with multi-organ failure and cardiac arrest leading to death.

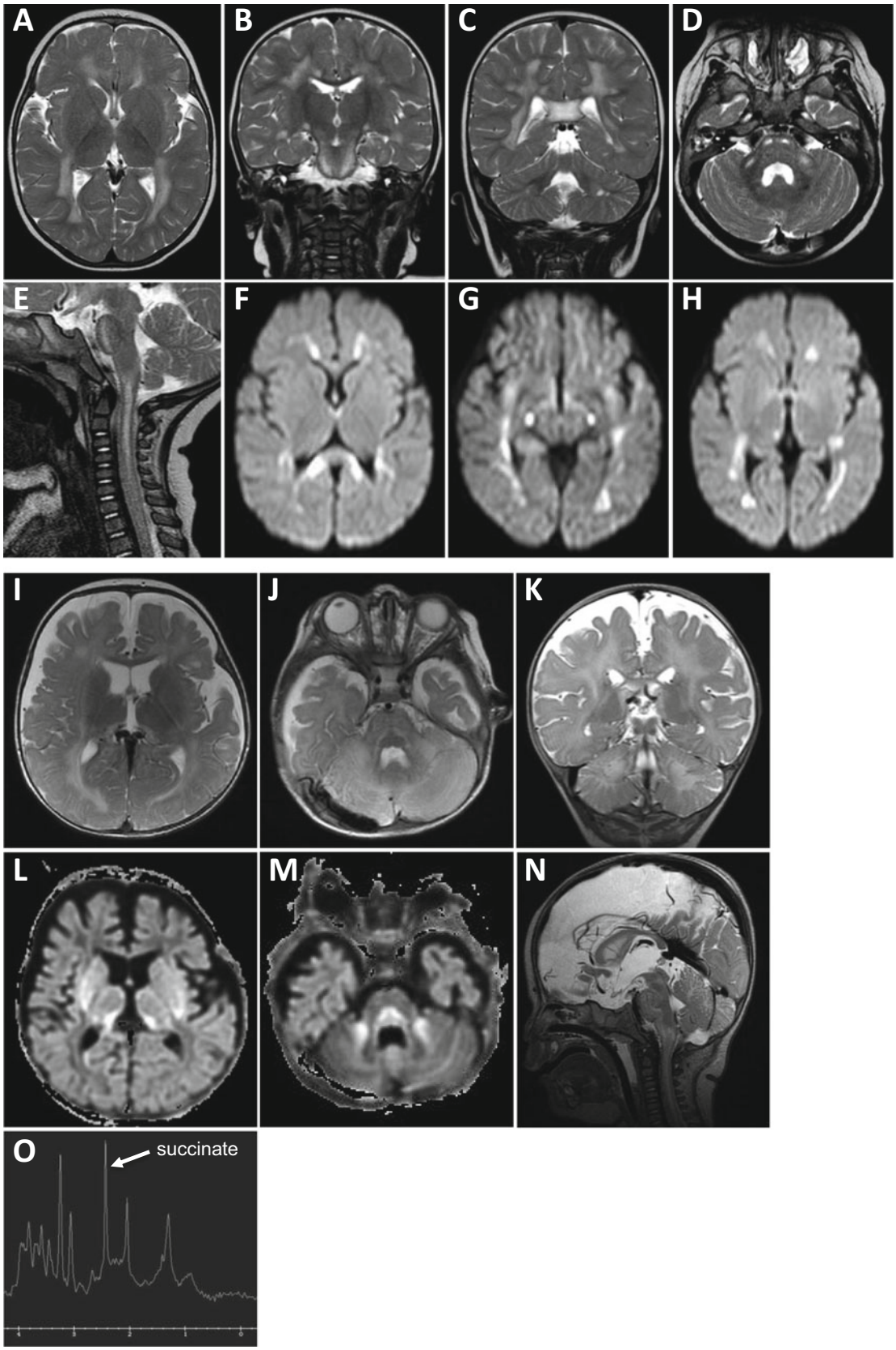

Fig. 2 Magnetic resonance imaging (MRI) of patient 1 (**a**–**h**) at 15 months of age and patient 2 (**i**–**n**) at 10 months of age including T2-weighted (T2W) (**a**–**e**; **i**–**k**, **n**) and diffusion-weighted (DWI) (**f**–**h**; **l**, **m**) imaging. MRI of patient 1 demonstrates increased T2W signal

Methods

Analysis of RC Enzyme Activity

Patient 1 Spectrophotometric analysis of RC enzyme activity in frozen muscle tissue and cultured fibroblasts was performed as previously described (Larsen et al. 2012).

Patient 2 Isolation of fresh muscle mitochondria, polarographic measurements, and spectrophotometric enzyme analyses were performed essentially as previously described (Tulinius et al. 1991).

Genetic Investigations

Patient 1 DNA from patient 1 was used for a genomewide search for homozygosity with the Affymetrix GeneChip 6.0 array (Affymetrix Inc., Santa Clara, CA), essentially as described (Ostergaard et al. 2011). *SDHB* and *SDHAF1* were PCR-amplified using intronic primer pairs flanking each exon. PCR products were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems).

Patient 2 Whole Exome Sequencing (WES) and WES data analysis were carried out as described (Sofou et al. 2015). Sanger sequencing of DNA from the patient and his parents verified the mutations identified by WES. Sequencing analysis was performed using an ABI PRISM 3100 Genetic analyzer and the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). All *SDHB* variants are numbered according to GenBank accession number NM_003000.2. The strategies for identification of the genetic background for the disorder differed, since the two patients were investigated in different laboratories.

Western Blot Analysis

Western blot analysis was performed essentially as previously reported (Ostergaard et al. 2007). Primary antibodies were against SDHB (Atlas Antibodies, Stockholm, Sweden; 1:1,000), SDHA (Thermo Fischer Scientific, Waltham, MA; 1:1,000), and against porin (Proteintech, Chicago, IL; 1:1,000) as loading control. The secondary antibody was goat anti-rabbit at a 1:1,000 dilution (Dako, Glostrup, Denmark).

Results

Muscle Histology

Histological analysis revealed normal fiber diameter variation with only few centralized nuclei in patient 1, while there was slightly increased fiber diameter variability and normally placed nuclei in patient 2. Both had normal amount of connective tissue and no vacuoles, inclusion bodies, or ragged red fibers in trichrome staining (not shown). Both patients had positive COX staining in all fibers, while there was a diffuse and severe lack of SDH staining (shown for patient 2 in Fig. 3a, I). Oil-red-O staining showed some fibers with lipid content, but within the normal range (patient 1, not shown). Sudan Black staining revealed some fibers with increased lipid content in patient 2 (not shown). Electron microscopy was performed in patient 2 only and was found pathological as presented in Fig. 3a, III.

RC Enzyme Activity

Polarographic (patient 2) and spectrophotometric (patient 1 and 2) analyses revealed a severe complex II deficiency in muscle from both patients (spectrophotometric 14 and 7% of control mean for patient 1 and 2, respectively; polarographic 17% of control mean for patient 2). Complex I activity was mildly affected, whereas the other enzyme activities were practically normal. The results were confirmed in fibroblasts of patient 1 with complex II activity at 6% of control mean (supplementary Table S1). Together with the finding of increased succinate concentration in urine of patient 1 and an abnormal succinate peak on ^{1}H MR spectroscopy of patient 2, this constellation confirmed complex II deficiency.

Fig. 2 (continued) intensity involving frontal, parietooccipital, and posterior temporal white matter sparing juxtacortical fibers (**a**–**c**), lateral geniculate bodies (**b**), corpus callosum with sparing of the inner and outer blades (**c**), middle cerebellar peduncles, corticospinal tracts in the anterior pons (**d**, **e**), and extensive involvement of the cervical medulla (**e**). Diffusion restriction is demonstrated in the periventricular white matter and corpus callosum (**f**), and particularly evident in the lateral geniculate bodies (**g**). Minimal changes are seen in the thalamic pulvinar (**h**) and bilateral ventral nuclei (**f**). MRI of patient 2 likewise reveals extensive T2 hyperintensities involving frontal, parietooccipital, and posterior temporal white matter with sparing of the juxtacortical fibers (**i**, **k**). T2 hyperintensity and restricted diffusion of the bilateral pulvinar and ventral thalamic nuclei (**i**, **l**) was prominent. Infratentorially, the pons including crossing fibers, middle cerebellar peduncles, and deep white matter of the cerebellar hemispheres (**j**, **k**) as well as medullary pyramids and central cervical medulla (**n**) are affected. Decreased diffusivity is demonstrated in the dorsal pons and cerebellar peduncles (**m**). (**o**) shows ^{1}H MR spectroscopy with short echo time and voxel of interest placed over centrum semiovale with an abnormal succinate peak at 2.4 ppm detected in patient 2

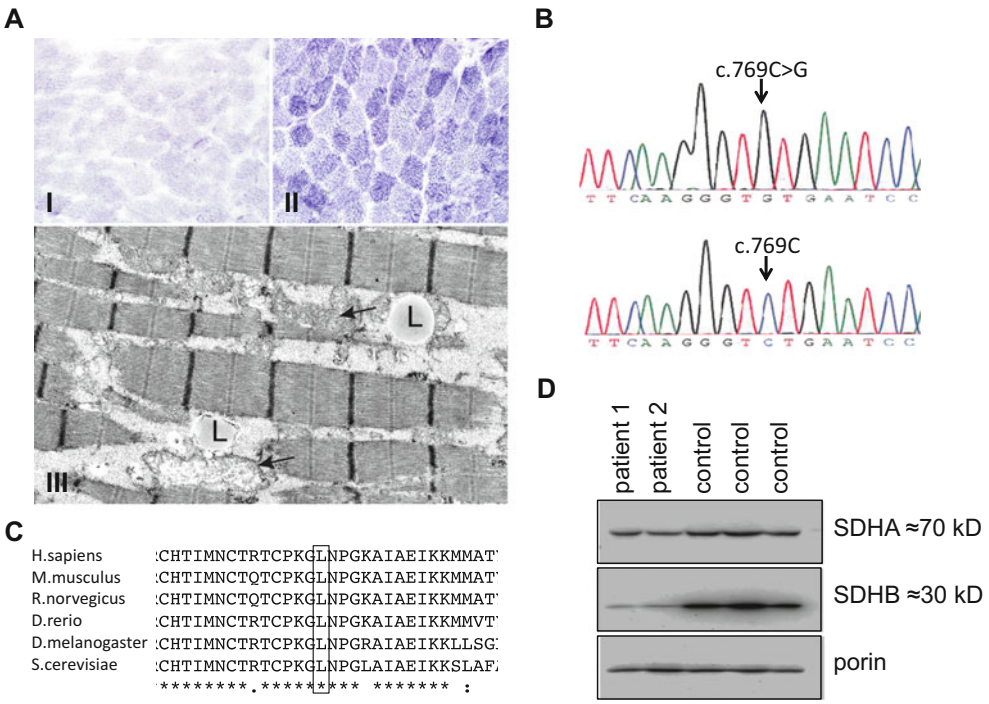

Fig. 3 Muscle histology, sequence analysis, and Western blot analysis in patients with *SDHB* mutations. (**a**) Muscle histology of patient 2: Muscle biopsy of the vastus lateralis muscle at age 1 year demonstrated reduced histochemical activity of succinate dehydrogenase (SDH) (*I*) compared to an age-matched control (*II*). Electron microscopy (*III*) demonstrated a few lipid droplets (L), some enlarged mitochondria with partly disrupted cristae (*arrows*), and abundant glycogen between the myofibrils. (**b**) DNA sequence analysis of *SDHB* in patient 1 indicates the position of homozygous missense mutation c.769C>G (p.Leu257Val) (*top*) compared to wild-type sequence in a control individual (*bottom*). (**c**) Alignment of a relevant stretch of amino acid sequence of SDHB in different species shows that leucine 257 (*black rectangle*) is evolutionary conserved. (**d**) Immunoblot analysis of fibroblast cells from patient 1, patient 2, and three controls with antibodies against SDHA, SDHB, and porin as loading control reveals reduced amount of SDHB and a lesser extent of SDHA in both patients

Molecular Genetic Analyses

Patient 1 Homozygosity mapping showed several homozygous regions, and *SDHB* and *SDHAF1* were the only complex II-related genes located in regions of homozygosity. Sequencing of the *SDHAF1* gene was normal while sequencing of the *SDHB* gene showed a homozygous missense mutation c.769C>G (p.Leu257Val), not previously reported in the literature (Fig. 3b). It has been reported in 1 out of 121,292 alleles in the ExAC database (http://exac.broadinstitute.org/), but it has to be noted that this database does not include any significant representation of persons of Lebanese descent. This mutation affects a highly conserved amino acid (Fig. 3c). According to in silico analysis with SIFT (http://sift.jcvi.org/), MutationTaster (http://www.mutationtaster.org/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), we assessed this mutation as likely pathogenic. Both parents as well as several other family members were heterozygous carriers of the mutation (Fig. 1).

Patient 2 The variants identified by WES were sequentially filtered according to four criteria: (1) variants located in genes encoding mitochondrial proteins in MitoCarta (Pagliarini et al. 2008), (2) low allele frequency in general population, as described previously (Sofou et al. 2015), (3) variants listed in Human Gene Mutation Database (HGMD), and (4) variants compatible with an autosomal recessive pattern of inheritance. This strategy rendered only two variants in one candidate gene, *SDHB*, namely c.143A>T (p.Asp48Val) and c.689G>A (p.Arg230His). The variants were verified by Sanger sequencing and the parents each carried one of the mutations (p.Asp48Val maternal and p.Arg230His paternal). Information on genetic testing of other family members was not available. Both mutations have been reported previously; c.143A>T (p.Asp48Val) has been found homozygous and compound heterozygous in patients with leukodystrophy and complex II deficiency (Alston et al. 2012; Helman et al. 2015), whereas the c.689G>A (p.Arg230His) mutation has been reported in heterozygous form in two patients with

apparently sporadic paraganglioma/pheochromocytoma (Amar et al. 2005), in a family with paraganglioma/pheochromocytoma and renal cell cancer (Ricketts et al. 2012), as well as in patients with head and neck paraganglioma (Cerecer-Gil et al. 2010; Peterson et al. 2014).

Western Blot Analysis

Western blot analysis of fibroblast cells showed a clearly decreased amount of SDHB protein in patient 1 and 2. The level of SDHA protein was only slightly reduced (Fig. 3d).

Discussion

While heterozygous *SDHB* mutations are a well-known cause of familial paraganglioma/pheochromocytoma (Ricketts et al. 2010), bi-allelic mutations in *SDHB* have been identified in only a few patients with complex II deficiency and a progressive neurological phenotype with onset in infancy (Alston et al. 2012; Helman et al. 2015). Here, we present two further patients with bi-allelic *SDHB* mutations causing complex II deficiency and a progressive neurological disease with leukoencephalopathy. Altogether three mutations were found, of which two have been reported previously. The novel missense mutation in *SDHB* (c.769C>G, p.Leu257Val) was assessed as most likely pathogenic since it was found in a patient with complex II deficiency as assessed by biochemical measurement, *SDHB* was one of the two complex II genes in the homozygous regions according to homozygosity mapping, the amino acid is highly conserved, and database analysis (in silico analysis and ExAC database) supported this assessment. More significantly, Western blot analysis showed decreased amount of SDHB protein in patient fibroblasts. The p.Asp48Val variant has been found in six out of seven reported patients with complex II deficiency due to *SDHB* mutations (Alston et al. 2012; Helman et al. 2015), whereas the p.Arg230His variant has been reported in heterozygous form in patients with familial paraganglioma/pheochromocytoma (Amar et al. 2005) and/or renal cell cancer (Ricketts et al. 2012). To our knowledge, this is only the second example in the literature where one specific *SDHx* mutation is associated with both recessive mitochondrial disease in one patient and familial paraganglioma/pheochromocytoma in others; previously, this has exclusively been described for the c.91C>T (p.Arg31*) variant in *SDHA* (Renkema et al. 2015).

Clinically, the phenotypic presentation of both *SDHB* patients in this report lies well within the phenotypic spectrum of complex II deficiency (Jain-Ghai et al. 2013) as well as of *SDHB* deficiency (Alston et al. 2012; Helman et al. 2015). Onset at age 6–12 months, progressive neurological involvement with spasticity, truncal hypotonia, and pathological brain imaging as in patient 1 and 2 are typically described for the more severely affected patients, and cardiomyopathy as found in patient 2 is likewise reported in about 25% of 37 complex II deficient patients reviewed by Jain-Ghai et al. (2013). Time of death at age 25 months (patient 1) and 12 months (patient 2) is relatively early compared to mortality in the complex II deficient cohort where only 5 of 37 patients reportedly deceased before the age of 2.5 years. On the other hand, outcome data were not available on at least 15 of the patients in this study. Of the six patients with SDHB deficiency, one patient died at age 1 year, whereas the remaining five patients are alive, with current ages from 19 months to 9 years (Alston et al. 2012; Helman et al. 2015). The ophthalmological features of patient 1 including optic atrophy, decreased vision, and nystagmus are in line with the expected phenotype of mitochondrial encephalomyopathy including previously reviewed patients with complex II deficiency (Jain-Ghai et al. 2013). No specific eye manifestations have been described in previous reports of complex II-related leukoencephalopathy (Alston et al. 2012; Jain-Ghai et al. 2013; Helman et al. 2015).

Regarding neuroradiological findings, both patients presented with encephalomyelopathy (Fig. 2) with extensive involvement of white matter in frontal, parietooccipital, and posterior temporal lobes with sparing of the U-fibers, involvement of corpus callosum, the middle cerebellar peduncles, and cerebellar deep white matter. In addition, the corticospinal tracts and cervical medulla were affected extensively, and pulvinar and ventral thalamic nuclei showed pathological signal in both patients. MR spectroscopy was only performed in patient 2 and revealed a succinate peak, a specific finding in complex II deficiency (Brockmann et al. 2002; Ghezzi et al. 2009). Recently, a distinct MRI pattern was defined for patients with SDH deficiency-related leukoencephalopathy (Helman et al. 2015), with a typical progression of MRI appearance during the course of disease, allowing differentiation from other mitochondrial leukoencephalopathies. It can be added to the list of recognizable MRI patterns, which serve as valuable diagnostic tools in the evaluation of genetic leukoencephalopathies (Schiffmann and van der Knaap 2009; Parikh et al. 2015). MRI pathologies of the patients in this study fit into this MRI pattern description, probably corresponding to early or intermediate stage of the disease. As MRI pattern recognition, MR spectroscopy as well as next generation sequencing technologies get more readily available, the diagnostic approach in patients with suspected mitochondrial leukoencephalopathy will lead to the establishment of diagnosis without the need for muscle biopsy.

