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JIMD Reports

Volume 31

SSIEM

Springer

JIMD Reports
Volume 31

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JIMD Reports
Volume 31

Springer

SSIEM

ISSN 2192-8304 ISSN 2192-8312 (electronic)
JIMD Reports
ISBN 978-3-662-54118-0 ISBN 978-3-662-54119-7 (eBook)
DOI 10.1007/978-3-662-54119-7

This Springer imprint is published by Springer Nature
The registered company is Springer-Verlag GmbH Germany
The registered company address is: Heidelberger Platz 3, 14197 Berlin, Germany

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JIMD Reports
DOI 10.1007/8904_2016_545

RESEARCH REPORT

Living with Intoxication-Type Inborn Errors of Metabolism: A Qualitative Analysis of Interviews with Paediatric Patients and Their Parents

Nina A. Zeltner · Markus A. Landolt · Matthias R. Baumgartner · Sarah Lageder · Julia Quitmann · Rachel Sommer · Daniela Karall · Chris Mühlhausen · Andrea Schlune · Sabine Scholl-Bürgi · Martina Huemer

Received: 14 October 2015 / Revised: 6 January 2016 / Accepted: 09 February 2016 / Published online: 13 August 2016

Abstract *Introduction*: Progress in diagnosis and treatment of patients with intoxication-type inborn errors of metabolism (IT-IEM) such as urea cycle disorders, organic acidurias or maple syrup urine disease is resulting in a growing number of long-term survivors. Consequently, health-related quality of life (HrQoL) of patients is increasingly regarded as a meaningful outcome parameter. To develop the first validated, disease-specific HrQoL questionnaire for IT-IEM, patients and parents were interviewed as content experts to identify major physical and psychosocial constraints and resources.

Methods: Focus group interviews with 19 paediatric IT-IEM patients and 26 parents were conducted in four metabolic centres in Austria, Germany and Switzerland. Disease-specific HrQoL categories were established by qualitative content analysis.

Results: Fourteen disease-specific topics related to the three well-established generic HrQoL dimensions of physi-

Communicated by: Pascale de Lonlay

Competing interests: None declared

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cal, mental and social functioning were derived from the interview transcripts. Both patients and parents perceived dietary restrictions and social stigmatisation as major burdens. Dietary restrictions and emotional burdens were more important for young (<8 years) patients, whereas cognition, fatigue and social issues were more relevant to older patients (≥8 years). Treatment-related topics had a significant effect on social and emotional HrQoL.

Discussion: By exploring patients' and parents' perspectives, 14 HrQoL categories were identified. These new categories will allow the development of a disease-specific, standardised questionnaire to assess HrQoL in paediatric IT-IEM patients. Age-appropriate information on the disease and psychosocial support targeted to patients' individual burdens are essential to the delivery of personalised care that takes account of physical, mental and social dimensions of HrQoL.

Introduction

Urea cycle disorders (UCD), organic acidurias (OA) and maple syrup urine disease (MSUD) are intoxication-type inborn errors of metabolism (IT-IEM) sharing main clinical and treatment characteristics. Patients must follow a low-protein diet, have to cope with the constant fear of life-threatening metabolic crises and frequently develop neurocognitive impairments.

Considerable progress in recent decades in the diagnosis and treatment of IT-IEM has resulted in a growing number of long-term survivors (de Baulny et al. 2005; Batshaw et al. 2014). The long-term medical care and support of patients and families requires insight into subjective psychosocial conditions and health-related quality of life (HrQoL) (Bullinger 2002). HrQoL is a multidimensional construct that represents "a patient's perception of the impact of disease and treatment on functioning in a variety of dimensions, including physical, psychological and social domains" (Varni et al. 1999, p. 126). As such, it is a meaningful outcome parameter for clinical trials and the evaluation of the quality and cost-effectiveness of treatment (Bullinger 2002). Three general types of HrQoL assessment measures exist. Generic HrQoL instruments (e.g. the PedsQL (Varni et al. 1999)) compare HrQoL between healthy individuals and patients, chronic generic instruments (e.g. DISABKIDS (The DISABKIDS Group Europe 2006)) serve to assess and compare HrQoL in patients with chronic diseases in general and disease-specific instruments (e.g. the PKU-QOL (Regnault et al. 2015)) address the characteristics of a particular disease or disease group. The latter are therefore the method of choice in clinical trials (Walterfang et al. 2013). Since the concept of HrQoL is based on subjective experience, self-assessment using age-appropriate instruments is the gold standard (Matza et al. 2013). Self and proxy reports are not interchangeable (Upton et al. 2008), but proxy reports are valuable in very young or cognitively impaired patients.

Although interest in psychosocial issues in IT-IEM has recently increased, research is still sparse and has methodological shortcomings (Zeltner et al. 2014). The first disease-specific assessment instrument for individuals with IT-IEM is currently under development by our research group. In this process, patients and parents are involved as "content experts" (Matza et al. 2004). Focus group interviews are the method of choice to identify topics, concerns and resources relevant in everyday life (Matza et al. 2013). This paper presents the qualitative content analysis of focus group and single interviews with IT-IEM patients and their parents. The two main aims of this study were (1) to identify HrQoL topics relevant for paediatric IT-IEM patients and (2) to investigate differences in statement frequencies of topics related to the informant (patient or parent) and to the age of the affected child.

Methods

Subject Recruitment

Physicians from four metabolic centres in Austria, Germany and Switzerland invited IT-IEM patients (≤18 years) and their parents by phone or during routine consultations for participation. If patients were younger than 8 years or unable to participate due to neurocognitive impairment, only their parents were invited.

Focus Groups and Single Interviews

Focus group interviews were conducted by a team of trained moderators with medical or psychological background in each centre. For patients and parents unable to attend, single interviews were arranged either in the hospital, by phone or at home. Focus groups of two to seven participants were composed based on the patient's age (<8 years, 8–12 years, 13–18 years). Patients younger than 8 years were represented by their parents. Parallel patients' and parents' focus groups were conducted for 8–12- and 13–18-year-olds. Each group was led by two trained moderators, who followed a manual specifically adapted for IT-IEM and based on the DISABKIDS methodology (The DISABKIDS Group Europe 2006). Single interviews were conducted by one moderator following the manual. The focus group procedure is presented diagrammatically in Fig. 1.

Data Processing and Analysis

Interviews were transcribed verbatim in German from audio recordings using transcription software (f4, 2014, Dr. Dresing & Pehl GmbH, Marburg, Germany). Statements used in the

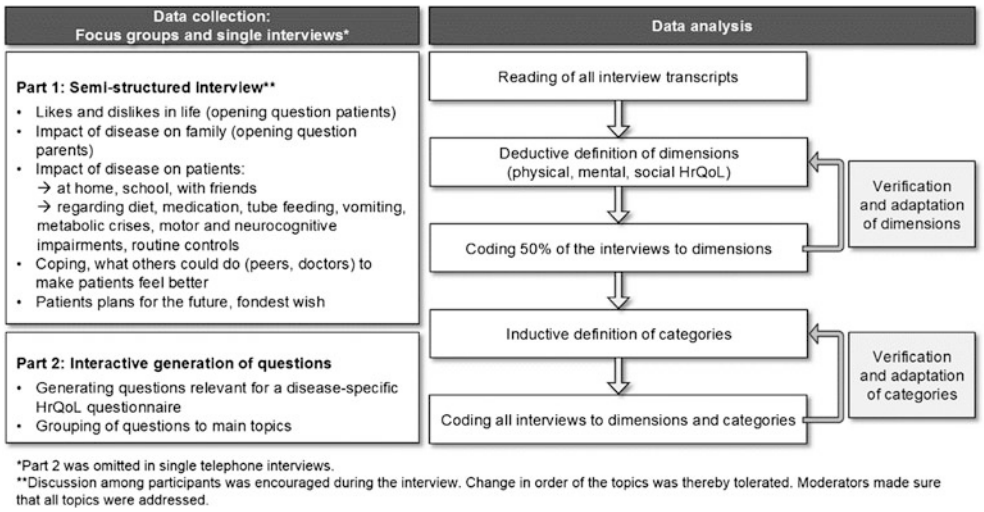

Fig. 1 Content of interviews based on the developed manual and content analysis based on Mayring (2010) and Kuckartz (2014)

result section of this paper were translated to English by a professional interpreter. Two coders (N.A.Z., S.L.) analysed the interviews according to the established qualitative data analysis procedure developed by Mayring (Mayring 2010; Kuckartz 2014) using the MAXQDA software (MAXQDA, version 11, 1989–2015, VERBI Software – Consult – Sozialforschung GmbH, Berlin, Germany).

Statements identified in the transcripts were assigned to the three HrQoL core dimensions *physical*, *mental* and *social* HrQoL (World Health Organisation 1947). Based on these core dimensions, categories were inductively defined. The system of categories was elaborated in several analytical cycles (Fig. 1). Coding disagreements were resolved by discussion. Two psychologists not otherwise involved in the study assigned 42 randomly chosen statements to the 14 categories. Inter-rater reliability (Cohen 1988) was "almost perfect" for one (Cohen's kappa = 0.94) and "substantial" for the second rater (Cohen's kappa = 0.79) (Landis and Koch 1977).

Educational status of parents was assessed using the ISCED Manual (OECD 1999) which differentiates seven internationally comparable levels. Sex distribution among age groups was examined by fisher's exact tests. Chi-square tests and standardised residuals were used to detect differences of statement frequencies per category between informant groups (patients/parents) and patients' age groups. Issues mentioned repeatedly by single participants resulted in multiple coded statements; length of statements was not considered. Stepwise comparisons were calculated between core dimensions, grouped categories and single categories (see Table 2). Analyses were performed using the statistical software package SPSS, version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

Results

Sample Characteristics

Thirty (71%) of 42 invited families participated, resulting in a study sample of 19 children and adolescents ($n = 9$ females, $n = 10$ males; $n = 9$ OA, $n = 9$ UCD, $n = 1$ MSUD; age range 9.5–16.5 years, mean = 13.0 ± 2.3 years) and 26 parents ($n = 25$ mothers, $n = 1$ father). All parents had at least one child affected by IT-IEM ($n = 12$ females, $n = 16$ males (from which $n = 2$ siblings); $n = 18$ OA, $n = 8$ UCD; patients' age range 0.9–16.8 years, mean = 10.3 ± 4.7 years) (see Table 1). Educational status of parents (mean of fathers' and mothers' educational status) was mean = 3.64 (±1.12, range 2–6). Distribution

Table 1 Number of interview participants in focus groups and single interviews

	Patient's age			
	<8 years	8–12 years	13–18 years	All age groups
Number of patients	–	9	10	19
Number of parents	9	9	8	26
Number of patients in focus group interviews	–	6	8	14
Number of patients in single interviews	–	3	2	5
Number of parents in focus group interviews	8	8	7	23
Number of parents in single interviews	1	1	1	3
Total participants	9	18	18	45

Table 2 System of disease-specific HrQoL categories based on content analysis of the interviews

Dimension	Group	Category	Description of category
Physical	Symptoms	Metabolic crises/ anticipation of crises	Experiencing metabolic crises, anticipation of crises (i.e. infections, hygiene), emergency admissions because of crisis
		Physical limitations	Motor limitations, organ problems
		Fatigue/nausea/vomiting	Fatigue, nausea and vomiting (events not immediately connected to metabolic crisis and emergency admissions)
	Treatment	Dietary restrictions	Dietary restrictions, regular food intake, low-protein food
		Medication/dietetics	Drug and dietetics intake, side effects
		Tube feeding	PEG tube, nasogastric tube
		Routine controls/ hospital	Routine controls in the hospital/by the physician, reactions to blood sampling
Mental	Emotions	Negative emotions	Statements with negative emotional content. Sadness, anger, crying, feeling different than friends, shame (not wanting to make the condition public), wanting the disease gone
		Positive emotions	Statements with positive emotional content. Being happy, satisfied, feeling good and "normal" with the condition
	Cognitive functioning	Cognitive functioning	Cognitive functioning, especially in school, disturbed concentration, mental disability
	Independent living	Independent living	Feeling restricted in conduct of life, choice of employment, planning of leisure time
Social	Social life, social support	Friends	Having friends, social support/social inclusion by friends
		Family	Statements concerning the family (parents, siblings, grandparents, uncles, aunts) and their handling of the condition
	Stigmatisation/exclusion	Stigmatisation/exclusion	Being treated differently (negative/positive) by peers and society based on the condition: teasing, interrogation regarding condition, being pitied, exclusion (i.e. difficulties finding a school, going on school excursions)

of patients' sex among age groups did not significantly differ for patients ($p = 0.18$) or parents ($p = 0.09$).

Categorical System

A total of 915 statements addressing patients' HrQoL were identified from 19 transcripts of five patient and six parent focus groups and five patient and three parent single interviews. Fourteen content categories were defined and allocated to one of the core dimensions (physical, mental and social HrQoL) (Table 2). Frequencies of statements per category for patients and parents are shown in Fig. 2.

Physical Dimension of HrQoL

Symptoms and treatment of the disease relate to the physical dimension of HrQoL. *Dietary restrictions* (intake of regular food and special low-protein food) were a main topic for both patients and parents (Fig. 2). However, attitudes varied widely from "being used to dietary restrictions" or even having an "aversion to proteins" to complaints about "missing forbidden food and freedom of food choice" and "required organisational effort". The latter were especially encountered in social situations such as barbecues or school camps. A 16-year-old girl commented: "When I go to school camp, I have to take a second suitcase along. It's all food. And a woman cooks especially for me".

According to parents, pre-schoolers did not experience diet as particularly burdensome. In contrast, parents experienced caring for a young child's diet as most difficult and stressful. They felt disburdened when their child was able to take over responsibility by self-monitoring or indicating need for their sick-day regimen. The mother of a 5-year-old girl stated: "When she was two or three years old, she would often snatch a piece of sausage and put it into her mouth (. . .). By now I know that she won't do this anymore, that's something I can rely on". However, diet places an incessant strain on family life. Parents felt children used food intake or vomiting to exert pressure or gain attention, as explained by the mother of a 16-year-old: "That's always some form of pressure of hers, although she often isn't able to express herself that well; but with food she's totally in control of me and that she knows very well". Against this background, parents considered tube feeding supportive, since arguing about food intake became less central: "As soon as he had received the PEG, we were happy. We were no longer at the hospital, the pressure

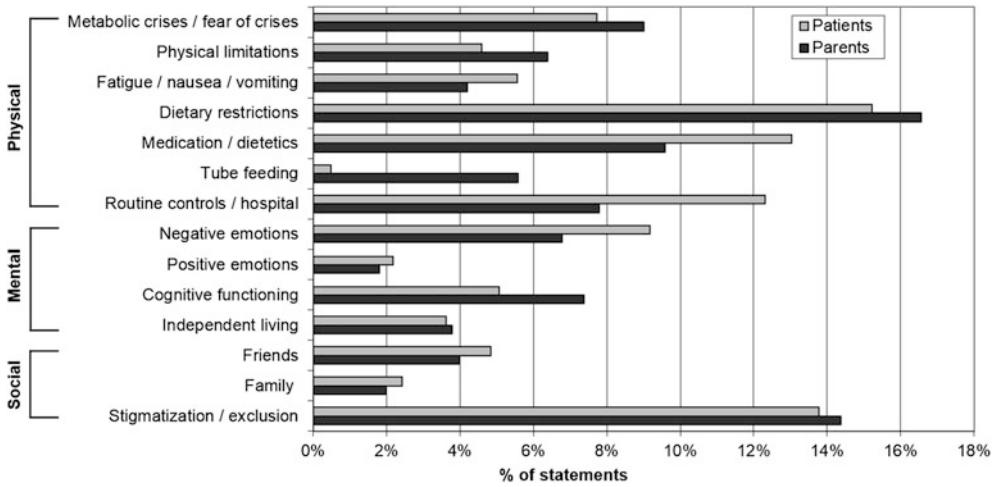

Fig. 2 Percentage of statements from total statements per category, for each patient and parent interviews (both for the age of 8–18 years)

on me was gone, I was no longer under pressure" (mother of a 13-year-old). In contrast, patients may hide their tube: "Absolutely nobody is allowed to see it [the tube] (. . .) and nobody is allowed to know about it, except close relatives and friends" (mother of a 10-year-old).

Although intake of dietetics and medication was a daily routine, the unpleasant taste of amino acid mixtures remained a major issue, and patients preferred pills over drinks whenever possible. Moreover, the lack of flexibility allowed by a tightly scheduled intake of medication and supplement interfered with everyday life and spontaneous engagement in social situations. Side effects such as diarrhoea or body odour, namely, the fishy odour of carnitine, were additional constraints in social situations.

The majority of patients felt blood sampling (category *routine controls/hospital*) to be extremely stressful and commented more frequently on this subject than parents (not statistically significant). Adolescents coped better with blood sampling but complained that hospital appointments reduced their leisure time.

Motor deficits and limitations in keeping up with peers were the most burdensome limitations for children with IT-IEM. One of the fondest wishes expressed by non-ambulatory children younger than 8 years was "being able to walk and run like others". *Fatigue/nausea/vomiting* were especially important for older patients comparing their performance with healthy peers'. "Because when I overdo it, I have to throw up. I simply don't manage as much as all the others" (16-year-old male patient working as apprentice).

Metabolic crises were traumatising experiences and caused constant fear of potential new crises, infections and other triggers. Most patients had experienced metabolic crises during infancy, and it was mainly their parents who remembered the details, as the father of a 7-year-old stated: "We had five crises with him, ended up five times in intensive care. Once we were abroad and the physicians said: You know, we can't do anything, the boy will die". The mother of a 16-year-old said: "Gastro-intestinal flu: all alarm bells ring, (. . .) this is a one-way ticket to hospital". Parents and patients reported always planning their annual leave in proximity to metabolic hospitals they trust to avoid encountering medical staff unfamiliar with the disease.

Comparing Frequencies of Statements

The frequency distribution of patients' and parents' statements significantly differed among the categories associated with treatment ($\chi^2(3) = 25.24,\ p < 0.05$). Patients mentioned *tube feeding* significantly less frequently than parents.

Comparison of patients' reports revealed a significant age difference between symptom and treatment statements ($\chi^2(2) = 11.44,\ p < 0.05$). Patients aged 8 to 12 made more statements about treatment, while 13–18-year-olds talked more frequently about symptoms and limitations. Comparing categories describing symptoms revealed that, in contrast to parents of older children, parents of patients younger than 8 years made no statements related to *fatigue/nausea/vomiting* ($\chi^2(4) = 15.18,\ p < 0.05$). In the categories describing treatment, parents of younger children talked more frequently about *dietary restrictions* and less frequently about *medication/dietetics* than parents of older children and adolescents ($\chi^2(6) = 22.19,\ p < 0.05$).

All other comparisons in the physical dimension between different age and respondent groups were not significant.

Mental Dimension of HrQoL

The mental dimension encompasses statements related to emotions, cognition and issues of independent living. *Negative emotions* were predominantly reported. The most frequent "fondest wish" of the patients was "not to have the disease" or to be "more like normal kids". The perception of not

being able "to do what other kids do" caused frustration. The impression of being "different" in some cases led to feelings of shame, making patients averse to others being informed about their condition. Since having a meal together is of high social significance, IT-IEM patients often felt socially excluded. The mother of a 7-year-old reported: "The kids [at Kindergarten] were not allowed to share [food] with him. This troubled him a lot, the fact that he now suddenly did not belong to the group. He cried bitterly then".

Cognitive functioning and school performance gained increasing significance for older children and adolescents. Children attending regular schools felt inferior when comparing their school performance with those of their healthy peers. Parents of children with severe cognitive impairment attending special schools worried about their children's performance but had the impression that the children themselves did not.

Comparing Frequencies of Statements

Parents' statements regarding *emotions*, *cognitive functioning* and *independent living* significantly differed between age groups ($\chi^2(2) = 29.70$, $p < 0.05$). In the younger age groups, more statements addressed *emotions*, while *cognitive functioning* was more important to the 13–18 years group.

All other comparisons in the mental dimension were not significant.

Social Dimensions of HrQoL

The social dimension refers to the impact of the disease on social life and stigmatisation experiences. Patients and parents generally felt supported by family and close friends. The second highest number of statements was attributed to the *social stigmatisation/exclusion* category (Fig. 2).

Peers teased patients because of physical or cognitive limitations or side effects of medication. A 9-year-old boy reported: "Actually, my disease is okay, except that some kids make fun of it (...). The medication smells of fish, so they call me fishhead and say that I stink of fish".

Patients of all age groups considered it difficult to explain their condition in a comprehensible way. "Well, my problem is simply that they keep asking: Why do you have that thing? And I don't know exactly what I'm supposed to answer" (10-year-old patient). Because of her strict diet, a 16-year-old girl was referred to as "the pickiest person we know" by her schoolmates. "I try to explain to them what it is about. But some of them just don't want to understand". Like their children, parents considered it almost impossible to explain the complex, rare disease to others in an understandable way. They felt frustrated because they perceived that their environment had no understanding of their child's condition. Criticism of or interference with parenting style (e.g. comments that mothers were "too strict" about diet or offers of inappropriate meals) was experienced as stressful. Attempts to avoid social exclusion, for instance, from school camps, exerted intense stress on families (escorting the child, precooking meals). Finding a sensitive social environment was considered of the utmost importance.

Comparing Frequencies of Statements

Compared to 8–12-year-old patients, 13–18-year-old patients commented less on *social life* and *social support* ($\chi^2(1) = 11.53$, $p < 0.05$).

All other comparisons in the social dimension were not significant.

Discussion

This study explored how IT-IEM affects daily life and identified 14 categories relevant for patients' HrQoL. The evolving categories could coherently be allocated to the core dimensions of *physical*, *mental* and *social* HrQoL, resulting in a structure consistent with well-established generic and chronic disease HrQoL questionnaires (Varni et al. 1999; The DISABKIDS Group Europe 2006; The KIDSCREEN Group Europe 2006). Categories within the *social* and *emotional* dimensions are similar to other questionnaires (Vogels et al. 1999; The KIDSCREEN Group Europe 2006), while categories within the *physical* dimension mostly reflect disease-specific issues. Notably, other questionnaires refer to more than three dimensions (e.g. Varni et al. 1999), while this study kept to the three core dimensions represented in almost all HrQoL questionnaires (Rajmil et al. 2004).

It is an important strength of this study that the individual perspectives of patients were considered and that the proxy reports from parents allowed insight into perspectives of young and/or cognitively impaired patients. Despite international cooperation, owing to the rarity of the diseases, the sample size is limited and patient recruitment was not free of selection bias.

Analyses of group differences demonstrated that the importance of specific HrQoL-related topics varied depending on the patients' age and between parents and patients. Diet and emotional contents were predominant when patients were young (<8 years), and both patients and parents had to build up knowledge and routine concerning food choice and preparation and intake of medication and dietetics. Furthermore, hospital stays were emotionally burdensome for young children, and families had to deal with uncertainty related to disease progression, neuro-

cognitive development and upcoming metabolic crises. Dealing with diet and uncertainty has been reported to be major burdens for parents with children with metabolic diseases before (Cederbaum et al. 2001; Pelentsov et al. 2015). As coping strategies significantly influence HrQoL in different chronic diseases (Graven and Grant 2013), tailored support may be helpful. Our results suggest that families with young children in particular would benefit from support targeting these uncertainties and coping with diagnosis and treatment. In addition, immediately at diagnosis, patients should be informed about support groups, which have been shown to be important informational resources in rare disorders (Hall 2013; Khangura et al. 2015). Accordingly, the majority of participants in this study greatly appreciated the exchange with others in the context of the interviews and expressed their wish to have this opportunity more frequently. Unfortunately, research shows that few families with children with rare diseases receive psychosocial support at the time of diagnosis (Anderson et al. 2013).

In this study, cognitive functioning and feeling fatigue or less productive gained significance for school-aged and adolescent patients, along with the increasing importance of school performance and social comparison with peers. Importantly, adolescents reported less frequently about social support than younger patients but still encountered experiences of stigmatisation, resulting in an imbalance of constraints and resources. Our results thereby suggest that transitional phases (to school, to adolescence), often combined with social and physical challenges, deserve special attention, as this has been reported before for other inborn errors of metabolism (Packman et al. 2012; Khangura et al. 2015). Sharing meals with others is known to have high social significance; hence, patients living with dietary restrictions experience numerous potentially stigmatising situations (Diesen et al. 2014). Since perceived stigmatisation is a predictor for poorer psychological adjustment (Masnari et al. 2013), it is of great importance that patients are supported in so-called stigma-handling strategies (Diesen et al. 2014), such as social skills training or school-based interventions, in which peers are provided with basic information about the condition, resulting in better understanding (Sharman et al. 2013).