The diagnosis of complex II deficiency due to *SDHB* mutations raises implications for genetic counseling that go beyond the recurrence risk in the family according to an autosomal recessive inheritance. Heterozygous mutations in *SDHB* have been described in familial paraganglioma/pheochromocytoma with a higher rate of malignancy and earlier onset than with the other *SDH* genes. *SDHB* mutations have also been associated with gastrointestinal stromal tumors, and renal cell carcinoma (Neumann et al. 2004; Ricketts et al. 2010). In one study, the lifetime risk (at age 70 years) of SDHB-related symptoms was ~80%, with a penetrance of ~60% for pheochromocytoma and ~45% for paraganglioma. The risk of renal cell cancer by age 70 years was 15–20% (average age-specific cumulative risk for cancer 50% by age 50) (Ricketts et al. 2010). Later studies have questioned this high penetrance and have suggested an overestimation because no correction was made for the ascertainment of mutation carriers. Substantially lower average penetrance was estimated when adjusting for ascertainment (average penetrance 13% at age 50; average lifetime penetrance (to age 80) 30%) (Schiavi et al. 2010). These results were confirmed in a more recent study based on a large pedigree with paraganglioma and pheochromocytoma with a Dutch founder mutation in *SDHB* (9% by age 50). The bias in this study is expected to be low, but an effect of this specific mutation cannot be excluded (Rijken et al. 2016). Also with these lower estimates, the need for expanded family testing and referral of heterozygous mutation carriers to a surveillance program remains unchanged. This should also be considered in the families of patients with bi-allelic mutations in *SDHD* and *SDHA* that also have been described with a dual role, with mitochondrial disease in patients with bi-allelic mutations and risk of paraganglioma/pheochromocytoma and cancer in heterozygotes (Ricketts et al. 2010; Kunst et al. 2011; Welander et al. 2011; Renkema et al. 2015).

There were no known cases of paraganglioma/pheochromocytoma in either of the two families reported here. In the family of patient 1, there are at least eight healthy carriers (five shown to be carriers and three obligate carriers) of the p.Leu257Val variant, and thus the penetrance of this variant can be assumed to be low. The p.Arg230His variant found in patient 2 has previously been reported in patients with paraganglioma/pheochromocytoma (Amar et al. 2005; Ricketts et al. 2012), but the penetrance is unknown. Due to the uncertainties regarding penetrance of different mutations, all heterozygous *SDHB* mutation carriers from family 1 were referred for a surveillance program comprising an annual clinical examination with measurement of blood pressure, urine catecholamines, and plasma metanephrines. In addition, MRI of the cranial basis, neck, thorax, abdomen, and pelvis was done every second year, alternating with MIBG scintigraphy. Children were tested for the mutation from age 10 years, and mutation carriers were referred to surveillance. Attention should especially be warranted in the case of the father of patient 2 who carries the *SDHB* mutation p.Arg230His that previously has been described to be associated with familial paraganglioma/pheochromocytoma and at least two cases of renal cell cancer. The parents were referred to a surveillance program consisting of a clinical examination and CT scan of the neck, thorax, and abdomen as well as screening with plasma metanephrines.

In this report, we present two additional patients with complex II deficiency with leukoencephalopathy caused by recessive *SDHB* mutations. We suggest that, in addition to *SDHA* and *SDHAF1*, also sequencing of *SDHB*, *SDHD*, and possibly *SDHC* should be included into the genetic work-up of patients with multisystem mitochondrial disorder and complex II deficiency, not least because of the potential implications regarding cancer surveillance in family members.

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Synopsis

Diagnosing leukoencephalopathy due to complex II deficiency and bi-allelic *SDHx* mutations has important implications for genetic counseling going beyond the recurrence risk in the family.

Author Contributions

S.G., N.D., and E.Ø. have planned the work; all authors have contributed to the evaluation of the patients, including analysis and interpretation of clinical, neuroradiological, biochemical, histological, and molecular results; all authors have contributed pertinent aspects to the reporting of this work.

Sabine Grønborg serves as a guarantor for the manuscript.

Conflict of Interest

Sabine Grønborg, Niklas Darin, Maria J. Miranda, Bodil Damgaard, Jorge Asin Cayuela, Anders Oldfors, Gittan Kollberg, Thomas V.O. Hansen, Kirstine Ravn, Flemming Wibrand, and Elsebet Østergaard declare that they have no conflict of interest.

For this Case Report ethics approval was not required at the participating authors' institutions.

The patients' families have given informed consent for the publication of these case reports.

References

Alston CL, Davison JE, Meloni F et al (2012) Recessive germline SDHA and SDHB mutations causing leukodystrophy and isolated mitochondrial complex II deficiency. J Med Genet 49:569–577

Amar L, Bertherat J, Baudin E et al (2005) Genetic testing in pheochromocytoma or functional paraganglioma. J Clin Oncol 23:8812–8818

Bricout M, Grevent D, Lebre AS et al (2014) Brain imaging in mitochondrial respiratory chain deficiency: combination of brain MRI features as a useful tool for genotype/phenotype correlations. J Med Genet 51:429–435

Brockmann K, Bjornstad A, Dechent P et al (2002) Succinate in dystrophic white matter: a proton magnetic resonance spectroscopy finding characteristic for complex II deficiency. Ann Neurol 52:38–46

Cerecer-Gil NY, Figuera LE, Llamas FJ et al (2010) Mutation of SDHB is a cause of hypoxia-related high-altitude paraganglioma. Clin Cancer Res 16:4148–4154

DiMauro S, Schon EA, Carelli V, Hirano M (2013) The clinical maze of mitochondrial neurology. Nat Rev Neurol 9:429–444

Ghezzi D, Goffrini P, Uziel G et al (2009) SDHAF1, encoding a LYR complex-II specific assembly factor, is mutated in SDH-defective infantile leukoencephalopathy. Nat Genet 41:654–656

Goldstein AC, Bhatia P, Vento JM (2013) Mitochondrial disease in childhood: nuclear encoded. Neurotherapeutics 10:212–226

Helman G, Caldovic L, Whitehead MT et al (2015) Magnetic resonance imaging spectrum of succinate dehydrogenase-related infantile leukoencephalopathy. Ann Neurol 79(3):379–386

Jackson CB, Nuoffer JM, Hahn D et al (2014) Mutations in SDHD lead to autosomal recessive encephalomyopathy and isolated mitochondrial complex II deficiency. J Med Genet 51:170–175

Jain-Ghai S, Cameron JM, Al Maawali A et al (2013) Complex II deficiency – a case report and review of the literature. Am J Med Genet A 161A:285–294

Kunst HP, Rutten MH, de Monnink JP et al (2011) SDHAF2 (PGL2-SDH5) and hereditary head and neck paraganglioma. Clin Cancer Res 17:247–254

Larsen S, Nielsen J, Hansen CN et al (2012) Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol 590:3349–3360

Morato L, Bertini E, Verrigni D et al (2014) Mitochondrial dysfunction in central nervous system white matter disorders. Glia 62:1878–1894

Neumann HP, Pawlu C, Peczkowska M et al (2004) Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. JAMA 292:943–951

Ostergaard E, Hansen FJ, Sorensen N et al (2007) Mitochondrial encephalomyopathy with elevated methylmalonic acid is caused by SUCLA2 mutations. Brain 130:853–861

Ostergaard E, Duno M, Batbayli M, Vilhelmsen K, Rosenberg T (2011) A novel MERTK deletion is a common founder mutation in the Faroe Islands and is responsible for a high proportion of retinitis pigmentosa cases. Mol Vis 17:1485–1492

Pagliarini DJ, Calvo SE, Chang B et al (2008) A mitochondrial protein compendium elucidates complex I disease biology. Cell 134:112–123

Parikh S, Bernard G, Leventer RJ et al (2015) A clinical approach to the diagnosis of patients with leukodystrophies and genetic leukoencephelopathies. Mol Genet Metab 114:501–515

Peterson LA, Litzendorf M, Ringel MD, Vaccaro PS (2014) SDHB gene mutation in a carotid body paraganglioma: case report and review of the paraganglioma syndromes. Ann Vasc Surg 28:1321 e9–1321 e12

Renkema GH, Wortmann SB, Smeets RJ et al (2015) SDHA mutations causing a multisystem mitochondrial disease: novel mutations and genetic overlap with hereditary tumors. Eur J Hum Genet 23:202–209

Ricketts CJ, Forman JR, Rattenberry E et al (2010) Tumor risks and genotype-phenotype-proteotype analysis in 358 patients with germline mutations in SDHB and SDHD. Hum Mutat 31:41–51

Ricketts CJ, Shuch B, Vocke CD et al (2012) Succinate dehydrogenase kidney cancer: an aggressive example of the Warburg effect in cancer. J Urol 188:2063–2071

Rijken JA, Niemeijer ND, Corssmit EP et al (2016) Low penetrance of paraganglioma and pheochromocytoma in an extended kindred with a germline SDHB exon 3 deletion. Clin Genet 89:128–132

Rutter J, Winge DR, Schiffman JD (2010) Succinate dehydrogenase – assembly, regulation and role in human disease. Mitochondrion 10:393–401

Schiavi F, Milne RL, Anda E et al (2010) Are we overestimating the penetrance of mutations in SDHB? Hum Mutat 31:761–762

Schiffmann R, van der Knaap MS (2009) Invited article: an MRI-based approach to the diagnosis of white matter disorders. Neurology 72:750–759

Sofou K, Kollberg G, Holmstrom M et al (2015) Whole exome sequencing reveals mutations in NARS2 and PARS2, encoding the mitochondrial asparaginyl-tRNA synthetase and prolyl-tRNA synthetase, in patients with Alpers syndrome. Mol Genet Genomic Med 3:59–68

Tulinius MH, Holme E, Kristiansson B, Larsson NG, Oldfors A (1991) Mitochondrial encephalomyopathies in childhood. I. Biochemical and morphologic investigations. J Pediatr 119:242–250

Welander J, Soderkvist P, Gimm O (2011) Genetics and clinical characteristics of hereditary pheochromocytomas and paragangliomas. Endocr Relat Cancer 18:R253–R276

Wong LJ (2012) Mitochondrial syndromes with leukoencephalopathies. Semin Neurol 32:55–61

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RESEARCH REPORT

Peak Jump Power Reflects the Degree of Ambulatory Ability in Patients with Mitochondrial and Other Rare Diseases

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Abstract Metabolic diseases that lead to neuromuscular, bone, and joint involvement can reduce ambulation and quality of life. Using jumping mechanography, we developed a novel assessment, peak jump power (PJP), and related this to ambulatory ability in patients either having a known or suspected underlying rare disease. From adults and children, we recruited 88 healthy controls and 115 patients (61 with mitochondrial disease and 54 with another diagnosis). Patients were categorized as having no complaints of weakness or ambulation (ambulatory competent; AC), weakness but able to ambulate without aids (ambulatory weakness; AW), or not able to ambulate without aids such as a walker, cane, or wheelchair (ambulatory assistance; AA). Subjects were asked to perform five successive jumps from a squat position. Instantaneous power (W; watts) was calculated and the highest result was divided by the body mass (kg) to calculate PJP (W/kg). Between healthy controls and AC patients, there was no difference in mean PJP (20.5 ± 7.0 W/kg vs. 19.0 ± 7.4 W/kg, $p = 0.601$; mean ± SD). Progressively lower results were found in patients with AW with a mean PJP of 11.7 ± 5.1 W/kg ($p < 0.001$ versus AC) and further those with AA with a mean PJP of 5.8 ± 3.2 W/kg ($p < 0.001$ versus AW). A subgroup analysis of subjects showed that those who did not use ambulatory aids all had a PJP above 10 W/kg. Using this threshold, the receiver operating characteristic curve (ROC) analysis showed PJP to be highly sensitive evaluation of ambulatory ability (sensitivity 95.8%, specificity 52.1%).

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Abbreviations

6MWT	Six-minute walk test
ANOVA	Analysis of variance
AUROC	Area under the ROC curve
DCMA	Dilated cardiomyopathy with ataxia
DMD	Duchene muscular dystrophy
F	Force
FSHD	Facioscapulohumeral muscular dystrophy
GSD	Glycogen storage disorder type V or VII
MPS-I or II	Mucopolysaccharide storage disease
MRC	Medical Research Council
MS	Multiple sclerosis
P	Power
PJP	Peak jump power
ROC	Receiver operating characteristic curve
SD	Standard deviation
SSC	Stretch shortening cycle
V	Velocity
W	Watts

Introduction

In patients with inborn errors of metabolism, ambulation can be impaired from a combination of factors involving the nervous system, skeletal muscle, bones, and joints (Kling et al. 2014; Mathiowetz et al. 1985). The most commonly reported measure in ambulatory subjects in the literature is the six-minute walk test (6MWT). The 6MWT has been shown to relate to the degree of impairment in patients with chronic respiratory disease and heart failure (Solway et al. 2001). While the 6MWT can distinguish patients with impaired ambulatory ability from healthy controls (Bohannon 2006; Enright and Sherrill 1998; Roig et al. 2009; McQuiddy et al. 2015), and is used in clinical trials to measure ambulatory ability in patients who are able to walk, it has not been demonstrated to distinguish between levels of assistance required with walking in more severely affected patients of any type metabolic disease (McDonald 2002; Phillips et al. 2009). In many patients with metabolic diseases such as mitochondrial disease, Pompe disease, glycogen storage myopathies, or lysosomal storage diseases, the 6MWT cannot be performed due to the severity of underlying weakness. Furthermore, these patients do not have enough proximal strength to get up from a chair or perform an isometric force contraction for other measures of function such as the timed-up-and-go test (Bohannon 2006) or stair climbing. However, in trials using novel therapies that can take months to years to change ambulation, early assessment of efficacy in patients who start treatment when they have difficulty with ambulation is lacking. In pivotal trials using enzyme replacement therapy in lysosomal diseases, the distance walked by patients is far below that of the pulmonary disease population for which it was designed for (Redelmeier et al. 1997) and no clinically meaningful correlate has been established (Harmatz et al. 2005; Hendriksz et al. 2014; van der Ploeg 2010). It may be one thing to measure changes in walk distance in a population of adults who had no ambulatory disability prior to the onset of pulmonary disease and try to use that measure in a rare disease population with different physiology, due to muscle, bone, and joint problems, who at best may only be expected to walk a fraction of that distance. Yet it's clear that in drug therapy trials, some patients report benefits in quality of life from increased ambulatory ability that are not captured by their 6MWT (Brands et al. 2013). Being able to maintain ambulation, or improve it with therapy, occurs in a range of muscle performance that appears to be below the threshold of traditional measures of neuromuscular function and questionnaires are not able to capture smaller differences nor apply to both the pediatric and adult populations. This prompted us to look for a measure of ambulatory ability that could be easily performed in patients with severe muscle weakness. In the literature, various terms are used to describe or measure the ability of a subject to move about to perform activities of daily living – terms such as ambulation, mobility, physical performance, and others. Since the clinical correlation in this study relates to whether subjects needed any aids, we will use the term ambulatory ability to reflect this function.

We adapted a method originally described by Giovanni Cavagna who used force plate ergometers to measure mechanical work by having subjects perform a vertical jump (Cavagna 1975). The ability of a muscle to perform work is determined by power (watts; W) (Josephson 1999). When an individual attempts a vertical jump, the combination of neuromuscular interactions and joint mechanics all contribute to the vertical height achieved which is the basis of peak jump power (PJP) (Bobbert and van Ingen Schenau 1988; Vanezis and Lees 2005). Studies in mobility-competent adults (Rittweger et al. 2004) and children (Taylor et al. 2010; Veilleux and Rauch 2010) have shown jump power to be highly reproducible and that declines in jump power are related to sarcopenia in aging adults (Buehring et al. 2010). Here we present the first application of jump mechanography in a clinical setting as a quantitative measure of ambulatory ability in patients with neuromuscular, bone, and joint diseases. We hypothesize that PJP has a number of advantages in measuring ambulatory ability in the clinical setting: (1) to plan which resources and treatments (such as ambulatory aids) are likely to help improve function, (2) to evaluate a response to treatment, (3) to measure disease progression, and (4) to facilitate future planning of resources for patients and their caregivers.

Methods

Patients (both children and adults) referred to the Metabolic Clinic at Alberta Children's Hospital, Calgary, Alberta for the investigation of an underlying metabolic disease were invited to participate in the study. Patients either had an existing diagnosis of mitochondrial disease or were sent for an evaluation to consider mitochondrial or other neuromuscular causes of their weakness. The muscle weakness was either apparent (e.g., subject was already using a walker) or perceived (subject may complain of muscle weakness but no weakness was apparent on physical exam). Subjects were screened in clinic regarding their ability to perform a jump maneuver from a standing position without falling but they did not necessarily have to be able to jump completely off the ground. Subjects who were not able to attempt a jumping maneuver or did not want to participate were not referred for a jumping power evaluation. The wheelchair-using patients could stand without support and push off the

ground. Healthy controls were recruited using a poster; their medical history was reviewed to exclude any medical condition in their history that or signs and symptoms related to muscle weakness, trauma, or injury, or the use of walking aids or wheelchairs. All healthy controls reported no perceived muscle weakness and no reduce function in activities of daily living. All subjects were examined by the principal investigator (AK) to collaborate their use of any walking aids with their self-report. Written informed consent was obtained by all participants prior to participation in the study with children requiring consent from a guardian. The majority of patients were referred for or had an existing diagnosis of mitochondrial disease. Patients also had other diagnoses of genetic or metabolic myopathy or nonspecific features of a muscle disease. Some patients had a disease primarily affecting ambulatory ability through bone, joint, or neurologic function. Patients recruited were globally classified as having a neuromuscular disease phenotype. All neuromuscular patients were separately characterized into the following categories based on their self-reported (and/or witnessed) ambulatory ability:

1. Ambulatory competent (AC) – no perceived physical impairment of daily activities
2. Ambulatory weakness (AW) – perceived impairment in physical ability
3. Ambulatory assistance (AA) – impairment in physical ability with use of walking aids or a wheelchair to ambulate

Inclusion criteria required participants to be able to perform the jump maneuver and have no preexisting condition that would impede ambulation. Inability to participate acted as the exclusion criteria. None of the volunteer subjects reported any degree of ambulatory disability or complaints of muscle weakness and were not observed to use any ambulatory aids. Each healthy subject was characterized into a single category: healthy control (HC).

All jump measurements were performed in the C.H. Riddell Movement Assessment Centre at Alberta Children's Hospital, Calgary, Alberta. Following recruitment, each subject was asked to stand on a force place (Advanced Medical Technologies Inc., Watertown, MA, USA) for the measurement of body mass (kg). The subject was then asked to perform a jump without countermovement. This modification is necessary because patients with muscle weakness are not always able to do a countermovement and are not always able to squat to the same level before a jump. Traditional jumping protocols require a flight phase to calculate jump height and forces; however, this was not possible in some of our patients due to severity of muscle weakness and their fear of falling. Therefore, we asked the patients to squat into a position that was comfortable for them and to then rapidly push to a standing position to reduce the variability caused by countermovement between patients. Through this modification, we were able to compute lower extremity power from the instantaneous force that exceeds the force due to body mass. That is, when the subject stands on the force plate, squats then push rapidly, and the difference between the force recording during the rapid push to extend the legs and that during the quiet standing is the amount of force (F) in Newtons generated by the lower extremity muscles during the rapid standing (Fig. 1). The peak velocity (V) in meters per second is also determined during this time. Power (P) in W is the product of F and V in Newton meters per second ($P = F \times V$ in Nm/s). The maximum power generated (PJP) was computed using custom-written computer code (Matlab from MathWorks, Natick, MA, USA) and divided by the subject body mass to give a result in W/kg. All participants were asked to perform at least five jumps for the subject's data to be included for analysis. The time frame for performing all of the jumps was self-selected by the patient and was usually completed within 3 min, with a pause between each jump. The highest PJP produced was used for the data analysis. A staff person flanked either side of the subject in case they were not able to stand by themselves, but each subject performed the maneuver using their own power and unaided by staff.