In contrast to parents, patients talked reluctantly about tube feeding. Content of statements indicates tube feeding meant considerable relief for parents in terms of reduced worries and struggles with children about food intake. Consistent with this, previous research showed that tube feeding has a positive impact on parental HrQoL (Fabre et al. 2013). In contrast, feelings of shame and embarrassment seemed to be a reason why patients did not want to talk about their tube in the focus groups – which goes along with the psychosocial impact of tube feeding mentioned in the literature (Burmucic et al. 2006). We are aware of the limitation that the perspectives of very young and/or severely compromised patients on this issue have not been included in this study.

Based on the results of the focus groups, two main clinical implications can be drawn. Firstly, patients with IT-IEM and their parents have significant need for comprehensible explanations and information concerning their disease. Both patients and parents reported difficulties in making the condition understandable to the social environment. For other diseases (e.g. diabetes type 1), this need has been met by age-appropriate, verbal information from medical professionals and attractive paper- or IT-based materials (Murphy et al. 2007; Årsand et al. 2012). Improved understanding of the disease may motivate patients to reach better compliance (Sharman et al. 2013). Being able to explain the disease may help patients and parents to cope better with stigmatising social situations such as the often-experienced exclusion from school or leisure activities. In addition, low-threshold access to information about IT-IEM (e.g. informative homepages, guidelines) and metabolic expert advice would help physicians who are not specialised in the metabolic field in their communication with families.

Secondly, beyond essential medical treatment, psychosocial care should be offered to IT-IEM patients and parents. It has been shown that a child's well-being is considerably influenced by family variables (Landolt et al. 2002), and although the interviews in this study focused on the well-being of patients, parents spontaneously reported high levels of distress, which require appropriate support.

Remarkably, of the 26 participating parents, only one was male. This highly unequal gender distribution narrows the breadth of the parent perspective but seems to reflect real-life patterns. In many families, it is still mothers who are the primary health carer. In their traditional role as major income earner of the family, fathers are less present in the hospital setting and take other functional roles in caring for the child, such as seeking information (Yeh 2004). The underrepresentation of fathers in research focusing on children with chronic diseases is a common fact that it is important to note (Goldstein et al. 2013).

Studies in other diseases clearly revealed that physicians underestimate the impact of disease and treatment on emotional and social domains (Srikrishna et al. 2009). Treatment issues were a main concern for patients in this study because they hampered everyday life and made the disease perceivable for others. Thus, the fondest wishes of the patients to "be normal" and "not have the disease" should motivate physicians and dieticians to consider even more highly that the most feasible and practical treatment protocol serves patients and families best. Side effects of medication, such as an unpleasant smell, diarrhoea and

frequent intake of disliked amino acid mixtures, may impair emotional and social well-being more than expected. Additionally, fear of blood sampling made hospital appointments extremely stressful for many patients. It is well established that, besides local anaesthetics, psychological techniques (e.g. relaxation techniques, developmentally appropriate distraction) are highly effective in reducing pain and anticipatory fear (Duff 2003). It has been shown in other chronic paediatric diseases that addressing social issues and emotional distress during regular hospital follow-ups is supportive and that the use of HrQoL questionnaires (Santana and Feeny 2013) significantly increases meaningful patient-physician communication and patient well-being (Velikova et al. 2004).

Conclusion

The categories identified describe HrQoL of IT-IEM patients and will allow the construction of a disease-specific HrQoL questionnaire. Care for IT-IEM patients can be improved by providing appropriate information about the disease and individualised psychosocial support to ameliorate the multifaceted effects of the disease on physical, mental and social well-being.

Acknowledgements We thank all patients and parents who participated in the study and shared their experiences of living with IT-IEM. We are indebted to Prof. Monika Bullinger, Hamburg, for her valuable input regarding the design of the study. Furthermore, we gratefully acknowledge all colleagues involved as focus group moderators, during transcription and inter-rater reliability testing: Tilla Aegerter, Michael Ertl, Anna Giammarco, Ann-Christin Haag, Ornella Masnari, Miriam Michel, Katharina Nitsche, Corinne Pellegrino and Sabine Weber.

The study was supported by radiz – Clinical Research Priority Program for Rare Diseases from the University of Zurich – and by Milupa Metabolics, Friedrichsdorf, Germany.

Sentence Take-Home Message

Taking patients' and parents' perspectives into account allows the definition of meaningful follow-up parameters and the development of personalised interventions.

Compliance with Ethical Guidelines

Conflict of Interest

Nina A. Zeltner and Martina Huemer have received research grants from Milupa Metabolics. Markus A. Landolt, Matthias R. Baumgartner, Sarah Lageder, Julia Quitmann, Rachel Sommer, Daniela Karall, Chris Mühlhausen, Andrea Schlune and Sabine Scholl-Bürgi declare that they have no conflict of interest.

Informed Consent

All procedures were followed in accordance with the Helsinki Declaration of 1975, as revised in 2000 and approved by the ethical committee in Zurich, Switzerland (KEK-ZH Nr. 2012.0020), and the local ethical committees in Hamburg, Düsseldorf and Innsbruck. Informed consent was obtained from all participants/their legal representatives to be included in the study.

Details of the Contributions of Individual Authors

N.A.Z. was involved in designing the study, collected and analysed the data and drafted the manuscript. M.A.L. was involved in designing the study, gave advice on data collection and analysis and critically reviewed the manuscript. M.R.B. was involved in designing the study, contributed patient data and critically reviewed the manuscript. S.L. assisted in collecting and analysing the data. J. Q. was involved in designing the study. R.S. was involved in designing the study and collecting the data. C.M., A.S., D.K. and S.S. contributed patient data. M.H. provided the original concept of the study, coordinated the study and revised the manuscript. All authors read and approved the final version of the manuscript.

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JIMD Reports
DOI 10.1007/8904_2016_551

CASE REPORT

Switch from Sodium Phenylbutyrate to Glycerol Phenylbutyrate Improved Metabolic Stability in an Adolescent with Ornithine Transcarbamylase Deficiency

Alexander Laemmle • Tamar Stricker • Johannes Häberle

Received: 11 January 2016 / Revised: 25 February 2016 / Accepted: 29 February 2016 / Published online: 22 March 2016

Abstract A male patient, born in 1999, was diagnosed with ornithine transcarbamylase deficiency as neonate and was managed with a strict low-protein diet supplemented with essential amino acids, L-citrulline, and L-arginine as well as sodium benzoate. He had an extensive history of hospitalizations for hyperammonemic crises throughout childhood and early adolescence, which continued after the addition of sodium phenylbutyrate in 2009. In December 2013 he was switched to glycerol phenylbutyrate, and his metabolic stability was greatly improved over the following 7 months prior to liver transplant.

Introduction

Urea cycle disorders (UCDs) are inherited enzyme and transporter deficiencies that impair urea cycle function and result in the accumulation of toxic levels of ammonia in the blood and brain (Brusilow and Maestri 1996). Hyperammonemia-related neurological injuries range from lethal cerebral edema to mild or subclinical cognitive impairment, depending on the severity of the defect (Gropman et al. 2008), with the most severely affected patients typically presenting early in life (Brusilow and Maestri 1996; Summar et al. 2008; Tuchman et al. 2008). Ornithine transcarbamylase (OTC) deficiency is the most common UCD, accounting for slightly more than half of all UCD cases (Summar et al. 2008; Tuchman et al. 2008; Batshaw et al. 2014).

Medical management of UCDs is aimed at controlling hyperammonemia by reducing waste nitrogen through the restriction of dietary protein, the use of essential amino acid supplements, as well as the supplementation of L-citrulline and/or L-arginine. Patients whose symptoms cannot be adequately controlled by the abovementioned diet and treatment strategies alone are usually treated with alternative pathway drugs such as sodium benzoate and/or sodium phenylbutyrate (sodium PB) (Häberle et al. 2012). The latter lowers ammonia by enhancing excretion of waste nitrogen in the form of phenylacetylglutamine (Batshaw et al. 1982; Brusilow 1991).

Glycerol phenylbutyrate (GPB; Ravicti®, Horizon Pharmaceuticals, Brisbane, CA) is approved in the USA for the treatment of UCD patients 2 years and older whose ammonia levels cannot be controlled by conventional therapy. GPB has the same mechanism of action as sodium PB, but is a pre-pro drug consisting of phenylbutyric acid (PBA) linked to glycerol. Following hydrolysis by pancreatic lipases, PBA is released and converted to the active moiety, phenylacetic acid (Lee et al. 2010; McGuire et al. 2010; Monteleone et al. 2013). In clinical trials, GPB has been shown to provide effective ammonia control in adult and pediatric UCD patients (Lichter-Konecki et al. 2011; Smith et al. 2013; Diaz et al. 2013; Berry et al. 2014).

Case Report

A 16-year-old male patient was diagnosed with OTC deficiency as a neonate and was hospitalized approximately

Communicated by: Carlo Dionisi-Vici, MD

Competing interests: None declared

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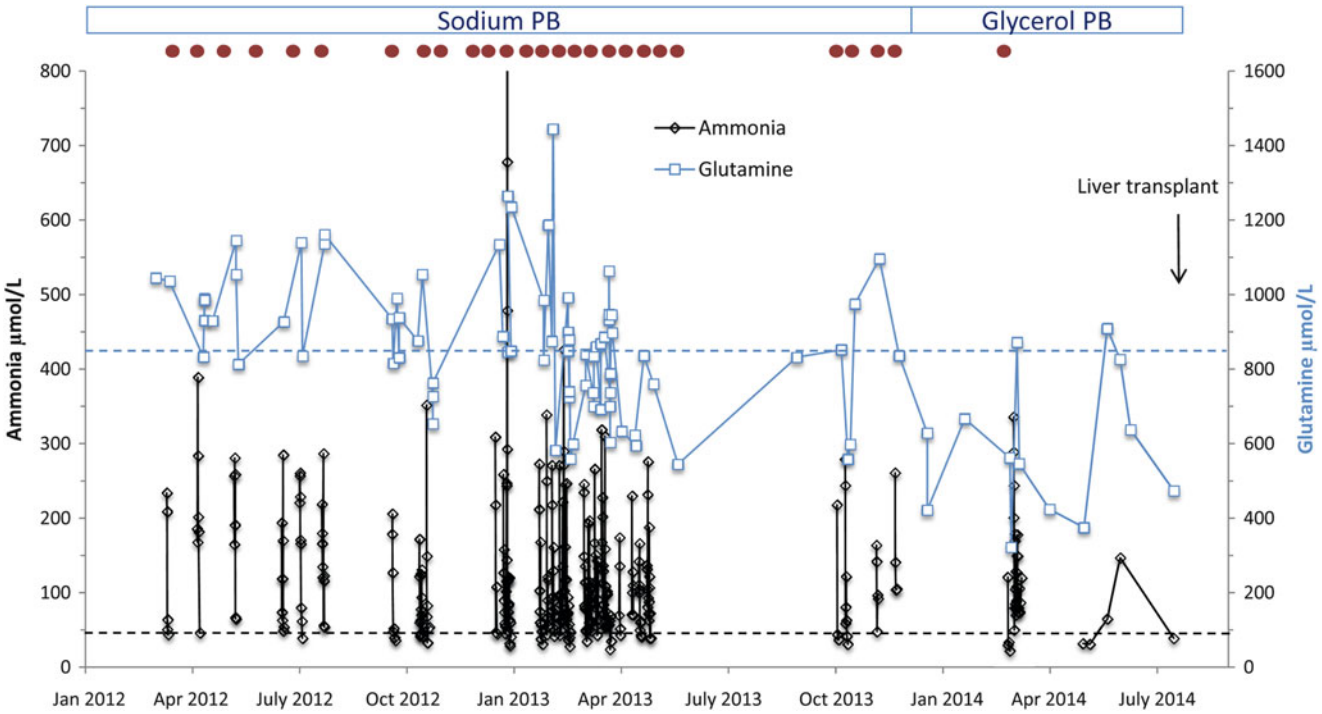

Fig. 1 Plasma glutamine and ammonia and hospitalizations in an adolescent with OTC deficiency. Illustration of main parameters of the course in the adolescent OTC deficient patient from January 2012 to July 2014. During this time, the patient received sodium phenylbutyrate (sodium PB) until he was switched to glycerol phenylbutyrate (glycerol PB) in December 2013. Plasma glutamine (in *blue*) and ammonia (in *black*) levels obtained during the entire period are shown; upper limits of normal are given as *dashed lines* in respective colors. Hospitalizations are indicated as *red dots* at the top of the figure

every 2 months for hyperammonemic crises as an infant. Hospitalizations were less frequent during childhood but still occurred several times a year. This report describes his treatments, hospitalizations, and laboratory results over a 7.5-year period, including the effect of GPB treatment for 7 months prior to liver transplant in 2014.