Statistical analysis for subject characteristics and PJP was performed using IBM SPSS Statistics for Windows, Version 20.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). A significance level threshold, alpha, was set at 0.05 for all statistical tests. Differences between the subject groupings were determined by a one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test and testing of assumptions. A receiver operating characteristic curve (ROC) was also generated to determine the suitability of PJP as a corresponding clinical threshold for determining patient disability. In order to generate an ROC threshold value, individuals within the healthy control and ambulatory competent groups were considered "negative" and the ambulatory weakness and ambulatory assistance groups considered "positive" for disability.

Results

Subject recruitment occurred between March 2009 and July 2012 with each healthy control or patient being represented only once (Table 1). A total of 88 healthy controls and 115 patients were recruited with each being able to adequately perform the testing protocol. The age of healthy controls ranged from 3 to 65 years of age. The age of neuromuscular

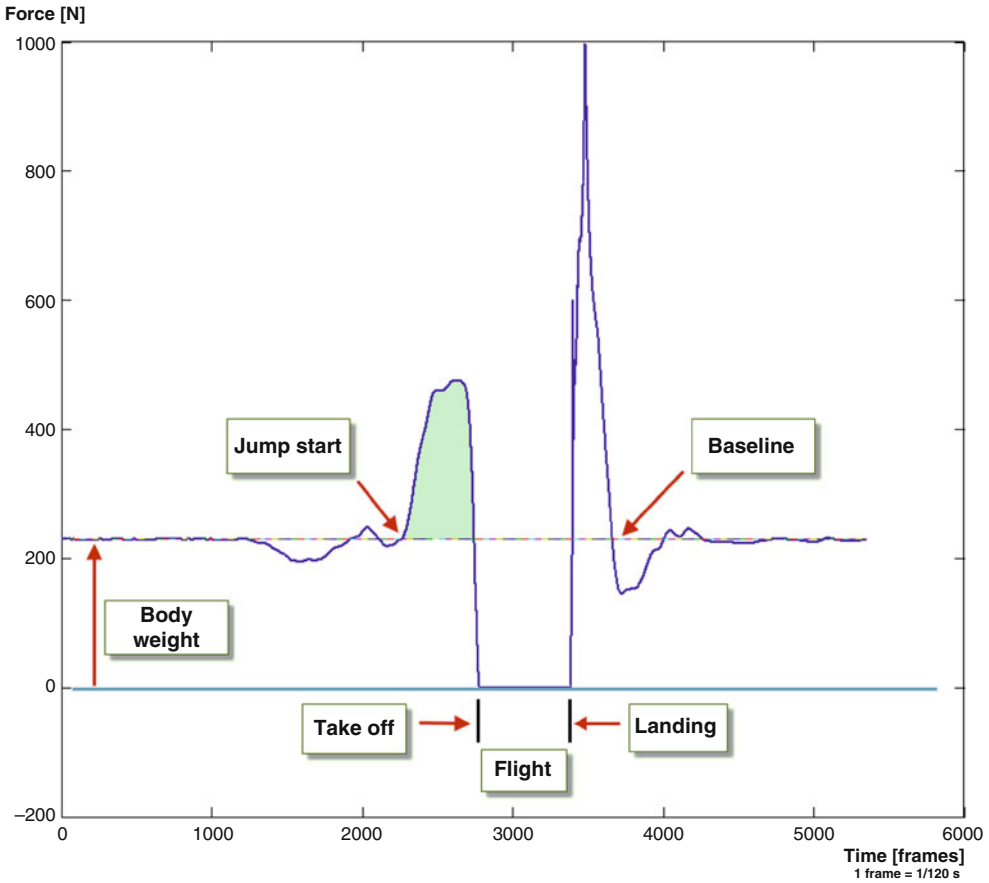

Fig. 1 Force (N; newtons) versus time (frames where 1 frame = 1/120 s) graph representing stages during a jump. All subjects were asked to perform a jump without countermovement. Some of our patients with declines in ambulatory ability were unable to perform the jump maneuver due to severity of muscle weakness – in which a modified jump was performed (absent flight phase). *Green area* highlighted following the initiation of the jump provides the force exceeding the individuals' body mass due to gravity. The area generated by this force is then quantified as power by using the velocity of the movement. Each subject's peak jump power (PJP) was computed using custom-written computer code and divided by the subject body mass to give a result in watts (W) per kilogram

Table 1 Subject characteristics at the time of peak jump power (PJP) assessment

Subject characteristics	Healthy controls	Ambulatory competent	Ambulatory weakness	Ambulatory assistance
Sample size	88	41	46	28
Age (years)	24.3 ± 17.0	35.5 ± 16.9	40.7 ± 17.0	40.7 ± 22.9
Gender	M = 37; F = 51	M = 24; F = 17	M = 22; F = 24	M = 10; F = 18
Body mass (kg)	53.7 ± 25.3	70.1 ± 25.0	66.4 ± 20.4	61.3 ± 25.6
Peak power output (W)	1,164.1 ± 758.8	1,334.0 ± 688.4	799.9 ± 496.2	404.4 ± 371.6
PJP (W/kg)	20.5 ± 7.0[a]	19.0 ± 7.4[a]	11.7 ± 15.1[b]	5.8 ± 3.2[c]

Peak power output and PJP were computed using custom-written computer code (Matlab, MathWorks). Ambulatory competent – no perceived physical impairment; ambulatory weakness – perceived physical impairment; and ambulatory assistance – use of a mobility aid. Ambulatory ability was self-reported by patients. A total of nine patients within the ambulatory assistance group operated a wheelchair in order to ambulate $p < 0.001$ if superscripts are dissimilar ([a], [b], and [c]) based on Tukey's, data are mean ± SD

patients ranged from 4 to 83 years of age. We found that children under 3 years of age in general were not able to jump on demand consistently in the setting of a clinical lab and therefore recommend 3 years of age as the lower limit for participation. Females comprised 58% of the healthy control group and 51% of the neuromuscular patients, respectively. Out of the 115 patients recruited, 61 had mitochondrial disease (diagnoses available in supplementary

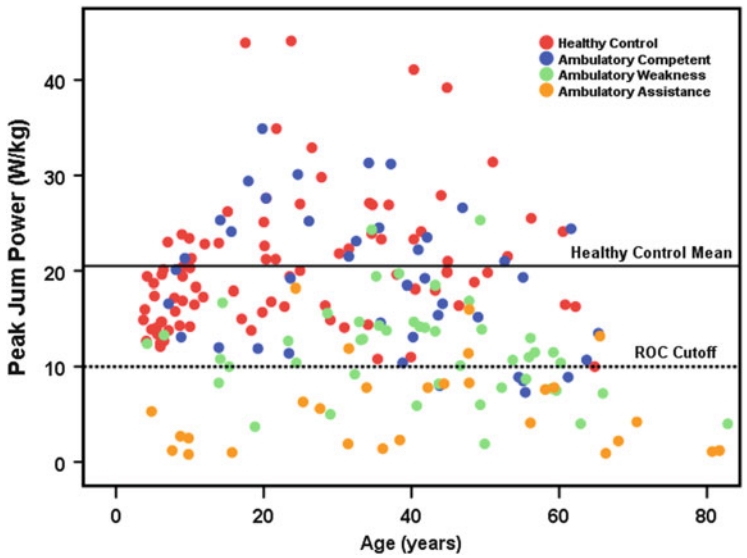

Fig. 2 Scatterplot of entire healthy control and patient population comparing PJP and age (years). Each subject grouping is denoted by a different color and is compared to the mean PJP for the healthy control group of 20.5 W/kg as well as the proposed PJP cutoff value of 10.0 W/kg

Table S1). The remainder of patients had DMD (Duchene muscular dystrophy), GSD (glycogen storage disease type V or VII), Pompe disease, Fabry disease, rhabdomyolysis, MPS-I or II (mucopolysaccharide storage disease), DCMA (dilated cardiomyopathy with ataxia), MS (multiple sclerosis), FSHD (facioscapulohumeral muscular dystrophy), or nonspecific myopathy. None of the patients had cause for muscle weakness localized to the spine such as spinal cord injury. A total of nine patients received no diagnosis ("other") but still had symptoms of weakness. Patients were subsequently divided based on their self-reported/observed ambulatory ability (Table 1). The total number of patients within the ambulatory competent group was 41, the ambulatory weakness group 46, and the ambulatory assistance group 28. There were no adverse events in any subject from performing the jumping maneuvers. Statistical assumptions were met as data from each ambulatory ability grouping was normally distributed and homogeneity of variances was verified.

A scatterplot of PJP across all ambulatory groups (Fig. 2) depicts representation of both children and adults in the control group, with all control subjects yielding a PJP above 10.0 W/kg. The highest measurement was 44.1 W/kg. The PJP in healthy controls was not significantly different to subjects within the ambulatory competent group (20.5 ± 7.0 W/kg vs. 19.0 ± 7.4 W/kg, $p = 0.601$; mean ± SD). Subjects in the ambulatory weakness group had a significantly lower PJP than both healthy and ambulatory competent groups, respectively (11.7 ± 5.1 W/kg, $p < 0.001$). Those subjects within the ambulatory assistance group demonstrated significantly lower PJP than all other groups (5.8 ± 3.2 W/kg, $p < 0.001$) (Table 1). A receiver operating characteristic curve (ROC) was generated using PJP and whether or not ambulatory assistance was needed for walking (Fig. 3). The area under the ROC curve was 0.888 with a 95% confidence interval of 0.841–0.935, which indicates that PJP is a good to excellent test to discriminate subjects who are ambulatory competent versus those in either ambulatory weakness or ambulatory assistance groupings (Fig. 3). Furthermore, utilizing the empirical PJP threshold value of 10.0 W/kg resulted in a highly sensitive clinical test (sensitivity 95.8%, specificity 52.1%).

Discussion

The range of PJP values for our healthy control group was between 10.0 and 44.1 W/kg which compares closely with other studies that have looked separately at adults (Rittweger et al. 2004; Dionyssiotis et al. 2010) and children (Taylor et al. 2010; Veilleux and Rauch 2010). In our healthy control group, values for maximum power (unadjusted for body mass) ranged from 210 to 3,402 W with lower values being found in children. However, normalizing power to body mass by using PJP (W/kg) showed that this method is highly comparable across age ranges and may be a more useful measure for longitudinal comparisons in clinic populations. A mild decline was seen in subjects over 60 years of age which likely represents early stages of declining jump power seen with sarcopenia as previously reported (Buehring et al. 2010). Overall, the results from the controls

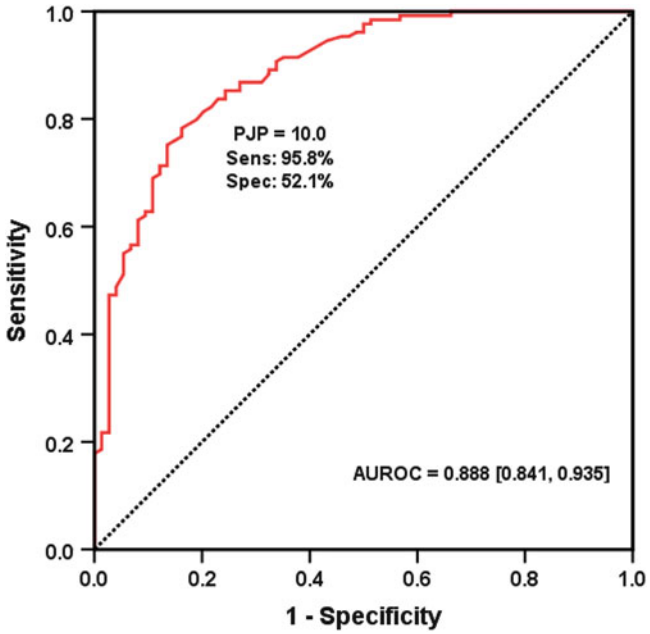

Fig. 3 Receiver operating characteristic curve (ROC) used to correlate PJP and level of subject disability. Sensitivity of PJP is plotted on the *y*-axis and 1-specificity (false positivity rate) is plotted on the *x*-axis. The established PJP cutoff value is listed along with the corresponding sensitivity (sens) and specificity (spec) values. AUROC; area under the ROC curve along with 95% confidence interval

show consistent application of the technique which is essential before comparison to the clinic population.

We show that jumping power reflects different degrees of ambulatory ability. Specifically, we found that if subjects possessed an underlying diagnosis of a neuromuscular disease but no physical limitation or symptoms, their PJP was no different from healthy controls. This finding is relevant since simply having a diagnosis of a neuromuscular disease does not imply that a patient had loss of ambulatory ability and their PJP can distinguish this. The perception of weakness, which is affected by many factors such fatigue, sleep, and pain, need not imply that the muscle is not able to perform work. In the clinical setting, this can direct care to providing relief of symptoms, encouraging treatments to sustain ambulation. Patients with impairments in ambulatory ability demonstrated a lower PJP compared to patients who did not report weakness. In this group, more detailed evaluations can be performed to evaluate for the risk of falling, effects on quality of life, and interventions undertaken to improve function depending on the underlying cause. Furthermore, patients who use ambulatory aids have PJP values even lower than those with just muscle weakness. Interventions used could be monitored using PJP to see if improvements are taking place or whether declines are still occurring that may confine a patient to a wheelchair.

The ROC analysis showed that PJP was an excellent discriminator of ambulatory ability. The threshold value of 10.0 W/kg adequately separates all healthy controls from patients possessing varying impairments in ambulatory ability – ambulatory weakness and ambulatory assistance groups. This threshold value, which applies to patients across a wide range of ages, may be used as a quick and easy quantitative clinical assessment of neuromuscular disease progression resulting in declines in ambulatory ability. The threshold value of 10.0 W/kg provides a high sensitivity of 95.8%. As with many tests, high sensitivity can come at the cost of specificity; this cutoff of 10.0 W/kg may select some subjects who do not show impairment in ambulation but also not select out any patient who does. The latter is typically the goal in the clinical setting. Depending on the clinical goal, whether it is high sensitivity to not miss any subjects who have disability versus high specificity to avoid false positives, different thresholds can be selected on the ROC curve.

Although various measures of ambulatory ability have been previously described, this is the first application of jump power in a clinical setting of patients with muscle, nerve, and joint disease. Furthermore, many of our patients are unable to perform existing measures of ambulatory ability such as the timed chair up and go or 6MWT. In all cases, PJP could be performed in a few minutes, requires no preparation and even some patients who were dependent on a wheelchair could perform the modified jump attempt (generating jumping power but with the feet still on the ground). No subjects developed problems from the repeated jumps including those with very limited ambulatory ability.

Our objectives in this study were to target ambulatory ability in the usual clinical setting. Studies in elderly patients show that PJP can decline with aging (Rittweger et al. 2004; Dionyssiotis et al. 2010; Runge et al. 2004) but in general those patients are much older than our control group where the oldest subject was 65 years of age. In the control population, we did not find a trend of PJP versus age. A longitudinal study may be better able to delineate this. In athletic Tunisian children, the fat-free body mass is related to jump power (Aouichaoui et al. 2012). Other studies have shown that in healthy controls, PJP has the lowest inter- and intra-subject variation and highest degree of reproducibility compared to traditional methods of assessing physical ability (Rittweger et al. 2003, 2004; Veilleux and Rauch 2010). Although we attempted to try and perform test retest analysis, many of our subjects with underlying disease reported gradually declining muscle performance with time and difficulty returning to the lab for repeated measures. However, test retest analysis on select patients exhibiting static disease showed an average of 8% variability in PJP ($n = 6$, unpublished data). The major limitation in this study was that some the PJP may have been overestimated because if subjects did a counter-movement during some of their jumps thereby decreasing their stretch shortening cycle (SSC; active stretch of a muscle-eccentric contraction followed by an immediate shortening of the same muscle-concentric contraction) (Gerodimos et al. 2008). Children and adults with these rare diseases, however, are going to naturally perform a jumping maneuver in the way that is most comfortable for them. In most cases, the biomechanics in an individual subject cannot be modified. Our data show that the clinical correlation still holds and the procedure can be applied in an actual clinic setting without patients being required to modify how they jump.

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Compliance with Ethics Guidelines

Conflict of Interest

Christopher Newell, Barbara Ramage, Ion Robu, and Aneal Khan declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (Enright and Sherrill 1998). Informed consent was obtained from all patients for being included in the study.

Author Contributions

Christopher Newell collected and analyzed data, generated figures, and helped develop the manuscript. Alberto Nettel-Aguirre assisted in the development of the primary study design, statistical analysis, interpretation of results, and contribution to the manuscript. Barbara Ramage, Ion Robu, and Aneal Khan contributed to the study design, methodologies described, administration of the study on subjects, data analysis, and manuscript content. Aneal Khan made the primary study design, was the principal investigator, and had primary responsibility for final content. All authors read and approved the final manuscript.

References

Aouichaoui C, Trabelsi Y, Bouhlel E, Tabka Z, Dogui M, Richalet JP et al (2012) The relative contributions of anthropometric variables to vertical jumping ability and leg power in Tunisian children. J Strength Cond Res 26(3):777–788

Bobbert MF, van Ingen Schenau GJ (1988) Coordination in vertical jumping. J Biomech 21(3):249–262

Bohannon RW (2006) Reference values for the timed up and go test: a descriptive meta-analysis. J Geriatr Phys Ther 29(2):64–68

Brands MM, Oussoren E, Ruijter GJ, Vollebregt AA, van den Hout HM, Joosten KF et al (2013) Up to five years experience with 11 mucopolysaccharidosis type VI patients. Mol Genet Metab 109 (1):70–76

Buehring B, Krueger D, Binkley N (2010) Jumping mechanography: a potential tool for sarcopenia evaluation in older individuals. J Clin Densitom 13(3):283–291

Cavagna GA (1975) Force platforms as ergometers. J Appl Physiol 39 (1):174–179

Dionyssiotis Y, Galanos A, Michas G, Trovas G, Lyritis GP (2010) Assessment of musculoskeletal system in women with jumping mechanography. Int J Womens Health 1:113–118

Enright PL, Sherrill DL (1998) Reference equations for the six-minute walk in healthy adults. Am J Respir Crit Care Med 158(5 Pt 1):1384–1387

Gerodimos V, Zafeiridis A, Perkos S, Dipla K, Manou V, Kellis S (2008) The contribution of stretch-shortening cycle and arm-swing to vertical jumping performance in children, adolescents, and adult basketball players. Pediatr Exerc Sci 20(4):379–389

Harmatz P, Ketteridge D, Giugliani R, Guffon N, Teles EL, Miranda MC et al (2005) Direct comparison of measures of endurance, mobility, and joint function during enzyme-replacement therapy of mucopolysaccharidosis VI (Maroteaux-Lamy syndrome):

results after 48 weeks in a phase 2 open-label clinical study of recombinant human N-acetylgalactosamine 4-sulfatase. Pediatrics 115(6):e681–e689

Hendriksz CJ, Burton B, Fleming TR, Harmatz P, Hughes D, Jones SA et al (2014) Efficacy and safety of enzyme replacement therapy with BMN 110 (elosulfase alfa) for Morquio A syndrome (mucopolysaccharidosis IVA): a phase 3 randomised placebo-controlled study. J Inherit Metab Dis 37(6):979–990

Josephson RK (1999) Dissecting muscle power output. J Exp Biol 202 (Pt 23):3369–3375

Kling JM, Clarke BL, Sandhu NP (2014) Osteoporosis prevention, screening, and treatment: a review. J Womens Health (Larchmt) 23(7):563–572

Mathiowetz V, Kashman N, Volland G, Weber K, Dowe M, Rogers S (1985) Grip and pinch strength: normative data for adults. Arch Phys Med Rehabil 66(2):69–74

McDonald CM (2002) Physical activity, health impairments, and disability in neuromuscular disease. Am J Phys Med Rehabil 81 (11 Suppl):S108–S120

McQuiddy VA, Scheerer CR, Lavalley R, McGrath T, Lin L (2015) Normative values for grip and pinch strength for 6- to 19-year-olds. Arch Phys Med Rehabil 96(9):1627–1633

Phillips M, Flemming N, Tsintzas K (2009) An exploratory study of physical activity and perceived barriers to exercise in ambulant people with neuromuscular disease compared with unaffected controls. Clin Rehabil 23(8):746–755

Redelmeier DA, Bayoumi AM, Goldstein RS, Guyatt GH (1997) Interpreting small differences in functional status: the Six Minute Walk test in chronic lung disease patients. Am J Respir Crit Care Med 155(4):1278–1282

Rittweger J, Mutschelknauss M, Felsenberg D (2003) Acute changes in neuromuscular excitability after exhaustive whole body vibration exercise as compared to exhaustion by squatting exercise. Clin Physiol Funct Imaging 23(2):81–86

Rittweger J, Schiessl H, Felsenberg D, Runge M (2004) Reproducibility of the jumping mechanography as a test of mechanical power output in physically competent adult and elderly subjects. J Am Geriatr Soc 52(1):128–131

Roig M, O'Brien K, Kirk G, Murray R, McKinnon P, Shadgan B et al (2009) The effects of eccentric versus concentric resistance training on muscle strength and mass in healthy adults: a systematic review with meta-analysis. Br J Sports Med 43 (8):556–568

Runge M, Rittweger J, Russo CR, Schiessl H, Felsenberg D (2004) Is muscle power output a key factor in the age-related decline in physical performance? A comparison of muscle cross section, chair-rising test and jumping power. Clin Physiol Funct Imaging 24(6):335–340

Solway S, Brooks D, Lacasse Y, Thomas S (2001) A qualitative systematic overview of the measurement properties of functional walk tests used in the cardiorespiratory domain. Chest 119 (1):256–270

Taylor MJ, Cohen D, Voss C, Sandercock GR (2010) Vertical jumping and leg power normative data for English school children aged 10–15 years. J Sports Sci 28(8):867–872

van der Ploeg AT (2010) Where do we stand in enzyme replacement therapy in Pompe's disease? Neuromuscul Disord 20 (12):773–774

Vanezis A, Lees A (2005) A biomechanical analysis of good and poor performers of the vertical jump. Ergonomics 48 (11–14):1594–1603

Veilleux LN, Rauch F (2010) Reproducibility of jumping mechanography in healthy children and adults. J Musculoskelet Neuronal Interact 10(4):256–266

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RESEARCH REPORT

RARS2 Mutations: Is Pontocerebellar Hypoplasia Type 6 a Mitochondrial Encephalopathy?

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Abstract Mutations in the mitochondrial arginyl tRNA synthetase (*RARS2*) gene are associated with Pontocerebellar Hypoplasia type 6 (PCH6). Here we report two patients, compound heterozygous for *RARS2* mutations, presenting with early onset epileptic encephalopathy and (progressive) atrophy of both supra- and infratentorial structures. Early pontocerebellar hypoplasia was virtually absent and respiratory chain (RC) defects could not be detected in muscle biopsies. Both patients carried a novel missense mutation c.1544A>G (p.(Asp515Gly)) in combination with either a splice site (c.297+2T>G) or a frameshift (c.452_454insC) mutation. The splice site mutation induced skipping of exon 4.

These two patients expand the phenotypical spectrum associated with *RARS2* mutations beyond the first report of PCH6 by Edvardson and colleagues. We propose to classify *RARS2*-associated phenotypes as an early onset mitochondrial encephalopathy, since this is more in agreement with both clinical presentation and underlying genetic cause.

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Introduction

Pontocerebellar hypoplasia (PCH) was used by Barth to describe a group of prenatal onset neurodegenerative disorders with clear hypoplasia or atrophy of cerebellar and pontine structures (Barth et al. 1990; Barth 1993). These patients present with severe mental and motor retardation, progressive microcephaly, and dystonia/chorea (Namavar et al. 2011a, b). MR imaging typically shows a "dragonfly" or "butterfly" configuration of the cerebellum (Namavar et al. 2011a). New subtypes of PCH, based on differences in phenotypes and/or genotype were described over the past years, and we showed that the same genetic variant can be responsible for different forms of PCH (Namavar et al. 2011b). The current nomenclature with ten subtypes does therefore not add to the diagnostic process (Table 1).

In an effort to create more clarity in the nomenclature, we focus here on PCH6 (OMIM 611523), a genetically and biochemically distinct form of PCH. Mutations in the mitochondrial Arginine tRNA-synthetase (*RARS2*) gene underlie PCH6, and the phenotype is similar to that of patients with mitochondrial respiratory chain (RC) defects (Edvardson et al. 2007). The canonical PCH6 phenotype consists of severe early onset epilepsy, progressive global atrophy including pons and cerebellum, lactic acidosis, and/or RC defects (Cassandrini et al. 2013).

In this study, we report two patients with previously unreported mutations in *RARS2*, in whom early pontocerebellar hypoplasia was virtually absent. RC defects could

Table 1 Overview of clinical features in patients with *RARS2*-gene mutations

Clinical feature	Number of patients affected[a] (%)
Developmental delay	29/29 (100%)
Seizures	25/29 (86%)[b]
Lactate elevated	15/22 (68%)
RC enzyme deficiency	8/17 (47%)
Early vermis hypoplasia	17/28 (61%)
Atrophy/hypoplasia of cerebellar hemispheres	24/28 (86%)
Pontine atrophy	16/28 (57%)
Cerebral atrophy	21/26 (81%)

[a] Not all data are available of all patients
[b] Three out of four patients without epilepsy died before the age of 2 months

not be detected in muscle biopsies taken from both patients. Based upon these two patients and a literature study, we conclude that *RARS2* mutations do not lead to a typical PCH phenotype. We therefore propose to categorize the *RARS2*-associated phenotypes as an early onset mitochondrial epileptic encephalopathy, since this is more in agreement with clinical presentation and underlying genetic cause.

Materials and Methods

Whole-Exome and Sanger Sequencing

DNA was extracted from peripheral blood samples using standard methods. In patient A *RARS2* was directly sequenced upon clinical suspicion using standard Sanger sequencing methods. In patient B, the *RARS2* mutations were identified by exome sequencing, which was performed as described before, and validated by standard Sanger sequencing methods (Neveling et al. 2013; Wortmann et al. 2015).

RT-PCR

Fibroblasts of patient A and parents were cultured on standard growth medium (DMEM (Lonza), 10% fetal calf serum (Bodinco B.V.), Penstrep (100 Units/mL Penicillin, 100 µg/mL Streptomycin, Gibco) and 10 µg/mL ascorbic acid). RNA was isolated with the RNeasy mini kit (Qiagen, Cat. No. 74106) from fibroblasts at 75% confluence or from whole blood using the PAXgene Blood RNA system (Qiagen, REF 762174, HB1051083). cDNA was synthesized with SuperScript III Reverse Transcriptase, according to the manufacturers protocol (Thermofisher). cDNA was amplified by PCR using the following M13 tailed *RARS2* primers: Exon 2–3 forward: 5′-AGCAGCTGGGATTGTAGAGA, Exon 5 reverse: 5′-CCACAATCTTCTTCTGGGGAA; exon 17 forward: 5′-TGTTTACAAGAGCCACAGTCTG; exon 19 reverse: 5′-CAGCCACTTCAGGAGGACTA. Fragment size was analyzed with agarose gel electrophoresis. DNA was extracted from gel using the silica beads method (Boom et al. 1990).

Results

Patient A

Clinical Description

Patient A is the second child of non-consanguineous parents, born at 39 weeks gestation after an uncomplicated pregnancy. Birth weight was 2,795 g (14th percentile), head circumference was 32 cm (9th percentile). Parents noted poor eye contact from birth. At 2 months of age, she was evaluated for feeding problems and jerking movements of the limbs. EEG showed an excess of slow activity with multifocal epileptiform discharges. Brain MRI showed bilateral symmetric white matter signal changes in the subcortical white matter, supratentorial atrophy, and mild vermal atrophy (Fig. 1a–d). Biochemical and metabolic investigations showed increased concentration of lactate in blood (3.2 mmol/L; normal <2.0 mmol/L), CSF (3.3 mmol/L; normal <2.3 mmol/L), and urine (lactic acid 742 µmol/L; normal <55 µmol/L). Enzyme activities of mitochondrial respiratory chain were normal in muscle, although a reduction in ATP production capacity was found (10.9 nmol/h.mUCS; reference range 15.4–30.2 nmol/h.mUCS).

At age 6 months, she continued to have epileptic seizures despite multidrug therapy, required tube feeding and had profound developmental delay. Examination showed progressive microcephaly, generalized hypertonia, and brisk reflexes. Brain MRI at age 6 months showed striking progression of infra- and supratentorial grey and white matter atrophy (Fig. 1e–g) as well as a bilateral frontoparietal and temporal subdural hygroma. Proton MR spectroscopy showed a lactate peak.

Genetic Analysis

Sanger sequencing of *RARS2* showed compound heterozygous mutations: c. 297+2T>G in intron 4 and c.1544A>G, p.(Asp515Gly) in exon 18 (NM_020320.3). Segregation analysis proved that the mutations were biallelic; father was heterozygous for the c.297+2T>G mutation and mother carried the c.1544A>G mutation.

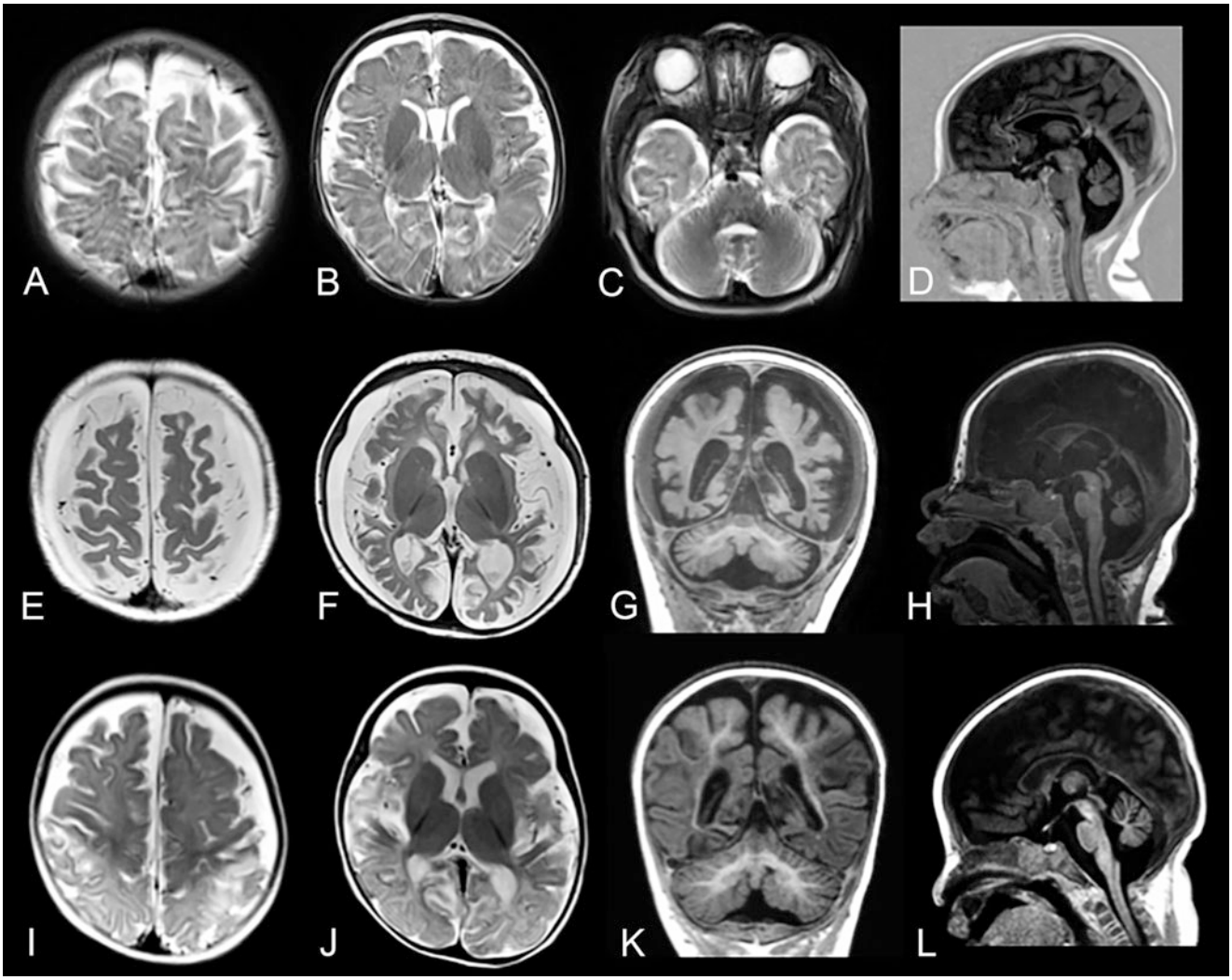

Fig. 1 Brain MRI of patients A and B. Axial T2-weighted (**a**–**c**, **e**, **f**, **i**, **j**), sagittal (**d**, **h**, **l**), and coronar (**g**, **k**) T1-weighted MR images of patient 1 aged 3 months (**a**–**d**) and 6 months (**e**–**f**) and patient 2 (aged 3 months, **i**–**l**). Note the bilateral subcortical T2 signal elevation of the central region in patient 1 (**a**) and of the central and parietooccipital cortex and white matter in patient 2 (**i**, **j**). Between the first and second MRI, severe supratentorial atrophy develops in patient 1 (**a**, **b** vs **e**–**g**). In patient 2, supratentorial structures also show global atrophy (**i**–**k**). Volume of pons and brainstem are normal in patient 1 (**d**), the vermis cerebelli is only slightly atrophic (**c**, **d**). At follow-up 3 months later, cerebellar volume appears slightly decreased with preserved pontine structures (**g**, **h**). In patient 2, there is no clear cerebellar, nor pontine atrophy (**k**, **l**)

The c.297+2T>G mutation was shown to perturb splicing by leading to a skip of exon 4 in the major *RARS2* transcript in fibroblasts of patient A and the father (Fig. 2).

Patient B

Patient B was the first child of non-consanguineous parents, born at 40 weeks of gestation after an uncomplicated pregnancy. Birth weight was 3,680 g (70th percentile). Head circumference was normal. At 3 months of age, she was admitted to the hospital for the evaluation of developmental delay and feeding problems. On examination she showed jerking movements, hypotonia, and nystagmus. EEG showed multifocal epileptiform activity and frequently focal clonic seizures. Brain MRI showed multifocal abnormalities of the parietal and occipital cortex and white matter and diffuse supratentorial atrophy (Fig. 1i–l). MRS showed a lactate peak whereas normal lactate levels were measured in blood (1.3 mmol/L; reference range 0.7–2.3 mmol/L) and CSF (1.7 mmol/L; reference range 0.7–2.3 mmol/L). Our first suspicion with this clinical and radiological presentation was Alpers disease due to *POLG* mutations, but genetic analysis of *POLG* was negative. Enzyme activities of mitochondrial respiratory chain were normal in muscle, although a reduction in ATP production capacity was found (5.8 nmol/h.mUCS; reference range

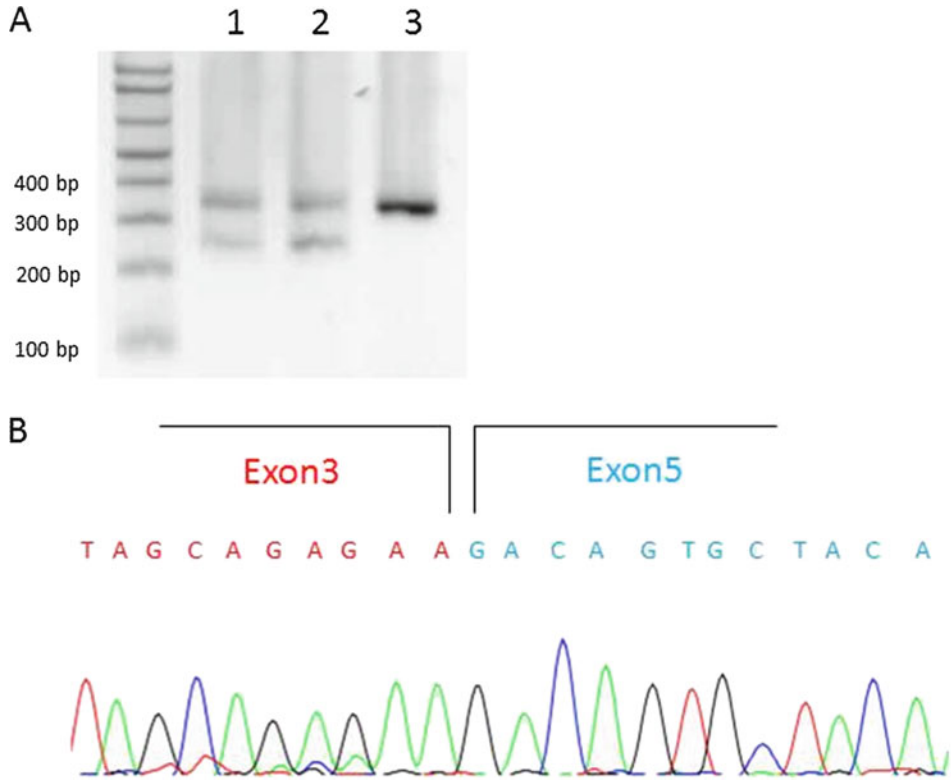

Fig. 2 The c.297+2T>G mutation causes skipping of exon 4. (**a**) PCR amplification product of *RARS2* cDNA, exon3–5. *Lane 1*, patient; *lane 2*, father; *lane 3*, mother. Aberrant splicing shown in the patient and father, suggesting exon 4 skipping. (**b**) Sanger sequencing of *RARS2* cDNA showing skipping of exon 4 in patient A

15.4–30.2 nmol/h.mUCS). She went on developing refractory status epilepticus and died 2 weeks after presentation.

Genetic Analysis

Two heterozygous mutations were identified in *RARS2* by whole-exome sequencing: c.1544A>G, p.(Asp515Gly), maternal, and c.453_454insC (p.(Asn152Lysfs*40), paternal; NM_020320.3).

In Silico Prediction of the c.1544A>G, p.(Asp515Gly) Mutation

Patient A and B both carried the c.1544A>G variant on one allele. This variant is located in exon 18 in the anticodon-binding domain, resulting in the amino acid substitution of a moderately conserved aspartic acid by glycine (p.(Asp515Gly)). In silico predictions by various programs were not uniform (SIFT: tolerated, PolyPhen HumDiv: probably damaging, with a score of 0.976, HumVar: possibly damaging with a score of 0.901). The mutation is not present in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), and with very low frequency (<1:10,000) in Exac (http://exac.broadinstitute.org/). Because the mutation is located in the Exonic Splice Enhancer domain of exon 18, an effect on splicing was considered. However, this could not be confirmed with RT-PCR (data not shown).