The period reported here starts in April 2007 (age 8 years and 2 months) when the patient was maintained on a low-protein diet (0.5 g/kg bodyweight (bw)/day) supplemented with essential amino acids (UCD2 and/or E-AM Anamix), L-citrulline (2–3 mmol/kg bw/day), and L-arginine (1.4–2.2 mmol/kg bw/day) and received sodium benzoate (166–311 mg/kg bw/day). In addition, to avoid prolonged fasting, the patient received overnight gastric-tube feeding receiving special protein-free infant formula and essential amino acids. Due to metabolic instability with recurrent hyperammonemic crises and hospitalizations, in June 2009, sodium PB (143–338 mg/kg bw/day) was added, which was replaced with GPB (296 mg/kg bw/day) in December 2013.

The patient was hospitalized for metabolic crises 15 times between April 2007 and May 2009 (7.2 hospitalizations/year, prior to receiving sodium PB) and 39 times between June 2009 and December 2013 (8.7 hospitalizations/year, while receiving sodium PB). The average length of stay was 5.4 days (range 2–27 days). Seven of the crises (four in 2008 and one each in 2007, 2010, and 2012) included 1–3 days in the intensive care unit. Most of the crises were of unknown etiology although six events were associated with infections (two respiratory tract infections, three gastroenteritides, one catheter infection), and three were associated with increased physical activity. Almost all events involved vomiting or nausea, and reduced vigilance was noted in 31 of 54 events (57%). Twenty-six of the hospitalizations occurred during the two calendar years prior to starting GPB (11 in 2012 and 15 in 2013, illustrated in Fig. 1).

Peak ammonia levels during metabolic crises for the period from April 2007 to December 2013 ranged from 81 to 1,106 µmol/L (mean, 261.2 µmol/L; reference range 12–48 µmol/L). Glutamine levels for the same period ranged from 346 to 1,445 µmol/L (mean 899 µmol/L; reference range 457–857 µmol/L). For the 2 years prior to starting GPB (2012 and 2013), mean peak ammonia levels were 359 and 257 µmol/L, respectively, and mean glutamine levels were 960 and 899 µmol/L, respectively (Fig. 1).

After starting GPB, during the first 7 months of 2014, the patient had a single hospitalization. This hospitalization (total duration 11 days) was for a metabolic crisis

associated with partial respiratory insufficiency due to pleuropneumonia. The patient finally received a successful liver transplant in August 2014.

Discussion

GPB is a recently approved treatment for UCD. It has been shown to significantly lower ammonia levels in pediatric UCD patients compared with sodium PB and to be associated with normal glutamine levels and fewer hyperammonemic crises (Berry et al. 2014). The lower ammonia levels likely reflect the slower absorption of PBA from GPB, presumably because GPB requires digestion by pancreatic lipases, compared with sodium PB, which is a salt and is more rapidly absorbed (Smith et al. 2013). The patient described here was diagnosed with OTC deficiency as a neonate and suffered from frequent hyperammonemic crises, many of unknown etiology, throughout his childhood and early adolescence, even after the introduction of sodium PB at 10 years of age. After switching to GPB short before the patient turned 15 years, his metabolic stability was clearly improved, with fewer and less severe hyperammonemic crises and lower glutamine levels. In fact, Fig. 1 shows that most plasma glutamine levels after introduction of GPB were within the reference range. Under GPB treatment for 7 months, there was only one hospitalization with a severe pleuropneumonia. In both years preceding start of GPB treatment, the patient required monthly hospitalizations. Although it cannot be excluded that the improvement was purely spontaneous, this is unlikely given the following considerations: patients with inherited metabolic disorders tend to become unstable during adolescence and numbers of decompensations often increase (MacDonald et al. 2012). Further, the response to the change in drug treatment was rather prompt rendering a purely age-related effect unlikely. Finally, the relatively mild course during the pleuropneumonia episode is highly suggestive for an actual improved metabolic stability.

Moreover, although not specifically evaluated, it was obvious that the significant reduction of metabolic crises and hospitalizations had a positive impact on the patient's and his family's quality of life. It would be of interest in future patients to quantify in more detail the positive effects of GPB treatment, on a biochemical level (plasma ammonia and glutamine) and regarding improvement of quality of life.

We conclude that GPB may be a useful therapeutic option in unstable UCD patients and helped to stabilize our patient until a liver transplant could be performed.

Acknowledgment Glycerol phenylbutyrate (Ravicti®) was provided free of charge on a compassionate use basis by Hyperion Therapeutics. Medical writing assistance was provided by Jacqueline Wu, PhD, with funding provided by Horizon Pharma (current license holder of Ravicti®), but this had no influence on the content of the report or on interpretation of data. Alexander Laemmle was supported by competitive research grants from the EMDO Foundation Zurich (Grant 851 to JH and AL) and by the Children's Research Center – University Children's Hospital Zurich (Grant 10511 to AL) while working on this study. The work on urea cycle disorders is supported by the Swiss National Science Foundation (grant 310030_153196 to JH).

Synopsis

An adolescent with ornithine transcarbamylase deficiency suffering from recurrent hyperammonemic crises showed a significant reduction of plasma ammonia and glutamine levels and a concomitant improvement of metabolic stability when therapy was switched from sodium phenylbutyrate to glycerol phenylbutyrate.

Compliance with Ethics Guidelines

Conflict of Interest

Alexander Laemmle and Tamar Stricker declare that they have no conflict of interest. Johannes Häberle declares the following conflict of interest: at the time of treatment with Ravicti®, he held a "Healthcare Professional Consulting Agreement" with Hyperion Therapeutics, then license holder of Ravicti®. This Consulting Agreement is continued with Horizon Pharma, the current license holder of Ravicti®.

However, all of the authors confirm full independence while treating the patient and while writing this article.

All of the authors participated in planning and performing conception, design, analysis and interpretation of data, drafting, and revising of the article.

Johannes Häberle is the responsible principle investigator and has decided to publish this case report which has not been previously reported elsewhere.

Written informed consent from the patient and the parents to use Ravicti® was obtained. Approval by the responsible ethical committee for using Ravicti® was not required as this was compassionate use.

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JIMD Reports
DOI 10.1007/8904_2016_548

RESEARCH REPORT

Inherited Metabolic Disorders: Efficacy of Enzyme Assays on Dried Blood Spots for the Diagnosis of Lysosomal Storage Disorders

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Received: 30 October 2015 / Revised: 13 February 2016 / Accepted: 16 February 2016 / Published online: 24 March 2016

Abstract High consanguinity rates, poor access to accurate diagnostic tests, and costly therapies are the main causes of increased burden of lysosomal storage disorders (LSDs) in developing countries. Therefore, there is a major unmet need for accurate and economical diagnostic tests to facilitate diagnosis and consideration of therapies before irreversible complications occur. In cross-country study, we utilized dried blood spots (DBS) of 1,033 patients clinically suspected to harbor LSDs for enzymatic diagnosis using modified fluorometric assays from March 2013 through May 2015. Results were validated by demonstrating reproducibility, testing in different sample types (leukocytes/plasma/skin fibroblast), mutation study, or measuring specific biomarkers. Thirty percent (307/1,033) were confirmed to have one of the LSDs tested. Reference intervals established unambiguously identified affected patients. Correlation of DBS results with other biological samples ($n = 172$) and mutation studies ($n = 74$) demonstrated 100% concordance in Gaucher, Fabry, Tay Sachs, Sandhoff, Niemann-Pick, GM1, Neuronal ceroid lipofuscinosis (NCL), Fucosidosis, Mannosidosis, Mucopolysaccharidosis (MPS) II, IIIb, IVa, VI, VII, and I-Cell diseases, and 91.4% and 88% concordance in Pompe and MPS-I, respectively. Gaucher and Pompe are the most common LSDs in India and Pakistan, followed by MPS-I in both India and Sri Lanka. Study demonstrates utility of DBS for reliable diagnosis of LSDs. Diagnostic accuracy (97.6%) confirms veracity of enzyme assays. Adoption of DBS will overcome significant hurdles in blood sample transportation from remote regions. DBS enzymatic and molecular diagnosis should become the standard of care for LSDs to make timely diagnosis, develop personalized treatment/monitoring plan, and facilitate genetic counseling.

Communicated by: Gajja Salomons

Competing interests: None declared

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Abbreviations

CK	Creatine phosphokinase
DBS	Dried blood spot
EQAS	External Quality Assurance Scheme
ERNDIM	European Research Network for evaluation and improvement of screening, diagnosis, and treatment of inborn errors of metabolism
GAGs	Glycosaminoglycans
LSDs	Lysosomal storage disorders
MPS	Mucopolysaccharidosis

Introduction

Enzyme assays have been the gold standard for providing definitive diagnosis of lysosomal storage disorders (LSDs) by demonstrating deficient enzyme activity in leukocytes/plasma/cultured fibroblast. This can be further confirmed

by mutation studies. Interference by isoenzymes in leukocytes and pseudo deficiency states in plasma can lead to misdiagnosis (Burin et al. 2000; Filocamo and Morrone 2011), whereas fibroblasts require an invasive skin biopsy and a long waiting period for the culture to obtain an adequate number of cells for testing (Coelho and Guigliani 2000). To overcome these limitations, enzyme estimation on dried blood spots (DBS) has been introduced in recent years (Civallero et al. 2006; Ceci et al. 2011). Although initiated for purpose of screening in newborns (Chamoles et al. 2001), DBS technique has now been adopted for early diagnosis of LSDs due to the requirement of a few drops of blood, easy transportation especially from remote areas and simultaneous measurement of multiple analytes without special storage requirements (Muller et al. 2010). Specific therapeutic intervention is available for MPS (I, II, IVa, and VI), Fabry, Pompe, and Gaucher, however, accurate and timely diagnosis is crucial for maximizing the benefit (Camelier et al. 2011; Kaminsky and Lidove 2014).

The burden of LSDs in India is severe because of large population, high consanguinity rate, lack of awareness and expertise, and limited genetic testing centers for providing correct diagnosis (Verma et al. 2012; Mistri et al. 2014; Sheth et al. 2014). Therefore, the present study was planned to develop evidence-based simple, reliable, and validated DBS methods to produce reproducible and accurate biochemical diagnosis for LSDs.

The previously established DBS enzymatic methods for the diagnoses of LSDs were modified to simplify assay procedures without compromising quality. Patients were diagnosed enzymatically for 18 different LSDs after validation. Diagnostic efficacy was proven by correlating results of DBS with those obtained from other biological samples/biomarkers/mutation studies. Additionally, testing accuracy was also ensured by participating in a pilot proficiency testing program (www.erndimqa.nl).

Materials and Methods

A DBS kit [customized card (Whatman 903) for blood sampling, lancet, and desiccant] with envelope was provided by Genzyme (India), a Sanofi company to clinicians in India and neighboring countries (Pakistan, Sri Lanka, and Bangladesh) for lysosomal enzyme testing in suspected cases of LSDs at our genetic center under "India Compassionate Access program." Dried blood specimens ($n = 1{,}033$) were received with brief clinical details, from March 2013 through May 2015. The mean age of patients (51% male) was 3 years (range 1 day to 65 years). For validation, blood samples of patients and normal subjects were collected in heparin tubes and on filter paper for leukocytes and DBS assays, respectively, where feasible. Plasma was separated from blood and stored at −80°C until use. For assays in skin fibroblast (SF), skin was biopsied from patients for culture followed by enzyme testing.

DBS protocols used to estimate various enzyme activities were modified with changes in elution buffer, molarities, pH of substrate buffers, and stopping solutions as given in Table 1 with references (a–n). Enzymes included were: β-glucosidase (EC:3.2.1.21), chitotriosidase (EC:3.2.1.14), sphingomyelinase (EC:3.1.4.12), β-galactosidase (EC:3.2.1.23), β-hexosaminidase A (EC:3.2.1.52), total hexosaminidase (EC:3.2.1.52), α-glucosidase (EC:3.2.1.20), α-galactosidase (EC:3.2.1.22), palmitoyl protein thioesterase (EC:3.1.2.22), tripeptidyl peptidase (EC:3.4.14.9), α-fucosidase (EC:3.2.1.51), α-mannosidase (EC:3.2.1.24), α-iduronidase (EC:3.2.1.76), iduronate 2 sulfatase (EC:3.1.6.13), α-*N*-acetylglucosaminidase (EC:3.2.1.50), galactose 6 sulfatase (EC:2.5.1.5), aryl sulfatase B (EC:3.1.6.12), and β-glucuronidase (EC:3.2.1.31).

One positive (affected case) and one negative (normal subject) controls were run with each assay. After incubation and stopping reactions as per modified protocols, fluorescence (excitation 365 nm, emission 455 nm) was read using Victor 2D multi-label counter (Perkin Elmer). Fluorescence readings were corrected for blanks (substrate incubated separately without DBS under identical conditions was added in blank tube immediately after stopping solution). A standard curve of 4 methylumbelliferone was used to extrapolate fluorescence count to moles of enzymatic product, and enzyme activity was calculated in nmol/h/mL of blood or nmol/h/mg of protein. Temperature effect on enzyme activity during transportation of DBS specimens from distant places was taken into account to rule out false positives by simultaneously testing either β-glucuronidase or β-galactosidase as a reference control enzyme in the same sample to verify sample integrity. In cases where the reference enzyme was deficient as well, repeat sample was requested.

To validate DBS methods for 18 lysosomal enzymes, reproducibility was demonstrated by intra- ($n = 3$) and inter-assay ($n = 10$) results using threshold of %CV <15 (Verma et al. 2015). DBS results of affected and normal cases ($n = 172$) were correlated by performing simultaneous assays in leukocytes/plasma/cultured SF samples. Since the transportation of liquid blood samples across the international borders/distant places was a big hurdle, DBS results of another 74 cases were confirmed by molecular studies. DNA was extracted from DBS (used previously for enzymatic analysis) and analyzed by PCR sequencing of all coding exons and flanking intronic regions (Yale University School of Medicine, New Haven, USA and ARCHIMED Life Science GmbH, Vienna, Austria; www.archimedlife.com). Bio-informatic interpretation was done to predict severity of mutations. These 246 cases including patients of 18 different LSDs (either confirmed by mutation or testing

Table 1 Modified protocols for the estimation of 18 lysosomal enzymes on dried blood spots with biological reference intervals

Enzymes	Procedure (*DBS* dried blood spot, *EL* elution buffer, *SB* substrate buffer, *SU* substrate, *ST* stopping buffer)	Biological reference interval		% Enzyme activity in affected = affected mean/ normal mean	References[a]
		Normal (nmol/h/ml)	Patients (nmol/h/ml)		
βGLU	DBS + 50 µL SB: 0.4 M CP, pH: 5.5 + SU: 100 µL 5 mM MβGLU containing 0.2% NaTC in 0.2 M SAB, pH 5.5 + 5 h at 37°C+ ST: 1,350 µL EDA	(n = 139): 2.3–18.4, mean = 5.0	(n = 35): 0.062–2.0, mean: 0.8	16.0	a, c
CHT	DBS+ EL: 40 µL 0.1 M SAB, pH 5.2+ SU: 40 µL 22 mM TAC in 0.1 M/ 0.2 M CP, pH 5.2+ 30 min at 37°C + 600 µL EDA	(n = 70): 0.062–100, mean = 23.2	(n = 35): 100–554.0, mean: 254.7	–	a, d
ASM	DBS+ EL: 60 µL D/W + 20 µL NaTC (0.2%) + 20 µL 0.1 M SAB, pH 5.2 + SU : 20 µL 0.68 mM HMP in 0.1 M SAB, pH 5.2 + 20 h at 37°C + ST: 380 µL AMP	(n = 20): 1.94–23.9, mean: 8.3	(n = 5): 0.062–0.8, mean: 0.4	4.8	c, e
βGal	DBS + 50 µL D/W+ SU: 50 µL 1 mM MβGal in 0.1/0.2 M CP, pH: 4.0 + 0.1 M sodium chloride + 3 h at 37°C + ST: 500 µL AMP	(n = 45): 20.7–69.0, mean: 32.8	(n = 7): 0.062–3.0, mean: 2.1	6.4	a, f
βHex A	DBS +EL: 50 µL D/W, SU: 30 µL 1 mM MβACGlu in 0.1/0.2 M CP, pH 4.4, + 3 h at 37°C + ST: 420 µL AMP	(n = 22) :26.9–186.5, mean: 85.5	(n = 7): 0.062–3.64, mean: 1.8 (n = 3): >100.0 (I-cell)	2.1	a, g
Total hex	DBS+ EL: 50 µL 0.2 M/0.4 M CP, pH: 4.5 + SU: 100 µL 3 mM MACDGlu + 2 h at 37°C + ST: 300 µL EDA	(n = 20) :24.0–191.6, mean: 78.6	(n = 5): 0.062–3.5, mean: 2.5	3.2	a, g
αGlu	Total acid αGlu (assay without acarbose inhibitor): DBS + 50 µL D/W	(n = 52): Total acid αGlu: 15.5–92.2; lysosomal acid αGlu: 5.5–29.6	(n = 15): Total acid αGlu: 4.8–38.3; lysosomal acid αGlu: 0.86–5.5,	25.0 (% ratio)	h
	Lysosomal acid αGlu (assay with acarbose (7.5 µM) inhibitor): DBS+ 10 µL D/W + 40 µL acarbose	Ratio[b]: 0.25–0.75, mean: 0.44	Ratio[b]: <0.22, mean: 0.11		
	Both sets kept for 2 h at RT + SU: 50 µL 4 mM MαGlu in 0.1/0.2 M CP, pH 4.0 in all tubes + O/N at 37°C + ST: 300 µL EDA	Poor control[c] (n = 15): total acid αGlu: 0.96–16.5, lysosomal acid αGlu: 0.29–6.7 Ratio: 0.27–0.8[b]			
αGal	DBS+ SU: 70 µL 5 mM MαGal in 0.1/0.2 M CP, pH 4.5 + O/N at 37°C + ST: 300 µL EDA	(n = 18):2.57–7.8, mean: 5.5	(n = 3): 0.062–1.0, mean: 0.4	7.2	a, b
PPT	DBS + EL: 100 µL D/W + 30 min. at 37°C. Take 10 µL + SU: 20 µL 0.64 mM MPβGlc + 48 h at 37°C + ST: 200 µL AMP	(n = 15): 5.0–12.5, mean: 8.2	(n = 5): 0.062–2.6, mean: 1.8	21.9	i
TPP	DBS + EL: 100 µL D/W + 30 min. at 37°C. Take 10 µL + SU: 100 µL 500 µM AAPAMC in 0.1 M SAB, pH 4.0 + 40 µL NaCl + 48 h at 37°C + 200 µL AMP	(n = 15): 30–80, mean = 51.2	(n = 2): 0.062–5.0, mean: 3.4	6.6	j
αFuco	DBS + EL: 40 µL D/W + SU: 40 µL 0.75 mM MαF in 0.1/0.2 MCP, pH 5.8 + 3 h at 37°C + ST: 200 µL AMP	(n = 15):20–65.0, mean: 38.9	(n = 3): 0.062–3.0, mean: 2.2	5.6	f
αManno	DBS + EL: 40 µL D/W + SU: 40 µL 5 mM MαM in 0.1/0.2 MCP, pH: 5.8 + 3 h at 37°C + ST: 200 µL AMP	(n = 15): 22–67.0, mean: 43.6	–		f
αIdu	DBS + EL: 30 µL D/W + 10 µL formate buffer, pH: 3.5 + 20 µL SL + SU: 20 µL 2 mM MαIdu in formate buffer, pH: 3.5 + O/N at 37°C + ST: 200 µL AMP	(n = 50): 2.4–12.0, mean: 4.5	(n = 12): 0.062–0.5, mean: 0.23 (n = 5):>25.0 (I-cell)	5.1	k
Idu2s	DBS+ EL: 100 µL D/W+ 30 min. at 37°C. Take 10 µL + SU: 20 µL MαIdu2S in 0.1 M SAB, pH 5.0 containing 10 mM lead acetate + 24 h at 37°C+ 40 µL 0.2M/0.4M CP + 10 µL LEBT+ 24 h at 37°C + ST: 200 µL AMP	(n = 18): : 11–35, mean: 17.5	(n = 5): 0.062–9.0, mean: 4.2	24.0	l
αHex	DBS + EL: 100 µL D/W + 37°C for 30 min. Take 10 µL supernatant + SU: 20 µL 0.25 mM MαNACGLU + O/N at 37°C + ST: 200 µL AMP	(n = 12): 2.4–3.8, mean: 2.8	(n = 2): 0.062–0.16, mean: 0.1	3.6	m

(continued)

Table 1 (continued)

Enzymes	Procedure (*DBS* dried blood spot, *EL* elution buffer, *SB* substrate buffer, *SU* substrate, *ST* stopping buffer)	Biological reference interval		% Enzyme activity in affected = affected mean/ normal mean	References[a]
		Normal (nmol/h/ml)	Patients (nmol/h/ml)		
G6S	DBS+ SU: 20 μL 10 mM MG6STEA in 0.1 M SAB, pH 4.3+ 48 h at 37°C + 5 μL 0.9 M phosphate buffer, pH 4.3+ 10 μL β-galactosidase + 6 h at 37°C + ST: 200 μL AMP	(n = 15): 7.5–43.5, mean: 15.4	(n = 7): 0.062–1.5, mean: 0.8	5.2	n
ASB	DBS + EL: 50 μL D/W + SU: 75 μL 10 mM MS in 0.2 MSAB, pH: 6.0 with 5 mM barium acetate + O/N at 37°C + ST: 450 μL AMP	(n = 15): 5.2–50.0, mean : 22.1	(n = 5): 0.062–1.2, mean: 0.9	4.0	f
βGlur	DBS+ SU : 50 μL of 5 mM MβGlr in 0.1 MSAB, pH: 4.0 + ST: 300 μL EDA	(n = 12): 5.5–45.8, mean: 23.8	(n = 5): 0.062–2.5, mean : 1.1 (n = 5): >200.0 (I-cell)	4.6	f

Enzymes: *βGLU* β-glucosidase, *CHT* chitotriosidase, *ASM* acid sphingomyelinase, *βGal* β-galactosidase, *βHex A* β-hexosaminidase A, *Total hex* total hexosaminidase, *αGlu* α-glucosidase, *αGal* α-galactosidase, *PPT* palmitoyl-protein thioesterase, *TPP* tripeptidyl peptidase, *α-Fuco* α-fucosidase, *α-Manno* α-mannosidase, *αIdu* α-iduronidase, *Idu2s* iduronate 2-sulfatase, *αHex* α-hexosaminidase, *G6S* galactose 6 sulfatase, *ASB* aryl sulfatase B, *βGlur* β glucuronidase

Reagents: *CP* citrate –phosphate buffer, *EDA* 0.3 M ethylene diamine, *SAB* sodium acetate buffer-adjust pH with 0.01 M acetic acid, *TAC* 4Mu β NNN tri-acetyl chitotriosidase, *Mβ-GLU* 4MU β-glucoside, *D/W* distilled water, *NaTC* sodium taurocholate, *HMP* 6-hexadecanoylamino 4-methylumbelliferyl –phosphorylcholine, *AMP* 0.1 M 2-amino, 2-methyl, 1-propanol, pH 10.3, *Mβ Gal* 4 Mu β-D-galactopyranoside, *MβACGlu* 4 Mu β-*N*—Ac-glucosaminide 6 sulfate, *MACDGlu* 3 mM 4 MU 2-acetamido 2-deoxy β-D glucopyranoside, *MαGlu* 4Mu α-D-glucopyranoside, *O/N* overnight (17 h), *MαGal* 4Mu α-D-galactopyranoside with 125 mM *N*-acetyl D-galactosamine, *MPβGlc* 4 MU -6S-palm-β-Glc, *AAPAMC* AAP-7-amido-4-methylcoumarin, *MαF* 4Mu α-L-fucoside, *MαM* 4Mu α-D mannopyranoside, *SL* saccharolactone, *MαIdu* 4 Mu α-L-iduronide (glycosynth), *MαIdu2S* 4- MU α-L iduronide 2 sulfate, *LEBT* lysosomal enzymes from bovine testis, *MαNACGLU*: 4-methylumbelliferyl α-*N*-acetyl glucosaminide, *MG6STEA* 4Mu-β-galactoside 6-sulfate.triethyl ammonium, *MS* 4Mu sulfate, *MβGlr* 4 Mu β-glucuronide