Discussion

Mutations in mitochondrial aminoacyl-tRNA synthetases (mt-aaRs) have emerged as an important cause of severe early onset neurologic disorders. Aminoacyl-tRNA synthetases are essential for protein translation as they catalyze the specific attachment of amino acids to their cognate tRNA. In eukaryotic cells, protein translation takes place both in the cytoplasm and mitochondria, and aaRs are therefore needed at both locations. The nuclear genome encodes 37 aaRs genes: 17 enzymes are exclusively active in the cytoplasma, 17 are mitochondrial enzymes, and 3 aaRs are bifunctional (GlyRS, LysRS, and GlnRS). Mutations in 15 out of 17 mt-aaRs are associated with human disease. Despite the ubiquitous expression and similar function mutations in mt-aaRs genes are each associated with tissue specific but overlapping phenotypes. Mutations in *FARS2*, *VARS2*, *TARS2*, and *CARS2* are associated with early onset mitochondrial encephalopathies, whereas *RARS2* mutations were first described in a sibship with PCH and RC defects and were designated as PCH6 (Edvardson et al. 2007).

In those patients pons and cerebellum were affected first, followed by general brain atrophy. The patients were homozygous for a splice site mutation causing a skip of exon 2 and resulting in a frameshift. Only a minimal amount of normal sized fragment was seen on RT-PCR. Correspondingly, the amount of charged mitochondrial tRNA-arg was severely reduced in patient fibroblasts (Edvardson et al. 2007).

We described two patients with novel *RARS2* mutations lacking the characteristic features of PCH on MRI. At an early stage, only mild cerebellar vermal atrophy with global supratentorial atrophy was present. Follow-up in patient A showed devastating progression of neurodegeneration affecting both supra- and infratentorial structures. Both patients were compound heterozygotes and carried the same c.1544A>G, p.(Asp515Gly) mutation. The other allele in both cases was predicted to be detrimental for protein function. The p.Asp515Gly mutation is located in the anticodon-binding domain and a possible pathogenic mechanism would be diminished recognition of arginine tRNA anticodons, leading to diminished or mis-acylation of arginine to noncognate tRNAs. Although we did not investigate the pathogenicity of this mutation with functional studies, the fact that both unrelated patients with a similar phenotype share this very rare variant in combination with a deleterious mutation on the other allele is strongly indicating a pathogenic effect. Patient A carried a splice site mutation on the other allele, resulting in skipping of exon 4, which was confirmed by RT-PCR. This exon contains 84 nucleotides and a skip is therefore not disturbing the reading frame. Some residual aminoacylation activity might be conserved in this shortened protein, as is the case in many of the other mutations identified in *RARS2*. Patient B had a frameshift mutation on the other allele, leading to a premature stop codon and a truncated protein with presumably little or no residual function.

Including the two patients we report here, 29 patients with *RARS2* mutations are described in literature so far (Namavar et al. 2011a; Cassandrini et al. 2013; Rankin et al. 2010; Glamuzina et al. 2012; Kastrissianakis et al. 2013; Joseph et al. 2014; Li et al. 2015; Lax et al. 2015; Nishri et al. 2016; Ngoh et al. 2016; Alkhateeb et al. 2016). Splice site, nonsense, or missense mutations are identified throughout the *RARS2* gene, but no patients with biallelic null mutations were identified, supporting the assumption that complete abolishment of tRNA-arg would be lethal. All patients who survived the neonatal period (24/29 patients, 83%) had severe developmental delay, and all but one patient older than the age of 2 months suffered from refractory epilepsy (25/26 patients, 96%). 15 out of 22 (68%) patients (lactate levels were unknown in seven patients) had elevated concentrations of lactate in either blood, urine, or CSF. Surprisingly, no RC defects could be detected in muscle or fibroblasts in 6 of those 15 patients (40%). Severity of cerebellar hypoplasia/atrophy was variable. MRIs made in a very early stage were either normal or showed relatively mild vermal hypoplasia, while follow-up MRIs often displayed rapidly progressive atrophy of both supra- and infratentorial structures (Cassandrini et al. 2013; Rankin et al. 2010; Kastrissianakis et al. 2013; Nishri et al. 2016). Remarkably, the basal ganglia often remained spared, a finding also endorsed by the patients we describe (Edvardson et al. 2007; Cassandrini et al. 2013; Glamuzina et al. 2012).

Recently three siblings with homozygous missense mutations in *RARS2* were reported with generalized spasticity and epilepsy, and a normal brain MRI. Unfortunately, little additional information regarding the clinical follow-up or age of brain MRI was provided (Alkhateeb et al. 2016).

Kastrissianakis and colleagues described two siblings with *RARS2* mutations with early MRIs showing marked supratentorial, instead of infratentorial, atrophy. Marked cerebellar atrophy developed later, but the pons remained relatively preserved throughout the disease course (Kastrissianakis et al. 2013). This finding is consistent with the imaging of the patients we report here, where atrophy of supratentorial structures is more striking than the pontocerebellar atrophy.

We conclude that the phenotypic spectrum associated with *RARS2* mutations has been significantly expanded beyond the initial report (Edvardson et al. 2007). As stated previously, mutations in other mt-aaRs genes have since then emerged as a cause of early onset mitochondrial encephalopathies. We therefore suggest that the name "PCH6" is no longer accurate to cover the phenotypes associated with *RARS2* mutations. A recent paper, describing two siblings with *RARS2* mutations and early onset epileptic encephalopathy without pontocerebellar hypoplasia, supports this conclusion (Nishri et al. 2016). Our proposal is to classify *RARS2*-associated phenotypes as an early onset mitochondrial encephalopathy, which is more in agreement with both clinical presentation and underlying genetic cause.

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Synopsis

PCH6 is a mitochondrial encephalopathy.

Compliance with Ethics Guidelines

Conflict of Interest

Tessa van Dijk, Fred van Ruissen, Bregje Jaeger, Richard J. Rodenburg, Saskia Tamminga, Merel van Maarle, Frank Baas, Nicole I. Wolf, and Bwee Tien Poll-The declare that they have no conflict of interest.

Informed Consent

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

Author Contributions

TvD collected data, performed the RT-PCR, and wrote the first draft of the manuscript. BTPT and NIW collected patient information and critically reviewed the manuscript. FvR and RjR performed diagnostic analysis and interpretation of results. BJ, ST, and MvM collected patient information. FB supervised the laboratory procedures and results, and critically reviewed the manuscript.

References

Alkhateeb AM, Aburahma SK, Habbab W, Thompson RI (2016) Novel mutations in *WWOX, RARS2*, and *C10orf2* genes in consanguineous Arab families with intellectual disabilities. Metab Brain Dis 31:901–907

Barth PG (1993) Pontocerebellar hypoplasias. An overview of a group of inherited neurodegenerative disorders with fetal onset. Brain Dev 15(6):411–422

Barth PG, Vrensen GFKM, Uylings HBM, Oorthuys JWE, Stam FC (1990) Inherited syndrome of microcephaly, dyskinesia and pontocerebellar hypoplasia: a systematic atrophy with early onset. J Neurol Sci 97(1):25–42

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noordaa J (1990) Rapid and simple method for the purification of nucleic acids. J Clin Microbiol 28:495–503

Cassandrini D, Cilio MR, Bianchi M et al (2013) Pontocerebellar hypoplasia type 6 caused by mutations in RARS2: definition of the clinical spectrum and molecular findings in five patients. J Inherit Metab Dis 36:43–53

Edvardson S, Shaag A, Kolesnikova O et al (2007) Deleterious mutation in the mitochondrial arginyl-transfer RNA synthetase gene is associated with Pontocerebellar Hypoplasia. Am J Hum Genet 81(4):857–862

Glamuzina E, Brown R, Hogarth K et al (2012) Further delineation of pontocerebellar hypoplasia type 6 due to mutations in the gene encoding mitochondrial arginyl-tRNA synthetase, *RARS2*. J Inherit Metab Dis 35:459–467

Joseph TJ, Innes AM, Smith AC et al (2014) Neuropathologic features of pontocerebellar hypoplasia. J Neuropathol Exp Neurol 73(11): 1009–1025

Kastrissianakis K, Anand G, Quaghebeur G et al (2013) Subdural effusions and lack of early pontocerebellar hypoplasia in siblings with RARS2 mutations. Arch Dis Child 98:1004–1007

Lax NZ, Alston CL, Schon K et al (2015) Neuropathologic characterization of pontocerebellar hypoplasia type 6 associated with cardiomyopathy and hydrops fetalis and severe multisystem respiratory chain deficiency due to novel RARS2 mutations. J Neuropathol Exp Neurol 74(7):688–703

Li Z, Schonberg R, Guidugli L et al (2015) A novel mutation in the promotor of RARS2 causes pontocerebellar hypoplasia in two siblings. J Hum Genet 60(7):363–369

Namavar Y, Barth PG, Kasher PR et al (2011a) Clinical, neuroradiological and genetic findings in pontocerebellar hypoplasia. Brain 134(1):143–156

Namavar Y, Chitayat D, Barth PG et al (2011b) TSEN54 mutations cause pontocerebellar hypoplasia type 5. Eur J Hum Genet 19: 724–726

Neveling K, Feenstra I, Gilissen C et al (2013) A post-hoc comparison of the utility of sanger sequencing and exome sequencing for the diagnosis of heterogeneous diseases. Hum Mutat 34:1721–1726

Ngoh A, Bras J, Guerreiro R et al (2016) RARS2 mutations in a sibship with infantile spasms. Epilepsia 57(5):95–102

Nishri D, Goldberg-Stern H, Noyman I et al (2016) *RARS2* mutations cause early onset epileptic encephalopathy without pontocerebellar hypoplasia. Eur J Paediatr Neurol 20(3):412–417

Rankin J, Brown R, Dobyns WB et al (2010) Pontocerebellar Hypoplasia type 6: a British case with PEHO like features. Am J Med Genet A 152A:2079–2084

Wortmann SB, Koolen DA, Smeitink JA, van den Heuvel L, Rodenburg RJ (2015) Whole exome sequencing of suspected mitochondrial patients in clinical practice. J Inherit Metab Dis 38: 437–443

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CASE REPORT

Missed Newborn Screening Case of Carnitine Palmitoyltransferase-II Deficiency

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Abstract Carnitine palmitoyltransferase-II (CPT-II) deficiency can be detected through newborn screening with tandem mass spectrometry. We report a 4-year-old patient with rhabdomyolysis due to CPT-II deficiency, which was initially missed by newborn screening. The patient presented with a 2-day history of fevers, upper respiratory infection, diffuse myalgia, and tea-colored urine. Her medical history was notable for frequent diffuse myalgia when ill. She was demonstrated to have homozygous mutation c.338C>T, p. S113L in *CPT2*, which is typically found in the adult-onset, myopathic form of the disease. An unknown number of CPT-II deficient patients with normal newborn screening have not yet presented to medical care with the adult-onset, myopathic form of disease. We conclude that (1) not all cases of CPT-II deficiency are currently detected through newborn screening, even when blood is appropriately collected on day 2 of life and (2) CPT-II deficiency should be kept on the differential for patients presenting with rhabdomyolysis, even if the newborn screening results were normal.

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Introduction

Patients with some inborn errors of metabolism, including long-chain fatty acid (LCFA) oxidation defects, can present with rhabdomyolysis, a clinical syndrome resulting from muscle breakdown (Chan et al. 2015). Carnitine palmitoyltransferase-II (CPT-II, EC 2.3.1.21) deficiency (OMIM: 255110) is one of the most common defects of LCFA metabolism (Bonnefont et al. 2004). CPT-II catalyzes formation of LCFA acyl-CoA species in mitochondria, allowing further oxidation and energy generation by other LCFA oxidation enzymes, including very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.8.9) (Bonnefont et al. 2004). Patients with CPT-II deficiency show elevated blood levels of long-chain acylcarnitine species, especially palmitoyl-carnitine (C16) and oleoyl-carnitine (C18:1). CPT-II deficiency can be categorized as the myopathic form (OMIM: 255110), the early infantile hepatocardiomuscular form (OMIM: 600649), or the lethal neonatal form (OMIM: 608836) with multisystem involvement (Bonnefont et al 2004). Patients with the myopathic form present with rhabdomyolysis, which may be complicated by life-threatening events, including acute renal failure from myoglobinuria, respiratory insufficiency due to diaphragm involvement (Smolle et al. 2001), and paroxysmal cardiac arrhythmia (Thuillier et al. 2000).

Screening for fatty acid oxidation defects, including long- and medium-chain defects, is recommended in the USA in accordance with the guidelines of the US Secretary of Health and Human Services' Recommended Uniform Screening Panel (http://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders /recommendedpanel/indes.html) and the American College of Medical Genetics Newborn Screening Expert Group (Watson et al. 2006). CPT-II deficiency is screened in almost all US newborn

Table 1 Acylcarnitine profile at birth (blood spot) and on presentation (plasma) in a 4-year-old girl experiencing acute rhabdomyolysis

	Newborn screen, bloodspot (μmol/L)		On presentation (μmol/L)	
Acylcarnitine species	Value	Cutoff[a]	Value	Reference range
Free carnitine (C0)	14.52	<8.00 (165)		
Acetyl-carnitine (C2)	13.13	<7.00 (89)	9.12	4.21–20.60
Propionyl-carnitine (C3)	0.38	<0.50 (66)	0.23	0–1.60
Palmitoyl-carnitine (C16)	3.44	>7.00 (162)	2.20	<1.00
Linoleyl-carnitine (C18:2)	0.13	>0.69 (91)	0.37	<0.30
Oleyl-carnitine (C18:1)	1.05	>2.99 (134)	0.81	<0.50
Octadecanoyl-carnitine (C18)	1.04	>2.00 (129)	0.39	<0.11
Dicarboxyoleyl-carnitine (C18:1-DC)	n/a	n/a	0.05	<0.03

n/a not available

[a] Median value of cutoffs posted on R4S website (https://www.clir-r4s.org; accessed on July 15, 2016), count of contributing laboratories shown between parentheses. Data reproduced with permission

screening (NBS) programs as a secondary target of NBS due to the lack of a proven efficacious treatment (Watson et al. 2006). Although newborns are routinely screened for fatty acid oxidation defects, some cases of LCFA oxidation defects (Schymik et al. 2006; Ficicioglu et al. 2010; Sahai et al. 2011) may be missed on NBS. Here, we report a case of rhabdomyolysis due to CPT-II deficiency in a child with normal NBS results.

Case Report

A previously healthy and developmentally normal 4-year-old girl was admitted to our general pediatrics service with acute rhabdomyolysis. Her parents reported a 2-day history of fevers (T_{max} 39.4°C), diffuse myalgia, tea-colored urine, upper respiratory infection, and two episodes of nonbloody, nonbilious emesis. The patient's medical history was notable for frequent diffuse myalgia when ill. There was no known family history of musculoskeletal, renal, or metabolic disorders, and her parents were nonconsanguineous. Her birth history was unremarkable. She was exclusively breastfed in the neonatal period without concern for hypoglycemia. An NBS obtained on day-of-life 2 revealed normal acylcarnitine profile results (Table 1).

On examination, the patient was nondysmorphic and exhibited nasal congestion, diffuse muscle aches in her arms that worsened with palpation, and a full range of motion of all extremities. Her initial creatine kinase level was 21,000 U/L, which peaked at 74,000 U/L. Her urine was dark. Although minimal red blood cells were seen on microscopy, initial urinalysis detected the presence of blood, consistent with rhabdomyolysis. A plasma acylcarnitine profile showed elevated levels of long-chain acylcarnitine species, which suggested the possibility of CPT-II or carnitine-acylcarnitine translocase (CACT) deficiency (OMIM: 212138, Table 1). Further genetic testing showed a homozygous mutation c.338C>T, p. S113L in *CPT2* and no mutations in *SLC25A20* gene.

Discussion

Tandem mass spectrometry analysis of acylcarnitine species is sensitive and specific for most fatty acid oxidation disorders (McHugh et al. 2011). The most sensitive indicator to detect CPT-II deficiency is an elevated (C16 + C18:1)/C2 ratio (Gempel et al. 2002; Marquardt et al. 2012). However, a study of simultaneous analysis of plasma and dried blood spot (DBS) from individuals with known metabolic diagnoses suggested that this indicator can sometimes be unreliable because some patients' (C16 + C18:1)/C2 ratio may be normal in DBSs (de Sain-van der Velden et al. 2013). Our patient's (C16 + C18:1)/C2 ratio of 0.34 was indeed below the 95th percentile of the reference range for DBSs reported in that paper (0.37). We also analyzed our patient's NBS acylcarnitine profile with the productivity and post-analytical interpretive tools of the Region 4 Stork (R4S) collaborative laboratory quality improvement of newborn screening by tandem mass spectrometry (https://www.clir-r4s.org/) (Marquardt et al. 2012; Hall et al. 2014). This produced the results presented in Fig. 1: (1) our patient's (C16 + C18:1)/C2 ratio was barely above the 99th percentile of the R4S cumulative reference range (0.33) but did not reach the lowest level (0.38) needed to contribute a score; (2) The overall case score was zero, but it was so because of an existing rule that forces a zero score whenever the C16 value is below the 90th percentile of the reference range (3.44 < 4.17 μmol/L). Because of this rule, which was

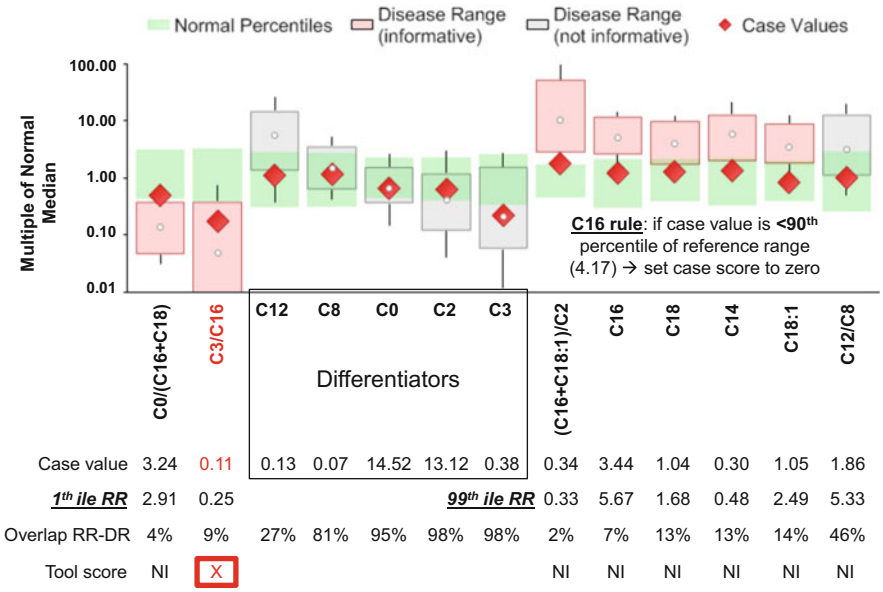

Fig. 1 Partial display of the post-analytical interpretive tool for CACT/CPT-II from the Region 4 Stork (R4S) collaborative laboratory quality improvement of newborn screening by tandem mass spectrometry website (https://www.clir-r4s.org/). The plot by condition panel is an overlay graph where for each marker and ratio the reference population, disease range and the case value are shown. All values are expressed as μmol/L and converted to multiple of the normal median on a log scale. *RR* reference range, *NI* not informative, *X* potentially informative score rejected by the C16 rule. Tool accessed on October 23, 2015, reproduced with permission

deemed adequate based on the analysis of 110 other cases, the R4S tool would have generated a zero score both at the time of birth and 4 years later; (3) Once the rule was modified in light of this case and propionylcarnitine was upgraded to informative marker, the overall profile would generate an informative score driven by a low concentration of propionylcarnitine and four new ratios (Fig. 2). In this case, either reliance on cutoff values or the R4S tool would have failed to detect CPT-II deficiency. On the other hand, this false negative event also represents an opportunity for continuing and evolving clinical validation, as similar cases could be detected by the revised tool.

There are several theoretical explanations for false-negative NBS results in disorders of fatty acid oxidation. These include (1) an anabolic state at the time of testing; (2) residual enzyme activity to overcome the catabolic stress of parturition; (3) depletion of carnitine, resulting in normalization of the long-chain acylcarnitine species; (4) excessively high cutoff values for acylcarnitine species that may disallow detection of all affected neonates; (5) sample mislabeling; and (6) laboratory error.

The stress of parturition is thought to be sufficient to expose biochemical abnormalities by day-of-life 2. To our knowledge, this is the first report of missed CPT-II deficiency on NBS when the blood sample was appropriately collected on day 2 of life. One prior report of missed CPT-II deficiency on NBS was attributed to the establishment of adequate nutrition by day-of-life 5, when the NBS sample was obtained (Kobayashi et al. 2007). However, other reports of missed cases of LCFA oxidation defects, especially VLCAD deficiency (Schymik et al. 2006; Ficicioglu et al. 2010; Sahai et al. 2011), suggest that false-negative NBS results can occur even when blood is appropriately collected on day 2 of life (Ficicioglu et al. 2010).

Our patient had a homozygous mutation c.338C>T, p. S113L in *CPT2*, which is typically found in the adult-onset, myopathic form of the disease (Bonnefont et al. 2004). It is possible that the c.338C>T, p. S113L mutation has sufficient residual activity to overcome the catabolic stress of parturition and to result in a normal NBS profile. Our patient had a normal NBS carnitine level, making carnitine depletion an unlikely cause of the false-negative result. Her NBS values were significantly below current cutoffs, but lowering NBS cutoffs to capture her values would result in unacceptably high false-positive rates. The only abnormal marker was the C3/C16 value based on the R4S post-analytical tool (McHugh et al. 2011) (Fig. 1). A series of new ratios, especially the (C16+C18:1)/C3 ratio, may be useful to enhance the chances of detection of mild CPT-II cases. However, more studies should be undertaken to understand the biochemical foundation for the consistently low concentration of propionylcarnitine in cases with CPT-II deficiency and to investigate whether adding C3-related cut offs would indeed increase sensitivity without negative impact on specificity and false positive rate. To test this hypothesis, three new ratios have already been added to the CPT-II tool in the new version of R4S named CLIR (Collaborative Laboratory Integrated Reports; https://clir.mayo.edu) (Fig. 2). It is unlikely that

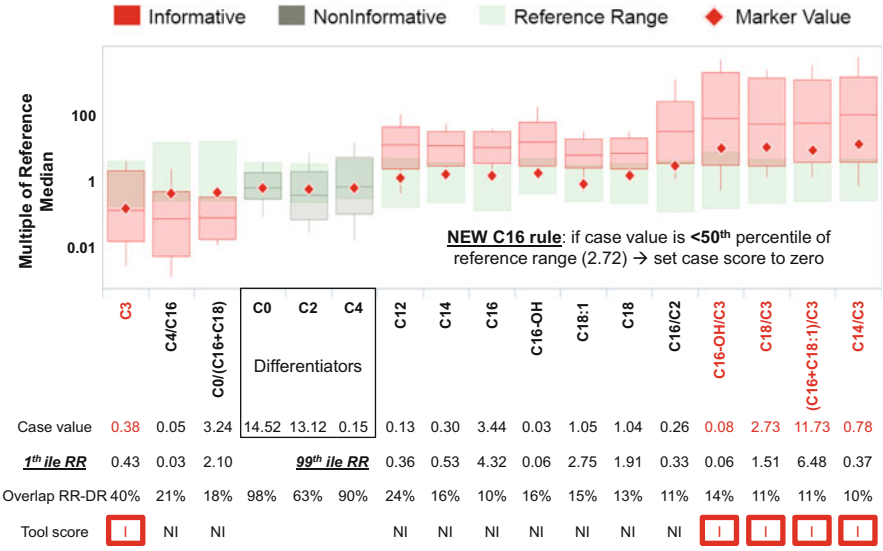

Fig. 2 Partial display of the post-analytical interpretive tool for CACT/CPT-II from the Collaborative Laboratory Integrated Reports (CLIR) website (https://clir.mayo.edu). See legend of Fig. 1 for details. *I* informative score. Tool accessed on July 15, 2016, reproduced with permission

our patient's NBS sample was mislabeled because the screening laboratory did not report a corresponding false-positive result around the same time. Laboratory error is also possible but unlikely, as repeat acylcarnitine analysis showed normalization of the patient's profile when not ill. In a previous case report, a patient with demonstrated heterozygous novel variants in *CPT2* and a positive CPT-II deficiency result on NBS on day-of-life 3 showed normalization of the acylcarnitine profile on day-of-life 9 (Illsinger et al. 2008). Given that newborns have only undergone regular NBS capable of detecting CPT-II deficiency since the late 1990s, there may be other patients with normal NBS results who have not yet presented to medical care with the adult-onset myopathic form of the disease.

Rhabdomyolysis can result in severe hyperkalemia, hypocalcemia, hepatic inflammation (Sauret et al. 2002), and acute renal failure (ARF) (Mannix et al. 2006; Bosch et al. 2009). In pediatric patients, rhabdomyolysis is typically attributable to viral myositis in the first decade of life and to trauma or a drug-related pathology in the second decade of life (Mannix et al. 2006). Rhabdomyolysis in CPT-II deficiency is caused by the accumulation of toxic long-chain acylcarnitine species, usually resulting from prolonged exercise, but also occasionally from prolonged fasting, excess fat intake, cold exposure, fever, or certain drugs (e.g., valproic acid, diazepam, general anesthesia, and ibuprofen) (Bonnefont et al. 2004).

Treatment of CPT-II deficiency includes avoiding triggers of increased fatty acid oxidation to prevent the accumulation of long-chain acylcarnitine species and rhabdomyolysis and supplementing the diet with medium-chain triglycerides to bypass the metabolic blockade (Bonnefont et al. 2004). A formulation of odd-chain fatty acids (triheptanoin) is under investigation as an alternative therapy to medium-chain triglycerides (Vockley et al. 2015). In cases of concurrent rhabdomyolysis, aggressive fluid therapy is recommended to avoid ARF. Furthermore, high-dose intravenous dextrose can be used to reverse catabolism and stop the production of long-chain acylcarnitine species. These approaches to the treatment of CPT-II deficiency, including nonstandard approaches for the prevention and treatment of rhabdomyolysis, raise the question of whether it would be timely to consider CPT-II deficiency for inclusion in the core NBS panel rather than as a secondary target in the USA. In terms of the natural course of disease, phenotype, and treatment, CPT-II deficiency closely resembles VLCAD deficiency, which is included in the core panel.

We offer two conclusions. First, not all cases of CPT-II deficiency are currently detected through NBS, even when blood is appropriately collected on day 2 of life. Second, CPT-II deficiency should be kept on the differential for patients presenting with rhabdomyolysis, even if the NBS results were normal, because the treatments, complications, and recurrence rates of rhabdomyolysis in CPT-II-deficient patients differ from those of standard rhabdomyolysis.

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Synopsis

CPT-II deficiency should be kept on the differential for patients presenting with recurrent rhabdomyolysis, even if the newborn screening results were normal, because this disorder can be missed on newborn screening, and the treatments, complications, and recurrence rates of rhabdomyolysis in CPT-II-deficient patients differ from those of standard rhabdomyolysis.

Compliance with Ethics Guidelines

Conflict of Interest

Dr. Andrew Edmondson, Dr. Jennifer Salant, Dr. Lynne Ierardi-Curto, and Dr. Can Ficicioglu declare that they have no conflicts of interest.

Informed Consent/Animal Rights

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

Details of the Contributions of Individual Authors

Dr. Edmondson drafted the initial manuscript and revised the manuscript.

Dr. Salant contributed to drafting the initial manuscript.

Dr. Ierardi-Curto critically reviewed and revised the manuscript.

Dr. Ficicioglu critically reviewed and revised the manuscript.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

References

Bonnefont JP, Djouadi F, Prip-Buus C, Gobin S, Munnich A, Bastin J (2004) Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Mol Aspects Med 25:495–520

Bosch X, Poch E, Grau JM (2009) Rhabdomyolysis and acute kidney injury. N Engl J Med 361:62–72

Chan EK, Kornberg AJ, Ryan MM (2015) A diagnostic approach to recurrent myalgia and rhabdomyolysis in children. Arch Dis Child 100:793–797

de Sain-van der Velden MG, Diekman EF, Jans JJ et al (2013) Differences between acylcarnitine profiles in plasma and blood-spots. Mol Genet Metab 110:116–121

Ficicioglu C, Coughlin CR 2nd, Bennett MJ, Yudkoff M (2010) Very long-chain acyl-CoA dehydrogenase deficiency in a patient with normal newborn screening by tandem mass spectrometry. J Pediatr 156:492–494

Gempel K, Kiechl S, Hofmann S et al (2002) Screening for carnitine palmitoyltransferase II deficiency by tandem mass spectrometry. J Inherit Metab Dis 25:17–27

Hall PL, Marquardt G, McHugh DMS et al (2014) Post-analytical tools improve performance of newborn screening by tandem mass spectrometry. Genet Med 16:889–895

Illsinger S, Lücke T, Peter M et al (2008) Carnitine-palmitoyltransferase 2 deficiency: novel mutations and relevance of newborn screening. Am J Med Genet A 146A:2925–2928

Kobayashi H, Hasegawa Y, Endo M, Purevsuren J, Yamaguchi S (2007) A retrospective ESI-MS/MS analysis of newborn blood spots from 18 symptomatic patients with organic acid and fatty acid oxidation disorders diagnosed either in infancy or in childhood. J Inherit Metab Dis 30:606

Mannix R, Tan ML, Wright R, Baskin M (2006) Acute pediatric rhabdomyolysis: causes and rates of renal failure. Pediatrics 118:2119–2125

Marquardt G, Currier R, McHugh DM et al (2012) Enhanced interpretation of newborn screening results without analyte cutoff values. Genet Med 14(7):648–655

McHugh D, Cameron CA, Abdenur JE et al (2011) Clinical validation of cutoff target ranges in newborn screening of metabolic disorders by tandem mass spectrometry: a worldwide collaborative project. Genet Med 13:230–254

Sahai I, Bailey JC, Eaton RB, Zytkovicz T, Harris DJ (2011) A near-miss: very long chain acyl-CoA dehydrogenase deficiency with normal primary markers in the initial well-timed newborn screening specimen. J Pediatr 158:172, author reply 172–173

Sauret JM, Marinides G, Wang GK (2002) Rhabdomyolysis. Am Fam Physician 65:907–912

Schymik I, Liebig M, Mueller M et al (2006) Pitfalls of neonatal screening for very-long-chain acyl-CoA dehydrogenase deficiency using tandem mass spectrometry. J Pediatr 149:128–130

Smolle KH, Kaufmann P, Gasser R (2001) Recurrent rhabdomyolysis and acute respiratory failure due to carnitine palmityltransferase deficiency. Intensive Care Med 27:1235

Thuillier L, Sevin C, Demaugre F et al (2000) Genotype/phenotype correlation in carnitine palmitoyl transferase II deficiency: lessons from a compound heterozygous patient. Neuromuscul Disord 10:200–205

Vockley J, Marsden D, McCracken E et al (2015) Long-term major clinical outcomes in patients with long chain fatty acid oxidation disorders before and after transition to triheptanoin treatment-A retrospective chart review. Mol Genet Metab 116:53–60

Watson MS, Mann MY, Lloyd-Puryear MA et al (2006) Newborn screening: toward a uniform screening panel and system. Genet Med 8:12S–252S

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CASE REPORT

Leigh-Like Syndrome Due to Homoplasmic m.8993T>G Variant with Hypocitrullinemia and Unusual Biochemical Features Suggestive of Multiple Carboxylase Deficiency (MCD)

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Abstract Leigh syndrome (LS), or subacute necrotizing encephalomyelopathy, is a genetically heterogeneous, relentlessly progressive, devastating neurodegenerative disorder that usually presents in infancy or early childhood. A diagnosis of Leigh-like syndrome may be considered in individuals who do not fulfil the stringent diagnostic criteria but have features resembling Leigh syndrome.

We describe a unique presentation of Leigh-like syndrome in a 3-year-old boy with elevated 3-hydroxyisovalerylcarnitine (C5-OH) on newborn screening (NBS). Subsequent persistent plasma elevations of C5-OH and propionylcarnitine (C3) as well as fluctuating urinary markers were suggestive of multiple carboxylase deficiency (MCD). Normal enzymology and mutational analysis of genes encoding holocarboxylase synthetase (*HLCS*) and biotinidase (*BTD*) excluded MCD. Biotin uptake studies were normal excluding biotin transporter deficiency. His clinical features at 13 months of age comprised psychomotor delay, central hypotonia, myopathy, failure to thrive, hypocitrullinemia, recurrent episodes of decompensation with metabolic keto-lactic acidosis and an episode of hyperammonemia. Biotin treatment from 13 months of age was associated with increased patient activity, alertness,

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and attainment of new developmental milestones, despite lack of biochemical improvements. Whole exome sequencing (WES) analysis failed to identify any other variants which could likely contribute to the observed phenotype, apart from the homoplasmic (100%) m.8993T>G variant initially detected by mitochondrial DNA (mtDNA) sequencing.

Hypocitrullinemia has been reported in patients with the m.8993T>G variant and other mitochondrial disorders. However, persistent plasma elevations of C3 and C5-OH have previously only been reported in one other patient with this homoplasmic mutation. We suggest considering the m.8993T>G variant early in the diagnostic evaluation of MCD-like biochemical disturbances, particularly when associated with hypocitrullinemia on NBS and subsequent confirmatory tests. An oral biotin trial is also warranted.

Introduction

Leigh syndrome (LS) is the most common paediatric presentation of mitochondrial disease with an estimated pre-school incidence of 1 per 34,000 births (Darin et al. 2001). Higher incidences have however been reported in specific populations in the Faroe Islands, 1:1700 (Ostergaard et al. 2007) and Saguenay Lac-Saint-Jean region of Quebec, Canada, 1:2000 (Morin et al. 1993) which have been attributable to founder mutations.

Since its initial description (Leigh 1951), LS has evolved from a distinct neuropathological disorder defined by post-mortem histopathological findings to a clinical entity characterized by progressive neurodegenerative disease with symptoms and signs of brainstem and/or basal ganglia disease, raised lactate levels in blood and/or cerebrospinal fluid (CSF), and typical neuroimaging and/or neuropathological abnormalities (Rahman et al. 1996). More recently, Baertling et al. (2014) refined the diagnostic criteria to include the three most commonly described features: (1) neurodegenerative disease with variable symptoms; (2) bilateral neuroimaging or CNS lesions and (3) a variety of nuclear or mitochondrially encoded genetic causes of deficient mitochondrial energy metabolism. The term "Leigh-like syndrome" was proposed when these diagnostic criteria are only partially met but highly suggestive for LS (Rahman et al. 1996; Baertling et al. 2014).

The clinical presentation of Leigh syndrome can be highly variable with disease onset ranging from the neonatal period through adulthood. Typically, onset occurs between age three and 12 months, often triggered by an acute infection. Prenatal expression of mitochondrial disease has been described with oligohydramnios, intrauterine growth restriction and abnormal brain neuroimaging (Kumakura et al. 2009; Sofou et al. 2014). Late-onset Leigh syndrome has been associated with predominant extrapyramidal features, slow progression, acute deterioration, usually after decompensation with illness, and atypical presentations including features of Guillain–Barré syndrome, hypertrophic cardiomyopathy, anaemia and leukopenia (Huntsman et al. 2005).

LS is a genetically heterogeneous mitochondrial disorder. Causative variants have been identified in up to 75 genes involved in energy metabolism, encoded by either the nuclear or mitochondrial genomes, including each of the five OXPHOS complexes, electron carrier coenzyme Q10 (CoQ10) and components of the pyruvate dehydrogenase complex (Lake et al. 2015). Mitochondrial DNA (mtDNA) mutations underlie approximately 10–20% of LS cases (Rahman et al. 1996; Sofou et al. 2014). Approximately 10% of individuals have either the *MT-ATP6* (mitochondrially encoded ATP synthase 6) m.8993T>G or m.8993T>C variants which represent the only established genetic cause of a Complex V-mediated LS (Santorelli et al. 1993; Rahman et al. 1996; Thorburn and Rahman 2014). The *MT-ATP6* gene encodes a subunit of the 550 kKDa multi-subunit Complex V (ATP synthase or F_1F_o ATPase), which is one of the key enzymes involved in the aerobic generation of energy, synthesizing ATP from ADP using the proton gradient generated across the mitochondrial inner membrane (Kucharczyk et al. 2009). Unlike many pathogenic mtDNA variants, the m.8993T>G and m.8993T>C variants display a strong genotype–phenotype correlation, with a lack of tissue or age-dependent variation in mutant load (White et al. 1999a) which enables accurate prediction of the probability of severe outcome and empirical recurrence risks (White et al. 1999b). Overall, LS develops whenever the m.8993T>G mutant load exceeds 90%, while the milder NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa) phenotype occurs with moderate levels of approximately 70–90% mutant loads (Thorburn and Rahman 2014). Other properties of m.8993T>G variant include *de novo* occurrences with rapid segregation toward homoplasmy, often within a single generation, observed in approximately 20% of families (White et al. 1999a). Likely explanations for these sporadic cases include spontaneous variants arising during oogenesis (Degoul et al. 1997); or presence of a mitochondrial genetic "bottleneck", in which only a small subpopulation of mtDNA molecules are preferentially amplified to "repopulate" the oocyte (Blok et al. 1997; White et al. 1999b).

Here, we describe a male patient with increased C5-OH on NBS, presenting with psychomotor delay, central hypotonia, failure to thrive, episodes of decompensation with keto-lactic acidosis and persistent elevations in C3, C5-OH, with urinary markers suggestive of multiple carboxylase deficiency (MCD). Hypocitrullinemia was an additional feature which was also detected on NBS. Leigh-

like disease was subsequently diagnosed secondary to a de novo homoplasmic m.8993T>G variant.

Materials and Methods

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. The family reported in this study has agreed to the publication and provided written consent as part of the informed consent process.

Case Report

The patient, a Caucasian male, was born at term with a birth weight of 3.2 kg (25th percentile) to non-consanguineous healthy parents. The patient had increased C5-OH (2.0 μmol/L, ref <0.6) on newborn screen and a similar bloodspot result at 3 weeks of age. His bloodspot citrulline on NBS was 7 μmol/L (1% in this age group on screening is 7 μmol/L; the 50% (median) is 14 μmol/L). Low citrulline is not used as a marker in newborn screening (NBS) in Western Australia. Plasma acylcarnitine analysis revealed increased C5-OH (2.1 μmol/L, ref <0.9) and C3 (1.1 μmol/L, ref <0.2). Plasma amino acid analysis at 3 weeks of age showed increased alanine (696 μmol/L, ref 130–460) and decreased citrulline (2 μmol/L ref 10–45). Venous blood gas was normal, apart from increased lactate (3.8 mmol/L, ref <1.5). Plasma ammonia and urine organic acid analysis were normal. Bloodspot biotinidase enzyme assays performed twice were normal.