Blank: One blank tube was always set for each sample in the assay in which all reagents were added and kept under identical conditions except substrate. Substrate was incubated under identical conditions in separate tube without DBS specimen. This substrate was added in the blank tube immediately after stopping before taking reading

Analytical sensitivity: Since analytical sensitivity of these fluorometric enzyme assays is 0.062 nmol/h/mL, the affected range is given from 0.062 (Verma et al. 2015)

Multiple sulfatase deficiency (MSD): MSD patients can be diagnosed by determining deficiency of iduronate 2 sulfatase, galactose 6 sulfatase, and aryl sulfatase B enzymes

[a] *References*: a: Civallero et al. (2006); b: Chamoles et al. (2001); c: Chamoles et al. (2002a); d: Hollak et al. (1994); e: van Diggelen et al. (2005); f: Kelly (1977); g: Chamoles et al. (2002b); h: Winchester et al. (2008); i: van Diggelen et al. (1999); j: Lukacs et al. (2003); k: Hopwood et al. (1979); l: Voznyi et al. (2001); m: Marsh and Fensom (1985), and n: Camelier et al. (2011)

[b] Ratio = lysosomal acid αGlu/total acid αGlu

[c] Normal cases with low levels of both total acid α-glucosidase and lysosomal acid α-glucosidase while ratio within normal range

in other biological materials) were used to evaluate diagnostic efficacy of DBS enzyme assays. Results were also correlated with abnormal biomarkers including chitotriosidase (CHT) activity in Gaucher disease, creatine phosphokinase (CK) in Pompe, and glycosaminoglycans (GAGs) in patients with mucopolysaccharidoses. To ensure testing quality and specificity, the laboratory participated in a pilot study of lysosomal enzymes testing on DBS run by ERNDIM, The Netherlands, and performance was evaluated. These validated DBS methods were further used to identify the patients of different LSDs.

Results

Of 1,033 cases, 307 were found to have enzyme deficiency for one of the LSDs tested (Fig. 1). The reference intervals established for patients and normal subjects (Table 1) were used to analyze these patients. The largest cohort of 138 cases was diagnosed for Gaucher, followed by Hurler ($n = 63$), Pompe ($n = 58$), Tay Sachs ($n = 12$), and GM1 gangliosidosis ($n = 12$). Among these, 209 patients were from India (Gaucher: 80, Pompe: 52, MPS-I: 30, Fabry: 4, MPS (II, IV, VI): 17, Tay Sachs/Sandhoff: 14, and GM1: 12) between the ages of 1 day to 65 years (66% male), 92 patients between the age group 5 m to 13 years (70% male) were from Pakistan (Gaucher: 55, MPS-I: 31, Pompe: 5, and Fabry: 1), and 5 patients were from Sri Lanka (Gaucher: 3, MPS-I: 1, and Pompe: 1,) in the age group 1–5 years (60% male). One case of MPS-I (2 years/F) was diagnosed from Bangladesh. Of 54 diagnosed cases from India, 22 positive cases with enzyme values ≤4% of the normal mean were enrolled for enzyme replacement therapy (ERT) [Gaucher (Cerezyme): 2, Pompe (Myozyme): 12, MPS-I (Aldurazyme): 6, and Fabry (Fabrazyme): 2]. At present, three Pakistani patients of Gaucher with enzyme values 12–14% of the normal mean are on Cerezyme therapy.

Before analyzing DBS samples for diagnostic purposes, reproducibility and robustness of enzyme assays were shown by inter-assay and intra-assay results (%CV: 10–14) obtained within acceptable limits (%CV: <15) for

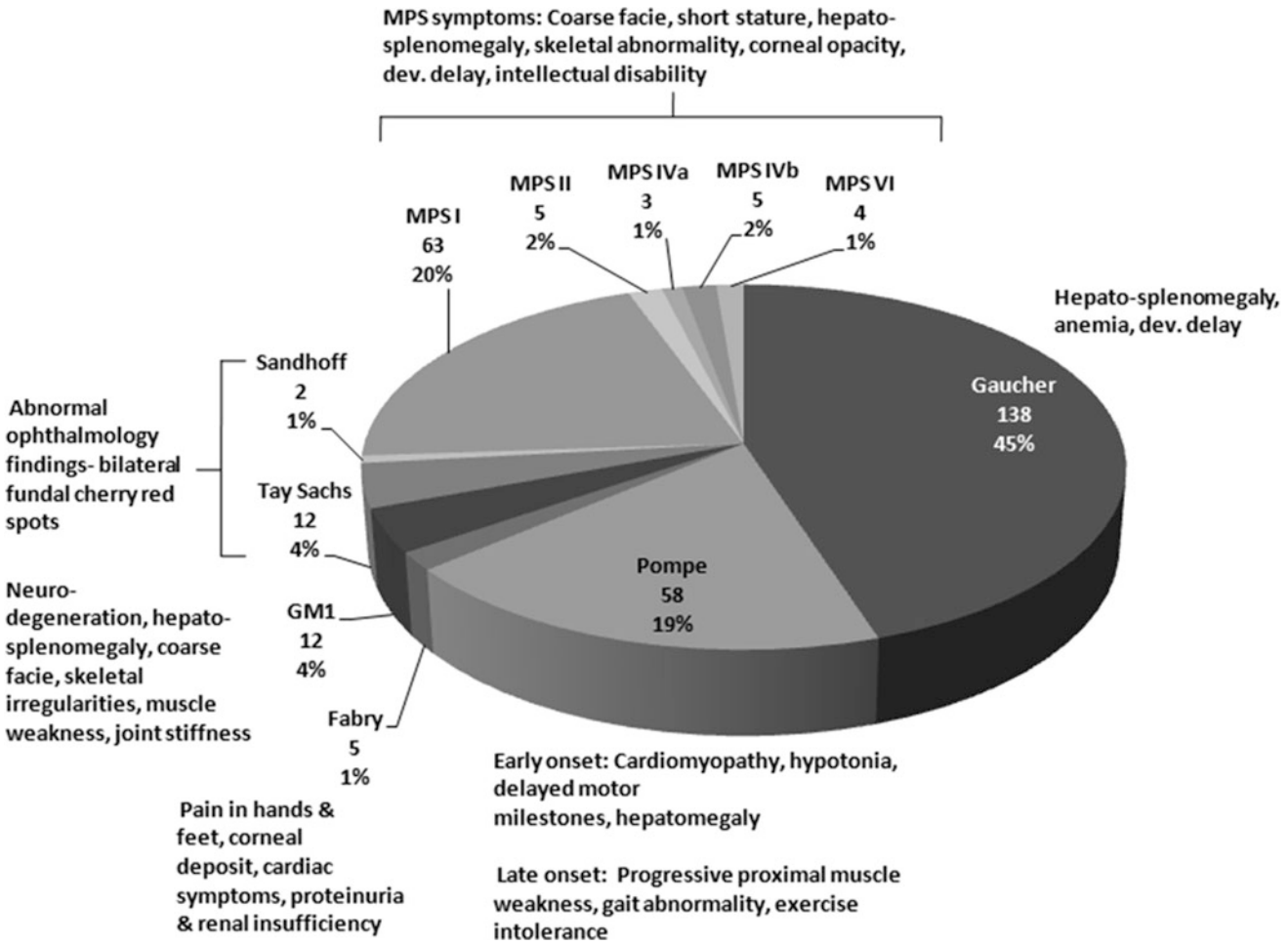

Fig. 1 Distribution of diagnosed cases of lysosomal storage disorders ($n = 307$) with clinical features

all 18 lysosomal enzymes. In correlation studies of 246 cases (demographic details are given in Table 2), 172 demonstrated 100% concordance between biochemical results of DBS and other biological materials [leukocytes ($n = 154$), plasma ($n = 13$), and SF ($n = 5$)]. Another 74 biochemically diagnosed cases on DBS were confirmed by mutation studies (Table 3). Among 44 affected cases, 38 showed pathogenic mutations in the corresponding gene. The remaining six cases (3 Pompe and 3 MPS-I) with low enzyme activity but no mutations were considered false positives because problems in transportation of liquid blood from remote regions along with financial incapability of patients to reach the laboratory made it difficult to verify the enzyme in leukocytes. As a result, overall study has 100% sensitivity (false negatives – nil), 95.2% specificity, and 97.6% diagnostic accuracy for Gaucher, Fabry, Pompe, Tay Sachs, Sandhoff, Niemann Pick, GM1, Neuronal ceroid lipofuscinosis (NCL), Fucosidosis, Mannosidosis, Mucopolysaccharidosis (MPS) type I, II, IIIb, IVa, VI, VII, and I-Cell diseases.

In the study on biomarkers, 35 out of 37 positive cases of Gaucher showed high chitotriosidase level while 2 were normal (Table 3). Similarly, eight cases with raised CK were biochemically affected for Pompe disease. All MPS-I affected cases ($n = 13$) either showed increased GAGs or disease specific band profiles on urine MPS electrophoresis. In three cases with α-iduronidase deficiency where raised GAGs and bands of dermatan sulfate and heparan sulfate were detected, mutation for MPS-I was not found. No biomarker was studied in patients of other disorders.

The overall satisfactory performance (91.7% correct analysis) in pilot study (DBS-lysosomal enzymes), ERN-DIM further ensured testing quality of DBS assays. This includes accurate diagnosis of Fabry, Hunter, Hurler, Gaucher, Pompe, MPS-IIIb, and late infantile-NCL patients with one false negative (Krabbe) and four false positives (Krabbe, Metachromatic leukodystrophy, MPS-IVa, and normal control).

Discussion

The present study demonstrates 100% sensitivity and 95.2% specificity of DBS enzyme assays to diagnose LSDs using modified protocols. One of the primary modifications was substitution of water for buffer to elute sample from filter paper, which has proven to be cost effective without compromising testing quality. Substrate (4MU sulfate) specificity for aryl sulfatase B was enhanced by including barium acetate in the buffer. Barium ions are a potential inhibitor of aryl sulfatase A (Bostick et al. 1978). For estimation of acid sphingomyelinase (ASM), 4MU bound fluorogenic substrate was used along with saccharolactone to inhibit the activity of β-glucuronidase since radiolabelled substrate used by Civallero et al. (2006) can cause health hazards. The procedures were further simplified by stopping reactions with 2-amino, 2-methyl, 1-propanol (0.1 M), pH 10.6 instead of more commonly used glycine-carbonate buffer, pH 9.6 (Willey et al. 1982). It is easy to prepare, stable for at least 6 months at room temperature without change in pH and microbial growth, and fluorescence remains stable up to 1 h at room temperature after stopping the reaction. The advantage of easy collection, transportation, storage, and dispatching of DBS especially from remote regions lacking specialized laboratories has been highlighted in this study which involved cross-country transportation of DBS samples. The reference intervals established for enzyme activities effectively discriminate between affected and normal subjects in South Asian population (Table 1). In most of the affected cases, enzyme activity was <8% of the normal mean except for Gaucher, Infantile-NCL, MPS II (16–24%), and Pompe (% ratio 25). Therefore, in the cases where enzyme activity remains 20–40% of normal mean, we recommend confirmatory testing (either in leukocytes or mutation studies).

Civallero et al. (2006) reported estimation of 12 lysosomal enzymes on DBS; however, their results were not validated with testing in other biological materials or biomarkers or molecular diagnosis. In the present study, these methods were used to validate the results achieved by using modified protocols. Absolute concordance was seen in Gaucher, Fabry, Tay Sachs, Sandhoff, Niemann Pick, GM1, Neuronal ceroid lipofuscinosis (NCL), Fucosidosis, Mannosidosis, MPS II, IIIb, IVa, VI, VII, and I-Cell diseases while 91.4% and 88% concordance was observed for Pompe and MPS-I, respectively. The six false positive results (unable to confirm in leukocytes) could be on account of mutations that were not detected by conventional sequencing or deterioration of enzyme (α-iduronidase being more sensitive to high temperature and humidity) in transportation as temperature in Indian Subcontinent varies between 35°C and 45°C during summer, and the mean duration of sample transportation was 10–12 days.