For the first week of life, the patient was exclusively breastfed and developed feeding intolerance with vomiting and loose stools. He was diagnosed with breast milk protein-induced enterocolitis syndrome (FPIES) and feeding was switched to a hypoallergenic, elemental formula (Neocate Infant, Nutricia); coincident with the feeding change, the feeding intolerance resolved. However, weight gain remained poor over the first 6 months of life; this was attributed to recurrent viral infections. He failed to achieve age-related developmental milestones by 6 months of age; Griffith's mental developmental assessment at 12 months confirmed global developmental delay with an overall mental age of 7 months and motor developmental age of 6 months. He was presented to the metabolic clinic at 13 months of age with failure to thrive, lethargy and central hypotonia. Physical examination at that time revealed brisk deep tendon reflexes, equivocal plantar reflexes and generalized muscle weakness. Laboratory investigations revealed mildly increased lactate (3.9 mmol/L, 0.5–2.0), with normal plasma ammonia, creatine kinase, liver enzyme tests and venous blood gas results. Plasma acylcarnitines showed increased C3 (3.1 μmol/L, ref <0.7) and C5-OH (0.97 μmol/L, ref <0.31); plasma amino acids revealed increased alanine (572 μmol/L, ref 143–439) and decreased citrulline (4 μmol/L, ref 10–45). Urine organic acid analysis showed mild to moderate increases in 3-hydroxyisovalerate, 3-hydroxypropionate, 3-methylcrotonylglycine, methylcitrate and 2-methyl-3-hydroxybutyrate. A biotin related inborn error was suspected, and oral biotin at 10 mg daily was initiated and subsequently increased to his current dose of 100 mg twice a day. Further investigations were carried out to exclude MCD including holocarboxylase synthetase (*HLCS*) gene sequencing, with deletion/duplication analysis; biotinidase (*BTD*) gene sequencing and fibroblast enzymology of propionyl-CoA carboxylase, methylcrotonyl-CoA carboxylase and pyruvate carboxylase using a low biotin culture medium (6 nmol/L). All these studies were normal.

Clinical improvements with increased patient activity levels, alertness and attainment of new developmental milestones were temporally associated with oral biotin treatment despite lack of significant correlation with biochemical improvements. Trio whole exome sequencing (WES) was carried out to exclude any novel genetic defects in the biotin pathway, and included analyses for any mutations in the mitochondrial carbonic anhydrase VA (*CA5A*) gene which presents with MCD phenotype (Karnebeek et al. 2014) but all tests were non-contributory.

At 15 months, he decompensated rapidly during an intercurrent pneumonia with human metapneumonia virus. He was ventilated in the ICU and had metabolic ketolacticacidosis with blood pH 7.27, bicarbonate 12 mmol/L, base excess −14, lactate 8.8 mmol/L, plasma beta-hydroxybutyrate (6.0 mmol/L, ref <0.3) and hyperammonemia (119 μmol/L, ref <50). CSF lactate (1.7 mmol/L, ref 0.7–1.8) and pyruvate (0.08 mmol/L, ref 0.03–0.1) were normal. Apart from a small lactate doublet on spectroscopy, brain MRI was normal. He was discharged home after a week with temporary regression of milestones, particularly gross motor which returned to pre-morbid state a few weeks later.

A repeat developmental assessment performed at 17 months showed steady progress with a 6 months gain over the preceding 6 months. Persistent plasma increases of C5-OH (1.1 μmol/L, ref <0.2), C3 (3.0 μmol/L, ref <0.6) and fluctuating urinary markers (3-methylcrotonylglycine, 3-hydroxyisovalerate, 3-hydroxypropionate, methylcitrate and 2-methyl-3-hydroxybutyrate) were observed, despite significant clinical progress. Fundoscopy and slit-lamp examination were normal. Quadriceps muscle biopsy showed well-orientated muscle fibres with variable fibre diameter ranges of 10–25 mm (age appropriate range 16–18 mm) and small subsarcolemmal aggregates of mitochondria in some fibres with modified Gömöri

trichrome stain. Electron microscopy displayed atrophic fibres, mild mitochondrial pleomorphism with lipid vacuoles and crowding of the cristae in rare fibres. Complex IV activity on snap frozen skeletal muscle homogenate was borderline low at 25% when expressed relative to citrate synthase, but in the lower end of the normal range 2.93 /min/mg (reference range 3.3–9.1) relative to protein. Complex V was not measured due to technical limitations. Identification of de novo homoplasmic (100%) m.8993T>G variant in muscle, blood and urine by next-generation sequencing of the mitochondrial genome led to the diagnosis of Leigh-like syndrome. Analysis of maternal blood and urine DNA tested negative for the m.8993T>G variant identified in her son. Therapy with riboflavin, thiamine, vitamin C, carnitine and coenzyme Q10 was started initially; oral supplementation of citrulline, creatine and alpha-lipoic acid was commenced at 24 months, 28 and 36 months, respectively. Repeat brain MRI at 31 months was again reported to be normal, apart from the presence of small lactate doublet. Early initiation of aggressive metabolic dietary support during unwell episodes has significantly decreased metabolic decompensations. Early institution of nasogastric feeding during intercurrent illness when oral feeding has been difficult has substantially reduced the frequency of hospital admissions and need to seek emergency care (from a 4 to 6 weekly frequency to approximately once in 6 months). The unwell dietary management plan includes increased caloric intake at 100 kcal/kg/day (fat 44%, protein 26% and carbohydrates 30%).

At 3 years 3 months of age, he has exhibited significant improvements in his overall developmental progress; particularly with gross motor milestones. He now has a healthy 2-month old sister. Prenatal testing through chorionic villus sampling for the sibling revealed no detectable m.8993T>G variant. She was born healthy with normal birth parameters, NBS and urine organic acids. She has shown normal weight gain and appears to be developing appropriately at 3 months of age.

Whole Exome Sequencing (WES)

After enrolment of the family within the TIDEX gene discovery project (UBC IRB approval H12-00067), WES was performed for the proband and his unaffected mother using the Agilent SureSelect kit and Illumina HiSeq 2000 (PerkinElmer, USA). The sequencing reads were mapped to the hg19 human reference genome. After multiple filtering steps (excluding sequencing errors, variants with MAF >0.01 etc.) and screening under multiple inheritance models, we uncovered eight genes harbouring homozygous recessive variants [one autosomal (*NTN5*) and seven X-linked (*SYTL5*, *CLCN5*, *BEND2*, *CNGA2*, *RBBP7*, *HEPH* and *TCEAL4*)], six compound heterozygous (*TNRC6A*, *NAV3*, *CLCN2*, *ALKBH2*, *UNC5CL* and *BSG*), and no nuclear de novo variants. None were deemed compatible with the patient's phenotype. Each of these variants were further analyzed for their impact on gene function using multiple bioinformatic tools (including, SIFT, Polyphen and CADD scores), as well as published literature on their function to ascertain a potential correlation with the proband's phenotype. The only variants of potential interest were the ones affecting CLCN5 and CLCN2; however, neither of these was decided to be explanatory for the observed MCD phenotype, and thus ATP6 was deemed the best fit.

Biotin Concentrations in Physiologic Fluids

All samples were initially assayed for biotin using a sequential solid phase avidin-binding assay as previously described (Mock 1997) that measures Total Avidin-Binding Substances (TABS) referred to hereafter as biotin unless noted otherwise.

Biotin Uptake Studies

Radioactive ^{3}H-biotin (specific activity: 60 Ci/mmol; radiochemical purity >97%) was purchased from American Radiolabeled Chemicals (ARC) (St. Louis, MO). Other reagents and chemicals used in these studies were purchased from commercial vendors; all were of either analytical or molecular biology grade. Specific primers used for PCR amplifications were from Sigma Genosys (Woodlands, TX).

Biotin uptake was performed in lymphocytes from the proband and normal controls as previously reported (Said et al. 1998) using Krebs–Ringer (KR) buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 $MgSO_4$, 0.85 $CaCl_2$, 5 glucose, 5 glutamine, 10 HEPES and 10 MES, pH 7.4). Briefly, lymphocytes were harvested by centrifugation and equal amount of cells were used for uptake studies for 5 min as described previously. The reaction was stopped by adding 2 ml of ice-cold KR buffer and cells were passed through PVDF membrane, rinsed twice with ice-cold buffer. The membrane was incubated further with scintillation fluid and radioactivity was measured in a scintillation counter (Beckman Coulter LS6500). Passive diffusion was quantified by performing uptake of ^{3}H-biotin in the presence of excess unlabeled biotin (1 mM). Protein concentrations were measured by DC protein assay kit (Bio-Rad). Uptake was expressed as fmol/mg protein/5 min.

Real-Time PCR Analysis

Total RNA was isolated from lymphocytes using TRIzol reagent (Invitrogen). RNA samples were then treated with

RNAse free DNAse I (Invitrogen) to remove contaminating DNA, and were reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). Relative expression of the sodium dependent multivitamin transporter (*SMVT; SLC5A6*) transcript was quantified by real-time PCR (Bio-Rad CFX 96 real-time PCR system) using gene-specific primers for hSMVT (Forward 5′-TGTCTACCTTCTCCATCATGGA-3′ and Reverse 5′-TAGAGCCCAATGGCAAGAGA-3′). β-Actin was used as an internal control (Forward 5′-AAATGGGTTCTAGACCGCGGAGA-3′ and reverse 5′-CATGCTCGATGCGGTACTTCA-3′) and data were calculated following relative relationship method.

Western Blot Analysis

To determine the protein level of hSMVT, lymphocytes were lysed in RIPA buffer containing protease inhibitors, and the supernatant was collected by brief centrifugation. Equal amounts of protein (approximately 60 μg) were loaded into NuPAGE 4–12% Bis-Tris gradient mini gels (Invitrogen). The gel was electro-blotted onto PVDF membrane and incubated overnight with blocking buffer (LI-COR). The blot was then probed with specific human polyclonal antibodies to SMVT and β-actin (Santa Cruz Biotechnology), followed by probing with labelled secondary antibodies (anti-mouse IRDye 800 and anti-rabbit IRDye 680 in 1:25,000 dilutions) for 1 h. Fluorescent intensity of the specific band was quantified using the Odyssey Infrared imaging system (LI-COR Biosciences) and Odyssey application software (version 3.0).

Results

Biotin Studies: In samples collected 10 days after initiating biotin therapy at 10 mg per day, the biotin level measured in plasma was ~1,500 times upper limit of reference range and the urine excretion rate was ~4,000 times upper limit of reference range (Table 1). These striking increases contrasted with the level in CSF, which was ~15 times upper limit of reference range. The discrepancies of biotin concentrations in the different fluids were of uncertain significance as the samples were collected post-biotin supplementation.

Carrier-mediated biotin uptake was studied in EBV-transformed lymphocytes from the proband, and compared with similar studies performed in EBV-transformed lymphocytes from normal adult controls (ABC) and with those from cord blood controls (CBC). The results (Fig. 1) showed considerable biotin uptake that was slightly but significantly ($p < 0.05$) lower than uptake by EBV-transformed lymphocytes from control cord blood samples, yet somewhat higher than that of adult EBV-transformed lymphocytes. The higher uptake in cord blood controls compared to adult EBV-transformed lymphocytes could be attributed to developmental maturation. This has previously been described in a rodent model with higher intestinal biotin uptake observed during the suckling period compared to adulthood (Said and Redah 1988).

SMVT Transcript and Protein Studies: SMVT mRNA (Fig. 2a) and protein levels (Fig. 2b) in EBV-transformed lymphocytes from the proband were not different than those for EBV-transformed newborn (cord blood) and adult controls. In the light of these findings, we speculate that the relatively lower increases in CSF might represent saturation of a normal carrier-mediated transport across the blood–brain barrier. There is evidence for such a carrier in animal studies (Spector and Mock 1987, 1988).

Table 1 Biotin determinations of urine, CSF and plasma samples

Sample	Proband	Normal range
Urine (pmol/mg creatinine)	429,355	45–118 ($n = 49$)
CSF (pmol/ml)	88.0	0.022–5.9 ($n = 55$)
Plasma (pmol/ml)	1,398	0.55–1.1 ($n = 80$)

Discussion

Persistent hypocitrullinemia has previously been reported in 13 patients with the m.8993T>G variant (Rabier et al. 1998; Parfait et al. 1999; Enns et al. 2006; Debray et al. 2010; Henriques et al. 2012; Mori et al. 2014). In an additional report, a female patient who was prenatally diagnosed with apparently mild ornithine transcarbamylase (OTC) deficiency succumbed during an acute febrile encephalopathic illness at 6 months of age, with neuroimaging suggestive of Leigh syndrome. Post-mortem genetic testing identified the m.8993T>G variant in the liver, muscle and blood with variant loads of 82–87% (Henriques et al. 2012). Persistent hypocitrullinemia has been described in other mitochondrial disorders including Pearson syndrome (Ribes et al. 1993), Leigh syndrome caused by isolated complex I deficiency (Debray et al. 2010), mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS syndrome) (Naini et al. 2005), mitochondrial neurogastrointestinal encephalopathy (MNGIE) disease (Bindoff 2006), and with secondary mitochondrial respiratory chain dysfunction caused by organic acidemias (Atkuri et al. 2009) and deficiency of mitochondrial pyrroline-5-carboxylate synthetase (P5CS) (Baumgartner et al. 2000). In a small series of 16 Leigh syndrome patients, sensitivity and specificity of hypocitrullinemia as a marker for the m.8993T>G variant were reported to be 66% and 85%, respectively (Debray et al. 2010). Hypocitrullinemia was also found to be a character-

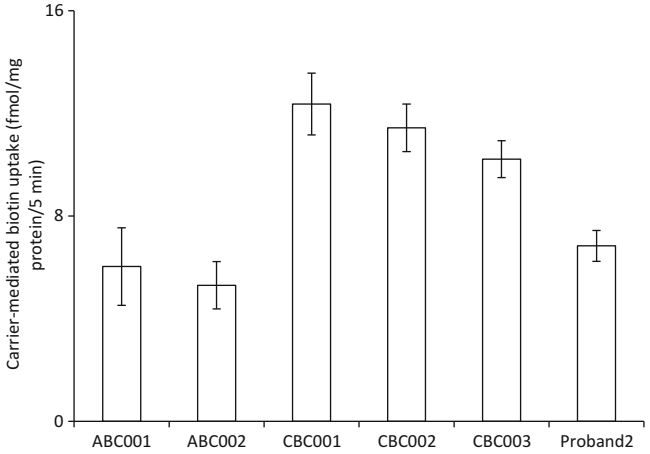

Fig. 1 Carrier-mediated biotin uptake measured in EBV-transformed lymphocytes of proband compared to EBV-transformed lymphocytes from adults (ABC) and cord blood control (CBC). Data were expressed as fmol/mg protein/5 min and are mean ± SE of three independent experiments

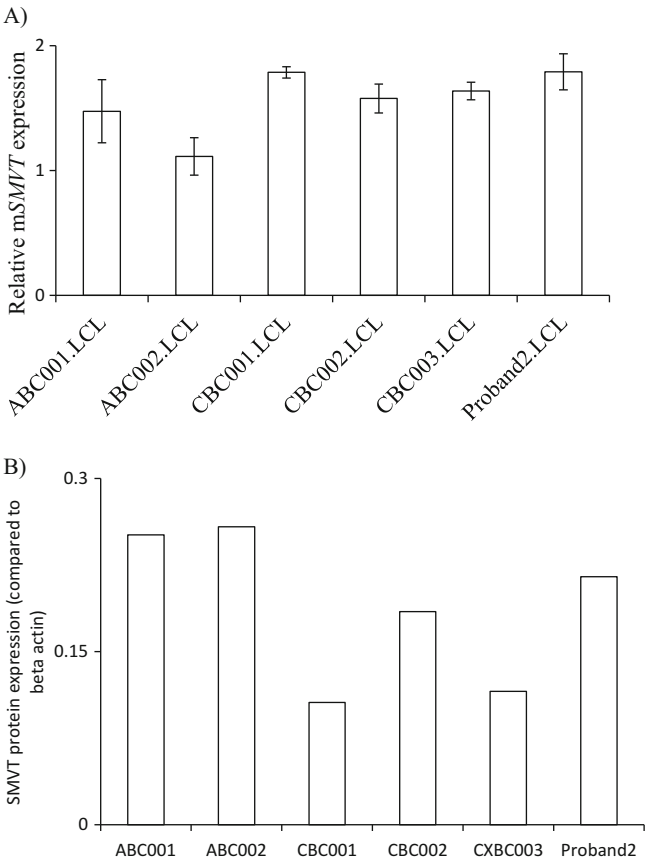

Fig. 2 SMVT transcript and protein studies. (**a**) *SMVT* mRNA expression: real-time PCR was performed using total RNA isolated from lymphocytes. The level of *hSMVT* expression was normalized relative to β-actin as described in "Materials and Methods". Data are mean ± SE of three independent experiments. (**b**) SMVT protein expression: total protein isolated from the lymphocytes was used for western blotting. The blot was probed with anti-SMVT antibody and expression was normalized relative to β-actin

istic unique to patients with the m.8993T>G mutation, seen in 90% of patients, as opposed to less than 20% for other respiratory chain deficiencies (Rabier et al. 1998). These findings are sufficiently convincing to consider hypocitrullinemia as a useful biochemical marker for the m.8993T>G mutation and, more generally, as a marker of impaired oxidative phosphorylation in the enterocyte (Rabier et al. 1998; Parfait et al. 1999; Debray et al. 2010; Henriques et al. 2012; Mori et al. 2014).

Citrulline is a non-protein amino acid that is not normally incorporated into proteins during protein synthesis. However, citrullinated proteins comprise citrulline residues that result from post-translational modification of arginine; these include keratinization-related proteins (Ishigami et al. 2002) and basic myelin protein, which makes up to 35% of the protein component of the central nervous system (Ishiyama et al. 2001). It is a non-essential amino acid, synthesized almost exclusively in the mitochondrial matrix of enterocytes, catalyzed by pathways involving P5CS, carbamoyl phosphate synthase 1 (CPS1) and OTC (Fig. 3). CPS I and P5CS activities are both tightly related to ATP concentration, while ADP acts as an inhibitor (Elliot and Tipton 1974). Citrulline metabolism within the urea cycle in the liver is strictly compartmentalized; involving ammonia detoxification and arginine synthesis.