Diagnosis of Pompe disease was made by considering the ratio of activities of lysosomal acid α-glucosidase to total acid α-glucosidase. In a number of our cases where individual values of lysosomal and total acid α-glucosidase were low, the ratio fell into the normal range. In view of normal ratio, these cases (poor controls) should be considered as normal and analyzed on the basis of an additional range set in our study (Table 1). Their unaffected status has been further proven by the absence of pathogenic mutations.

Recently, Cobos et al. (2015) suggested for correlating biochemical results with a second enzyme assay and/or

Table 2 Demographic details of the cases ($n = 246$) used in correlation studies

Diseases with enzyme	Total cases	Countries	Region	Cases	Age	Gender	Positive	Normal
Gaucher (β-glucosidase)	54	India	North India	23	3 months–7 years	12 M, 11 F	15	8
			South India	13	1–10 years	7 M, 6 F	8	5
			East India	1	13 years	F	1	0
			West India	3	2–5 years	1 M, 2 F	2	1
		Pakistan		11	11 months–13 years	8 M, 3 F	9	2
		Sri Lanka		3	2.6–9 years	3 M	2	1
Pompe (α-glucosidase)	35	India	North India	10	10 months–35 years	8 M, 2 F	3	7
			South India	15	3 months–22 years	7 M, 8 F	8	7
			East India	2	9 months–1 year	2 F	2	0
			West India	5	5–12 years	4 M, 1 F	3	2
		Pakistan		1	1 year	M	1	0
		Sri Lanka		2	6 years, 7 years	2 F	0	2
Fabry (α-galactosidase)	11	India	North India	7	8–65 years	4 M, 3 F	2	4
			South India	4	11–35 years	3 M, 1 F	1	4
Tay Sachs (β-hexosaminidase A)	14	India	North India	9	3 months–2.5 years	6 M, 3 F	6	3
			South India	5	6 months–4 years	3 M, 2 F	3	2
Sandhoff (Total hexosaminidase)	10	India	North India	4	1–3.5 years	2 M, 2 F	2	2
			South India	3	2–4 years	1 M, 2 F	2	1
			West India	3	3–5 years	2 M, 1 F	1	2
GM1 (β-galactosidase)	11	India	North India	8	1–5 years	4 M, 4 F	4	4
			West India	3	1.5–8 years	3 M	2	1
Niemann Pick (sphingomyelinase)	5	India	North India	5	2.5 months–2.5 years	3 M, 2 F	3	2
NCL1 (Infantile) (palmitoyl-protein thioesterase)	10	India	North India	4	1–3 years	1 M, 3 F	2	2
			South India	6	4 months–1 year	3 M, 3 F	3	3
NCL2 (late infantile) (tripeptidyl peptidase)	8	India	North India	8	7–15 years	6 M, 2 F	3	5
Fucosidosis (α-fucosidase)	7	India	North India	5	2–4 years	2 M, 3 F	2	3
			East India	2	1–3 years	2 M	0	2
Mannosidosis (α-mannosidase)	5	India	North India	5	3.5–8 years	3 M, 2 F	0	5
MPS-I (α-iduronidase)	25	India	North India	8	2–4.5 years	3 M, 3 F	4	4
			South India	9	1–13 years	4 M, 5 F	6	3
		Pakistan		6	1–15 years	3 M, 3 F	3	3
		Sri Lanka		2	1–1.6 years	1 M, 1 F	0	2
MPS-II (iduronate 2 sulfatase)	10	India	North India	5	1–12 years	4 M, 1 F	4	1
			West India	5	2.5–15 years	4 M, 1 F	1	4
MPS-IIIb (α-hexosaminidase)	9	India	North India	5	2–5 years	2 M, 3 F	0	5
			South India	4	2–2.5 years	2 M, 2 F	2	2
MPS-IVa (galactose 6-sulfatase)	10	India	North India	5	2–7 years	4 M, 1 F	3	2
			South India	5	5–10 years	2 M, 3 F	2	3
MPS-VI (aryl sulfatase B)	11	India	North India	4	6 months–6 years	2 M, 2 F	2	2
			South India	5	1–5.3 years	3 M, 2 F	2	3
			East India	2	1.5–2.5 years	2 F	2	0
MPS-VII (β-glucuronidase)	8	India	North India	5	12–18 years	4 M, 1 F	1	2
			West India	3	14–23 years	3 F	2	3
I-Cell	3	India	North India	3	3–5 years	3 F	3	0
Total	246			246			127	119

Table 3 Correlation studies ($n = 246$): DBS with other biological samples using same enzymatic assays (A), DBS with biomarkers status (B), and DBS biochemical diagnoses with molecular diagnoses (C)

				Biological samples				
Diseases with enzyme	Total cases	Diagnoses	DBS[a] nmol/h/mL	Leukocytes/plasma/skin fibroblast ($n = 172$) (A)	Ref. interval nmol/h/mg	Biomarkers/metabolites on DBS/plasma/serum/urine ($n = 95$) (B)	Mutation/sequence data ($n = 74$) (C)	Evaluation
Gaucher (β-glucosidase)	54	Affected ($n = 37$)	($n = 37$): 0.062–1.87	($n = 12$): 1.8–4.5 (β-glucosidase in leuko.)	Leuko.: 0.062–6.0	DBS – CHT ($n = 23$): 151–3,425	*Total ($n = 25$):*	FP and FN – not found
					DBS-CHT: >100.0 nmol/h/mL	($n = 2$): 0.013, 6.29	[$n = 16$]-L444P (homozygous),	54/54 = (100% satisfactory)
					Pl.- CHT: >150 nmol/h/mL	Pl. – CHT ($n = 12$): 433–4,251.0	[$n = 3$]- F2131 (TTT-ATT) (homozygous),	
							[$n = 6$]- {S356F (homozygous), L444P/S42N, L444P (homozygous)/A456P (heterozygous), L444P (heterozygous)/S356F (homozygous), L420(CTT->CTA), R120W(CGG->TGG) (heterozygous)/E326K(GAG->AAG)}	
		Normal ($n = 17$)	($n = 17$): 2.0–8.1	($n = 13$): 11.2–43.2 9 (β-glucosidase in leukocytes)	Leuko.: 10–84.0	DBS – CHT ($n = 4$): 4.5–43.2	($n = 4$): Mutation not available	
					DBS-CHT: <100 Pl.-CHT <150.0	Pl. – CHT ($n = 1$): 2.9		
Pompe (α-glucosidase)	35	Affected ($n = 17$)	($n = 17$) : 0.005–0.16 (ratio)	Leukocytes ($n = 5$): <0.2 (ratio)	<0.22 (ratio)	Serum CK: ($n = 8$): Raised	($n = 6$): (homozygous) c.1799G>A, (homozygous) c.1820G>A, (homozygous) c.1447G>A, (heterozygous) c.1726G>A/c.2065G>A, (heterozygous) c.2780C>T/c.2783A>G, (homozygous) c.2560C>T	FP[b] – three, FN – not found
				Skin fibroblast: ($n = 3$): 0.062–0.89	0.062–5.0		($n = 3$): Mutation not available (FP)	32/35 = (91.4% satisfactory)
		Normal ($n = 18$)	($n = 18$):0.25–0.8 (ratio)	Leukocytes ($n = 5$): 0.31–0.62 (ratio)	0.25–0.75 (ratio)	($n = 11$): CK raised in four	($n = 8$): Mutation not available	
							($n = 5$): (Heterozygous) c. 2780C>T	
Fabry (α-galactosidase)	11	Affected ($n = 3$)	($n = 3$): 0.062–1.0	($n = 3$): 0.062–2.0	0.062–4.0	No biomarker	Not done	FP and FN – not found
		Normal ($n = 8$)	($n = 8$): 3.2–5.23	($n = 5$) 25–59.4	22–85.5	No biomarker	($n = 1$): (Heterozygous) c.166T>C, ($n = 2$): Mutation not available	11/11 = 100% satisfactory
Tay Sachs (β-hexosaminidase A)	14	Affected ($n = 9$)	($n = 9$):0.062–3.4	($n = 5$): 0.062–2.54	0.062–5.4	No biomarker	($n = 4$): 1277_1278insTATC/1277_1278insTATC	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 28.9–90.6	($n = 5$): 95–264	74–323	No biomarker	Not done	14/14 = 100% satisfactory
Sandhoff (total hexosaminidase)	10	Affected ($n = 5$)	($n = 5$): 0.062–2.1	Plasma ($n = 5$) : 0.062–312	0.062–330	No biomarker	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 31–228.1	Plasma enzyme ($n = 5$): 800–2,250	660–5,000	No biomarker	Not done	10/10 = 100% satisfactory

GM1 (β-galactosidase)	11	Affected ($n = 6$)	($n = 6$): 0.062–1.29	($n = 6$) : 0.062–8.3	0.062–14	No biomarker	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 23–63.08	($n = 5$): 65–494.9	58–676	No biomarker	Not done	11/11 = 100% satisfactory
Niemann Pick (A/B) (sphingomyelinase)	5	Affected ($n = 2$)	($n = 2$): 0.062–0.75	($n = 2$): 0.062–3.5 (nmol/17 h/mg)	0.062–5.0	No biomarker	Not done	FP and FN – not found
		Normal ($n = 3$)	($n = 3$): 4.5–21.4	($n = 3$): 13.7–18.2 (nmol/17 h/mg)	10–32	No biomarker	Not done	5/5 = 100% satisfactory
Neuronal ceroid lipofuscinosis (NCL-infantile) (PPT)	10	Affected ($n = 5$)	($n = 5$): 0.062–2.6	($n = 5$): 0.062–12.0	0.062–13	No biomarker	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 5.8–9.2	($n = 5$): 102–204.0 (nmol/17 h/mg)	25–224.0	No biomarker	Not done	10/10 = 100% satisfactory
Neuronal ceroid lipofuscinosis (NCL-late infantile) (TPP)	8	Affected ($n = 3$)	($n = 3$): 0.062–3.52	($n = 3$): 0.062–1.2	0.062–10	No biomarker	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 35.5–61.2	($n = 5$): 36–74	25–198	No biomarker	Not done	8/8 = 100% satisfactory
Fucosidosis (α-fucosidase)	7	Affected ($n = 2$)	($n = 2$): 0.062–0.23	($n = 2$): 0.062–0.098	0.062–2.0	No biomarker	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 32–45.95	($n = 5$): 243–512.5	200–735.0	No biomarker	Not done	7/7 = 100% satisfactory
Mannosidosis (α-mannosidase)	5	Normal ($n = 5$)	($n = 5$): 32–59.0	($n = 5$): 54–229.0	40.0–398.0	No biomarker	Not done	FP and FN – not found
								5/5 = 100% satisfactory
MPS-I (α-iduronidase)	25	Affected ($n = 13$)	($n = 13$); 0.062–0.71	($n = 5$): 0.062–1.2	0.062–3.0	($n = 10$): Raised GAGS,	($n = 2$): (Homozygous) c.223G>A/ c.299+1G>A	FP[b] – Three, FN – not found
				Skin fibroblast: ($n = 2$):13.5, 20.0	0.062–25.0	($n = 3$): EP positive: (Dermatan sulfate + Heparan sulfate)	($n = 1$): (Homozygous) c.314G>A ($n = 3$): Mutation not available (FP)	3/25 = 88% satisfactory
		Normal ($n = 12$)	($n = 12$): 1.45–7.26	($n = 5$): 37.5–147.85	21–150.0	($n = 7$): Normal EP	($n = 6$): Mutation not available ($n = 1$): (Heterozygous) c.314G>A	
MPS-II (Iduronate-2-sulfatase)	10	Affected ($n = 5$)	($n = 5$): 0.062–5.0	($n = 5$): 0.062–4.2 (nmol/4 h/mg)	0.062–5.0	($n = 3$): Raised GAGs	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 12–25.5	($n = 5$): 19.9–45.5	18–94.0	($n = 3$): Normal GAGs	Not done	10/10 = 100% satisfactory
MPS-IIIb (α-hexosaminidase)	9	Affected ($n = 2$)	($n = 2$): 0.062, 0.09	($n = 2$): 0.062, 0.2 (nmol/17h/mg)	0.062–1.0	No biomarker	Not done	FP and FN – not found
		Normal ($n = 7$)	($n = 7$): 2.8-3.5	($n = 5$): 37–77.3	31–102	($n = 2$): GAGs normal	($n = 2$): Mutation not available	9/9 = 100% satisfactory
MPS-IVa (galactose 6 sulfatase)	10	Affected ($n = 5$)	($n = 5$): 0.062–0.57	($n = 5$):0.062–0.24 (nmol/17 h/mg)	0.062–5.0	($n = 5$): Raised GAGs	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$): 10.3–20.5	($n = 5$): 59–236.85	45–443.0	No biomarker	Not done	10/10 = 100% satisfactory
MPS-VI (Aryl sulfatase B)	11	Affected ($n = 5$)	($n = 5$): 0.062–0.93	($n = 5$): 0.062–3.99	0.062–6.0	No biomarker	Not done	FP and FN – not found
		Normal ($n = 6$)	($n = 6$): 7–32.0	($n = 5$): 43.4–121.0	14–133	($n = 1$): GAGs normal	($n = 1$): Mutation not available	11/11 = 100% satisfactory