Reduced oxidative phosphorylation can lead to deficient activities of both P5CS and CPS1 in the enterocyte with subsequent enterocyte dysfunction and impairment of the intestinal citrulline biosynthetic pathway (Rabier et al. 1998). Studies have demonstrated citrulline as an efficient marker of the active small bowel mass (Crenn et al. 2000), as well as in a wide range of pathologies; villous atrophy-

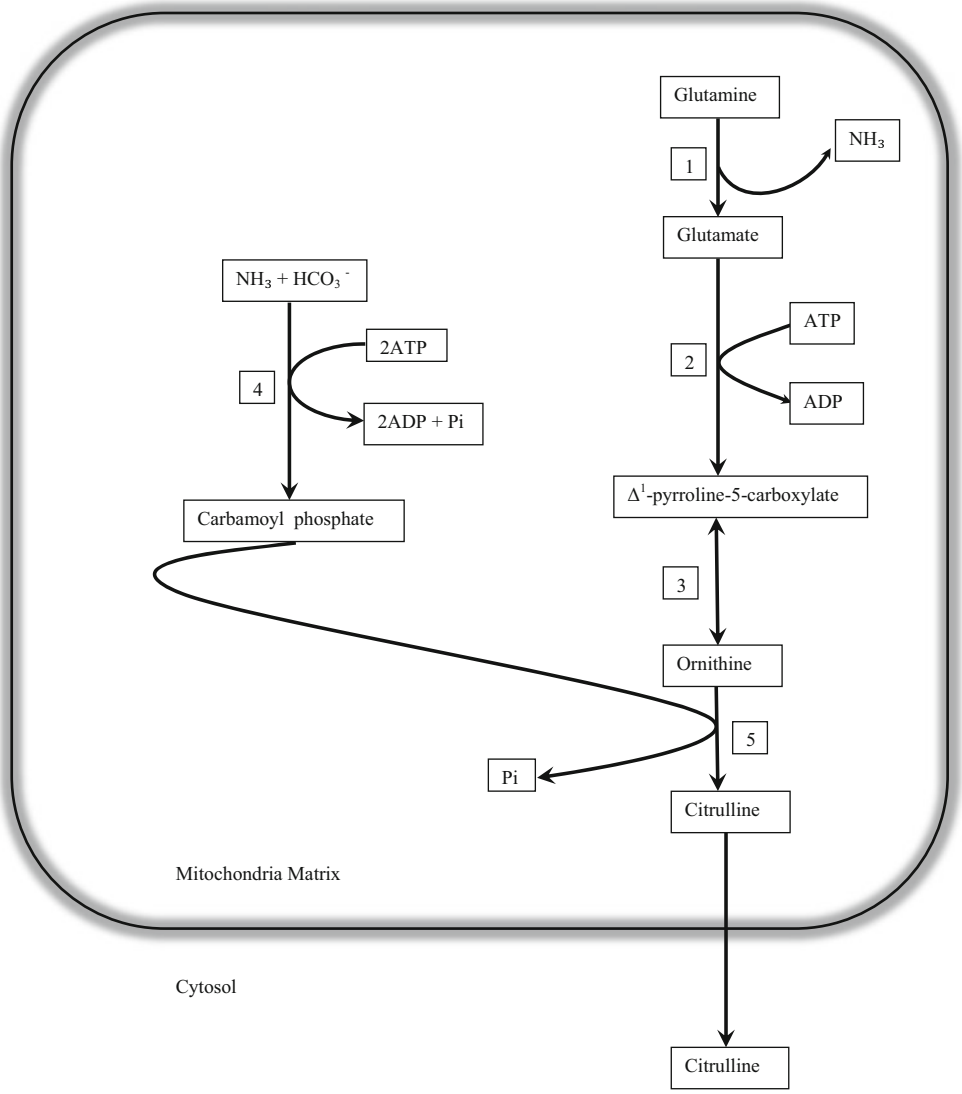

Fig. 3 Citrulline synthesis in mitochondrial enterocyte: *1* glutaminase, *2* Δ^1-pyrroline-5-carboxylate synthetase (P5CS), *3* ornithine δ-aminotransferase, *4* CPS I and *5* OTC

associated small bowel disease (Crenn et al. 2003); radiation-induced intestinal epithelial damage (Lutgens et al. 2003) or other myeloablative therapy (Blijlevens et al. 2004). The utility of citrulline as an effective marker of enterocyte mitochondrial dysfunction may correlate with the heteroplasmy in gut mucosa. A 97% heteroplasmy in gut mucosa was demonstrated in a patient with hypocitrullinemia (Rabier et al. 1998).

Abnormal acylcarnitine concentrations, particularly persistent elevations of C3 and C5-OH, have only been described in one other patient with the m.8993T>G variant (Hauser 2014). Our patient presented biochemically with profiles suggestive of MCD; however, extensive enzymatic and genetic analyses excluded that group of disorders. Biotin uptake and transport studies failed to identify any contributory cause. We speculate that defective oxidative phosphorylation not only affected citrulline biosynthesis, resulting in hypocitrullinemia, but also compromised ATP dependent carboxylases, causing the unusual biochemistry exhibited by this patient. Interestingly, this abnormal acylcarnitine profile has been associated with other *MT-ATP6* variants, specifically m.8959G>A and m.9155A>T (unreported data, personal communication, A Mattman), hence raising the possibility this may be a feature specific to defects of the ATPase 6 subunit of Complex V. While citrulline is among the metabolites measured by expanded newborn screening in Western Australia, low citrulline values are not officially reported for further work-up due to low positive predictive value.

Larger studies are needed to ascertain the true prevalence of these abnormal biochemical parameters which may be detected as early as in NBS, and to determine the pathogenesis connecting the m.8993T>G mutation as well as other *MT-ATP6* mutations and these metabolic abnormalities. We suggest expanding the differential diagnosis for persistent elevations in C3 and C5-OH detected on NBS, particularly when associated with hypocitrullinemia as potential NBS markers for mitochondrial disorders due to m.8993T>G associated Leigh-like syndrome and other *MT-ATP6* mutations. This may facilitate rapid diagnosis through targeted mutational analysis and minimize invasive tissue biopsies for respiratory chain studies. Presymptomatic diagnosis of mitochondrial disorders may be of therapeutic benefit, particularly with this biochemical phenotype when oral biotin therapy is warranted along with close monitoring of relevant biochemical and neurodevelopmental parameters.

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Compliance with Ethics Guidelines

Conflict of Interest

All authors declare that they have no conflicts of interest.

Details of the Contributions of Individual Authors

SB was the physician in charge of the family. BL, DMM, HMS, MTG, AM, CDvK, DRT and RJR performed/supervised/interpreted laboratory investigations. JC advised on the overall diagnostic work-up and management of the proband. SB and JC drafted the original manuscript. All authors have read/critically revised the manuscript.

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References

Atkuri KR, Cowan TM, Kwan T, Ng A, Herzenberg LA, Enns GM (2009) Inherited disorders affecting mitochondrial function are associated with glutathione deficiency and hypocitrullinemia. Proc Natl Acad Sci U S A 106(10):3941–3945

Baertling F, Rodenburg RJ, Schaper J et al (2014) A guide to diagnosis and treatment of Leigh syndrome. J Neurol Neurosurg Psychiatry 85:257–265

Baumgartner MR, Hu CA, Almashanu S et al (2000) Hyperammonemia with reduced ornithine, citrulline, arginine and proline: a new inborn error caused by a mutation in the gene encoding delta (1)-pyrroline-5-carboxylate synthase. Hum Mol Genet 9 (19):2853–2858

Bindoff L (2006) Mitochondrial gastroenterology. In: DiMauro S, Hirano M, Schon EA (eds) Mitochondrial medicine. Informa Healthcare, Abingdon, pp 143–159

Blijlevens NMA, Lutgens LCHW, Schattenberg AVMB, Donnelly JP (2004) Citrulline: a potentially simple quantitative marker of intestinal epithelial damage following myeloablative therapy. Bone Marrow Transplant 34:193–196

Blok RB, Gook DA, Thorburn DR, Dahl H-HM (1997) Skewed segregation of the mtDNA nt 8993 (T→G) mutation in human oocytes. Am J Hum Genet 60:1495–1501

Crenn P, Coudray-Lucas C, Thuillier F, Cynober L, Messing B (2000) Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. Gastroenterology 119:1495–1505

Crenn P, Vahedi K, Lavergne-Slove A, Cynober L, Matuchansky C, Messing B (2003) Plasma citrulline: a marker of enterocyte mass

in villous atrophy-associated small bowel disease. Gastroenterology 124:1210–1219
Darin N, Oldfors A, Moslemi AR, Holme E, Tulinius M (2001) The incidence of mitochondrial encephalomyopathies in childhood: clinical features and morphological, biochemical, and DNA anbormalities. Ann Neurol 49(3):377–383
Debray FG, Lambert M, Allard P, Mitchell GA (2010) Low citrulline in Leigh disease: still a biomarker of maternally inherited Leigh syndrome. J Child Neurol 25(8):1000–1002
Degoul F, Francois D, Diry M et al (1997) A near homoplasmic T8993G mtDNA mutation in a patient with atypic Leigh syndrome not present in the mother's tissues. J Inherit Metab Dis 20(1):49–53
Elliot KRF, Tipton KF (1974) Product inhibition studies on bovine liver carbamoyl phosphate synthetase. Biochem J 141:817–824
Enns GM, Bai RK, Beck AE, Wong LJ (2006) Molecular-clinical correlations in a family with variable tissue mitochondrial DNA T8993G mutant load. Mol Genet Metab 88(4):364–371
Hauser N (2014) Persistent elevations in C3 and C5OH, heralding signs for an underlying mitochondrial defect: neurogenic weakness with ataxia and retinitis pigmentosa (NARP). 64th Annual meeting of the American Society of Human Genetics, San Diego
Henriques M, Diogo L, Garcia P, Pratas J, Simoes M, Grazina M (2012) Mitochondrial DNA 8993T>G mutation in a child with ornithine transcarbamylase deficiency and Leigh syndrome: an unexpected association. J Child Neurol 27(8):1059–1061
Huntsman RJ, Sinclair DB, Bhargava R, Chan A (2005) Atypical presentations of Leigh syndrome: a case series and review. Pediatr Neurol 32(5):334–340
Ishigami A, Ohsawab T, Asagab H, Akiyama K, Kuramotoa M, Maruyamaa N (2002) Human peptidylarginine deiminase type II: molecular cloning, gene organization, and expression in human skin. Arch Biochem Biophys 407(1):25–31
Ishiyama N, Bates IR, Hill CM et al (2001) The effects of deimination of myelin basic protein on structures formed by its interaction with phosphoinositide-containing lipid monolayers. J Struct Biol 136(1):30–45
Kucharczyk R, Zick M, Bietenhader M et al (2009) Mitochondrial ATP synthase disorders: molecular mechanisms and the quest for curative therapeutic approaches. Biochim Biophys Acta 1793 (1):186–199
Kumakura A, Asada J, Okumura R, Fujisawa I, Hata D (2009) Diffusion-weighted imaging in preclinical Leigh syndrome. Pediatr Neurol 41(4):309–311
Lake NJ, Compton AG, Rahman S, Thorburn DR (2015) Leigh syndrome: one disorder, more than 75 monogenic causes. Ann Neurol. doi:10.1002/ana.24551
Leigh D (1951) Subacute necrotizing encephalomyelopathy in an infant. J Neurol Neurosurg Psychiatry 14:216–221
Lutgens LCHW, Deutz NEP, Gueulette J et al (2003) Citrulline: a physiologic marker enabling quantitation and monitoring of epithelial radiation-induced small bowel damage. Int J Radiat Oncol Biol Phys 57:1067–1074
Mock DM (1997) Determinations of biotin in biological fluids. In: McCormick DB, Suttie JW, Wagner C (eds) Methods in enzymology. Academic, New York, pp 265–275
Mori M, Mytingerm JR, Martin LC, Bartholomew D, Hickey S (2014) 8993T>G-Associated Leigh syndrome with hypocitrullinemia on newborn screening. JIMD Rep 17:47–51
Morin C, Mitchell G, Larochelle J et al (1993) Clinical, metabolic, and genetic aspects of cytochrome C oxidase deficiency in Saguenay-Lac-Saint-Jean. Am J Hum Genet 53:488–496
Naini A, Kaufmann P, Shanske S, Engelstad K, De Vivo DC, Schon EA (2005) Hypocitrullinemia in patients with MELAS: an insight into the "MELAS paradox". J Neurol Sci 229–230:187–193
Ostergaard E, Hansen FJ, Sorensen N et al (2007) Mitochondrial encephalomyopathy with elevated methylmalonic acid is caused by *SUCLA2* mutations. Brain 130:853–861
Parfait P, de Lonlay P, von Kleist-Retzow JC et al (1999) The neurogenic weakness, ataxia and retinitis pigmentosa (NARP) syndrome mtDNA mutation (T8993G) triggers muscle ATPase deficiency and hypocitrullinaemia. Eur J Pediatr 158:55–58
Rabier D, Diry C, Rotig A et al (1998) Persistent hypocitrullinaemia as a marker for mtDNA NARP T8993G mutation? J Inherit Metab Dis 21:216–219
Rahman S, Blok RB, Dahl HH et al (1996) Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39:343–351
Ribes A, Riudor E, Valcarel R et al (1993) Pearson syndrome: altered tricarboxylic acid and urea-cycle metabolites, adrenal insufficiency and corneal opacities. J Inherit Metab Dis 16(3):537–540
Said HM, Redah R (1988) Ontogenesis of the intestinal transport of biotin in the rat. Gastroenterology 94(1):68–72
Said HM, Ortiz A, McCloud E, Dyer D, Moyer MP, Rubin S (1998) Biotin uptake by human colonic epithelial NCM460 cells: a carrier-mediated process shared with pantothenic acid. Am J Physiol 275(5):C1365–C1371
Santorelli FM, Shanske S, Macaya A, DeVivo DC, DiMauro S (1993) The mutation at nt 8993 of mitochondrial DNA is a common cause of Leigh's syndrome. Ann Neurol 34:827–834
Sofou K, De Coo IF, Isohanni P et al (2014) A multicenter study on Leigh syndrome: disease course and predictors of survival. Orphanet J Rare Dis 9:52
Spector R, Mock DM (1987) Biotin transport through the blood–brain barrier. J Neurochem 48:400–404
Spector R, Mock DM (1988) Biotin transport and metabolism in the central nervous system. Neurochem Res 13:213–219
Thorburn DR, Rahman S (2014) Mitochondrial DNA-associated Leigh syndrome and NARP. In: Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP (eds) GeneReviews™[Internet]. University of Washington, Seattle, 1993–2013; (updated 2014 Apr 17, http://www.ncbi.nlm.nih.gov/books/NBK1173/)
van Karnebeek CDM, Sly WS, Ross CJ et al (2014) Mitochondrial carbonic anhydrase VA deficiency resulting from CA5A alterations presents with hyperammonemia in early childhood. Am J Hum Genet 94:453–461
White SL, Shanske S, McGill JJ et al (1999a) Mitochondrial DNA mutations at nucleotide 8993 show a lack of tissue or age-related variation. J Inherit Metab Dis 22:899–914
White SL, Collins VR, Wolfe R et al (1999b) Genetic counselling and prenatal diagnosis for the mitochondrial DNA mutations at nucleotide 8993. Am J Hum Genet 65:474–482

JIMD Reports
DOI 10.1007/8904_2017_587

ERRATUM

Erratum: Missed Newborn Screening Case of Carnitine Palmitoyltransferase-II Deficiency

Erratum to: JIMD Reports
DOI: 10.1007/8904_2016_528

Errata to:

Chapter 528: Missed Newborn Screening Case of Carnitine Palmitoyltransferase-II Deficiency, doi: 10.1007/8904_2016_528

Andrew C. Edmondson, Jennifer Salant, Lynne A. Ierardi-Curto, and Can Ficicioglu

The print and online versions of this chapter contain some of the errors, and the corrections are given below:

Figures were reanalyzed and interpreted with the guidance of Piero Rinaldo MD, PhD. The result, tables, and discussion points also were modified based on reanalysis of figures.

Case report

Line 15: *The following sentence added*

Retrospective evaluation of the NBS results obtained on day-of-life 2 revealed the acylcarnitine profile also shown in Table 1.

Discussion

Line 2: Reference changed to McHugh et al. 2011
Line 3: Reference added (Gempel et al. 2002)
Line 6: *Modified paragraph based on reanalysis of figures*.

Our patient's (C16 + C18:1)/C2 ratio of 0.34 was indeed below the 95th percentile of the reference range for DBSs reported in that paper (0.37). We also analyzed our patient's NBS acylcarnitine profile with the productivity and post-analytical interpretive tools of the Region 4 Stork (R4S) collaborative laboratory quality improvement of newborn screening by tandem mass spectrometry (https://www.clir-r4s.org/) (Marquardt et al. 2012; Hall et al. 2014). This produced the results presented in Fig. 1: (1) our patient's (C16 + C18:1)/C2 ratio was barely above the 99th percentile of the R4S cumulative reference range (0.33) but did not reach the lowest level (0.38) needed to contribute a score; (2) The overall case score was zero, but it was so because of an existing rule that forces a zero score whenever the C16 value is below the 90th percentile of the reference range ($3.44 < 4.17$ μmol/L). Because of this rule, which was deemed adequate based on the analysis of 110 other cases, the R4S tool would have generated a zero score both at the time of birth and 4 years later; (3) Once the rule was modified in light of this case and propionylcarnitine was upgraded to informative marker, the overall profile would generate an informative score driven by a low concentration of propionylcarnitine and four new ratios (Fig. 2). In this case, either reliance on cutoff values or the R4S tool would have failed to detect CPT-II deficiency. On the other hand, this false negative event also represents an opportunity for continuing and evolving clinical validation, as similar cases could be detected by the revised tool.

Line 51: *The following sentence added*

To test this hypothesis, three new ratios have already been added to the CPT-II tool in the new version of R4S named CLIR (Collaborative Laboratory Integrated Reports; https://clir.mayo.edu) (Fig. 2).

Table 1 modified and Table 2 deleted

Figure 1 modified and Figure 2 added

Figure 1 Legend

Partial display of the post-analytical interpretive tool for CACT/CPT-II from the Region 4 Stork (R4S)

collaborative laboratory quality improvement of newborn screening by tandem mass spectrometry website (https://www.clir-r4s.org/). The plot by condition panel is an overlay graph where for each marker and ratio the reference population, disease range and the case value are shown. All values are expressed as μmol/L and converted to multiple of the normal median on a log scale. Abbreviations are as follows: *RR* reference range, *NI* not informative, *X* potentially informative score rejected by the C16 rule. Tool accessed on October 23, 2015, reproduced with permission

Figure 2 Legend

Partial display of the post-analytical interpretive tool for CACT/CPT-II from the Collaborative Laboratory Integrated Reports (CLIR) website (https://clir.mayo.edu). See legend of Fig. 1 for details. Additional abbreviation is as follows: *I* informative score. Tool accessed on July 15, 2016, reproduced with permission

The updated original online versions of the original chapters can be found under DOI 10.1007/8904_2016_528

Acknowledgements

The following sentence added:

The authors also thank Piero Rinaldo, MD, PhD, Mayo Clinic, for his assistance in the preparation of the figures and for granting permission to reproduce material from the R4S and CLIR websites.

References

The following references added:

Gempel K, Kiechl S, Hofmann S et al (2002) Screening for carnitine palmitoyltransferase II deficiency by tandem mass spectrometry. J Inherit Metab Dis 25:17–27

Hall PL, Marquardt G, McHugh DMS et al (2014) Post-analytical tools improve performance of newborn screening by tandem mass spectrometry. Genet Med 16:889–895

JIMD Reports
DOI 10.1007/8904_2017_588

ERRATUM

Erratum: Leigh-Like Syndrome Due to Homoplasmic m.8993T>G Variant with Hypocitrullinemia and Unusual Biochemical Features Suggestive of Multiple Carboxylase Deficiency (MCD)

Erratum to: JIMD Reports
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Errata to:

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The publisher regrets to inform that one of the author name was incorrect. The correct name is C.D. van Karnebeek (wrong: C.D. van Karneebek).

The updated original online versions of the original chapters can be found under DOI 10.1007/8904_2016_559