(continued)

Table 3 (continued)

Diseases with enzyme	Total cases	Diagnoses	DBS[a] nmol/h/mL	Biological samples: Leukocytes/plasma/skin fibroblast ($n = 172$) (A)	Ref. interval nmol/h/mg	Biomarkers/metabolites on DBS/plasma/serum/urine ($n = 95$) (B)	Mutation/sequence data ($n = 74$) (C)	Evaluation
MPS-VII (β-glucuronidase)	8	Affected ($n = 3$)	($n = 3$): 0.062–2.5	($n = 3$): 0.062–15	0.062–20.0	No biomarker	Not done	FP and FN – not found
		Normal ($n = 5$)	($n = 5$):3.9–25.32	($n = 5$): 85–218.5	72.7–777.7	No biomarker	Not done	8/8 = 100% satisfactory
I-Cell (α-iduronidase, β hexosaminidase A, β-glucuronidase)	3	Affected ($n = 3$)	($n = 3$): Raised enzymes	($n = 3$): Plasma enzymes raised	Plasma range is different for different enzymes	No biomarker	Not done	FP and FN – not found 3/3 = 100% satisfactory
Total	246		246	($n = 172$)		($n = 95$) (overlapping of A & C)	($n = 74$)	Correct analysis: 240/246 (97.6% accuracy)

PPT palmitoyl-protein thioesterase, *TPP* tripeptidyl peptidase, *GAGS* glycosaminoglycans, *EP* urine MPS electrophoresis, *Pl.* Plasma, *Leuko* leukocytes, *CHT* chitotriosidase, *FP* false positive, *FN* false negative

Pl. Note: In the present study, primarily cases of Gaucher, Pompe, MPS-I, and Fabry were chosen for molecular confirmation because treatment is commercially available for these disorders [Genzyme (India), a Sanofi company]; however, some cases of Tay Sachs, MPS-VI, and MPS-IIIb were also included. Confirmation of DBS results in leukocytes/skin fibroblasts was not possible always as samples were transported from different countries and transportation of liquid samples was a big hurdle. The prohibitive cost of gene sequencing and insufficient samples has been the main reasons for not confirming all biochemically diagnosed cases by molecular studies

[a] *Reference intervals for DBS are given in* Table 1

[b] False positive samples after molecular confirmation were not tested in leukocytes (gold standard assay) due to cross-border liquid blood transportation problem. However, second control enzyme was tested to check the sample integrity. Hence, with no other option to verify, these samples were considered false positives to evaluate the diagnostic efficacy of DBS assays

mutation study. They reported mutations in 64.7%, 100%, 100%, and 62.5% patients that were putatively diagnosed by biochemical tests for MPS I, II, VI, and mucolipidosis II/III, respectively. Absence of pathognomonic IDUA mutations in two cases with low α-iduronidase activity was ascribed to the fact that aside from false positive results, certain mutations that may impact enzyme activity including exon spanning inversions, duplications, or deep intronic mutations are not detected by conventional sequencing. Similarly, in our study, untraceable mutations could be one of the reasons for false positives.

Another approach to corroborate our results on DBS was to demonstrate correlation with related biomarkers (Table 3). The raised level of CHT in all except two patients afflicted with Gaucher supports its relevance as a prognostic marker for disease progression. Normal chitotriosidase activity with low β-glucosidase activity can be explained by highly prevalent CHIT1 null polymorphism in 5–6% of Gaucher patients (Hollak et al. 1994). Normal mutation results in three of MPS-I cases with increased GAGs in MPS quantification/abnormal electrophoretic pattern in urine could in fact be patients suffering from another type of MPS where deficiency of α-iduronidase could be explained by low enzyme stability or undetectable mutations as described above. Similarly, elevated CK levels in biochemically diagnosed Pompe patients were significantly associated with Pompe disease.

Success of DBS technology in diagnosis of LSDs is underpinned by validation through participation in EQAS schemes. Importantly, our experience emphasizes the need for individual laboratories to establish and validate their own biological reference intervals for affected and normal subjects by analyzing a large number of samples so as to take into account varying local environmental and laboratory conditions as well as different ethnicities. In comparison to MS/MS technology with multiplex ability (Chace et al. 2003), our modified DBS methods are simple, sensitive, and inexpensive to set up. In addition, in developing countries, diagnoses are made on a case to case basis by relying on clinical phenotypes and laboratory test results. As a consequence, biochemical diagnosis of LSDs by single analyte analysis remains the preferable approach for reliable diagnosis of LSDs.

In India, the most prevalent LSDs were found to be Gaucher, Pompe, and MPS-I accounting for 38%, 25%, and 14.3% of the patients, respectively, in accordance with earlier studies (Verma et al. 2012; Sheth et al. 2014). In comparison to Pompe and Fabry, a higher incidence of Gaucher (60%) and MPS-I (34%) was seen among patients from Pakistan. Pathogenic mutation L444P was detected in all Gaucher positive cases from Pakistan. Traditional consanguinity in Pakistan may be one of the contributing factors to the high prevalence of these LSDs in comparison to other South Asian countries.

Patients presenting with one or more clinical features presented for a particular disease in Fig. 1 should be investigated for LSDs. Bilateral fundal cherry-red spot was observed in all 12 patients of Tay Sachs, 2 of Sandhoff, and 12 of GM1 Gangliosidosis disease. All 25 cases who are currently receiving ERT have demonstrated a gradual amelioration of symptoms. In many patients ERT has been transformative and even life-saving; in others, however, the impact has been limited, often due to delay in starting therapy. Unfortunately access to enzyme therapy in India and South East Asia as a whole is severely limited due to the lack of central state funding, as occurs for rare diseases in other counties.

We recommend the utilization of DBS-based fluorescent enzyme assays as diagnostic tools for timely identification of LSDs patients accurately; however, where feasible, molecular testing on DNA isolated from the same DBS is encouraged as part of the diagnosis.

Acknowledgement We thank all patients and normal subjects whose blood was used as a control/reference material to generate the data for this study. We extend our thanks to Genzyme (India) who has chosen our center as a referral laboratory for lysosomal enzymes testing. We express our gratitude to doctors from India and neighboring countries for referring patients to our genetic center. We appreciate Genzyme (India), a Sanofi company for extending support to patients for testing and treatment. We also thank Chairman and Director Administration, Sir Ganga Ram Hospital, for their cooperation in setting up genetic testing facility.

Take Home Message

Dried blood spots (DBS) have potential to substitute the established liquid bio-matrices (plasma/leukocytes/cultured fibroblasts) for reliable, accurate, and timely diagnosis of lysosomal storage disorders (LSDs) as demonstrated by 100% sensitivity and 95.2% specificity obtained after biochemical (using liquid biological samples) and molecular confirmation of the DBS-based enzymatic diagnoses to facilitate prognostication and genetic counseling and its implementation will also overcome challenges in cross-country transportation of conventional biological samples.

Compliance with Ethics Guidelines

Authors' contribution

Concept, *design, and method standardization*: Jyotsna Verma

Experimentation/*Acquisition of data*/*analysis*/*interpretation*: Jyotsna Verma, Divya C. Thomas, Sandeepika Sharma, Pramod K. Mistry, David C. Kasper

Patients' *management*/*clinical assessment*: I. C. Verma, Sunita Bijarnia, Ratna D. Puri

Drafting article or revising or reviewing it critically for important content: Jyotsna Verma, Divya C. Thomas, Pramod K. Mistry, David C. Kasper, I. C. Verma, Ratna D. Puri

Final approval for publication: All authors

Conflict of Interest

Jyotsna Verma and David C. Kasper received travel grant from Genzyme, a Sanofi company. Same company also supported I. C. Verma for organization of continuing medical education (CME). Divya C. Thomas, Sandeepika Sharma, Ratna D. Puri, Sunita Bijarnia-Mahay, and Pramod K. Mistry 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) of the World Medical Association. Informed consent was obtained from all patients/normal subjects for being included in the study.

Funding

Patients for enzymatic testing of four disorders (Pompe, Gaucher, Fabry, and MPS1) were sponsored by Genzyme (India), a Sanofi company via doctors from India and other neighboring countries to help in providing treatment on compassionate ground under their "India Compassionate Access Program." Genzyme has under no circumstance extended funds to us under any project head/grant number nor has it provided us with any financial support to facilitate the functioning of our laboratory so as to influence the results of our study.

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JIMD Reports
DOI 10.1007/8904_2016_544

RESEARCH REPORT

Parent Coping and the Behavioural and Social Outcomes of Children Diagnosed with Inherited Metabolic Disorders

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Received: 25 August 2015 / Revised: 21 December 2015 / Accepted: 02 February 2016 / Published online: 24 March 2016

Abstract *Objective*: To explore the level of coping and management of parents of children with inherited metabolic disorders (IMD) and the relationship with children's cognitive, behavioural and social functioning.

Methods: Parents of children ($n = 22$) with confirmed IMD (glutaric aciduria type I, methylmalonic aciduria, propionic aciduria, isovaleric aciduria, glycogen storage disease, maple syrup urine disease, ornithine transcarbamylase or very long-chain acyl-CoA dehydrogenase deficiency) completed standardised questionnaires regarding psychological distress, coping and family management. Children completed cognitive assessments and parents rated their behavioural and social functioning on standardised questionnaires. Scores were compared with normative data.

Results: Most parents were coping well; 4/22 reported high levels of psychological distress. Exploratory analysis found that parent coping variables were correlated to the child's internalising symptoms, whereas family management was related to children's externalising behaviours and social skills. No relationship was found between parent variables and cognitive functioning.

Conclusions: Parental coping and family management impact on the child's internalising symptoms and externalising behaviours, respectively. Early identification of issues in these domains may enhance referral for therapeutic interventions and family support programmes.

Inherited metabolic disorders (IMD) are individually rare but overall are diagnosed in 1:318–1:3670 live births, depending on the population (Hutchesson et al. 1998; Dionisi-Vici et al. 2002; Aygen et al. 2014). The implementation of newborn screening and initiation of earlier treatment have decreased morbidity and mortality rates (Waisbren et al. 2003; Wilcken et al. 2009), yet some children remain vulnerable to ongoing 'metabolic crises'. Parents need to constantly manage and monitor their children since poor management can affect physical and cognitive development (Hood et al. 2014), and some children may experience poor outcomes despite early treatment (Grünert et al. 2013; Waisbren et al. 2013). Thus, parents are faced with multiple stressors throughout the diagnosis period and beyond.

Parents may experience a range of emotions at the diagnostic stage, such as guilt, anger, grief, sadness and worry for the future (Weber et al. 2012). Long-term burden includes changes in lifestyle due to dietary constraints, medication regimes and frequent visits to the medical centre – all of which affect family routines, relationships and parenting styles (Cederbaum et al. 2001; Hatzmann et al. 2009). IMD can lead to financial burden as much as emotional burden (Gramer et al. 2013). Some parents also report a type of 'social burden' regarding what friends, family and society may think about them and their parenthood

Communicated by: Verena Peters

Competing interests: None declared

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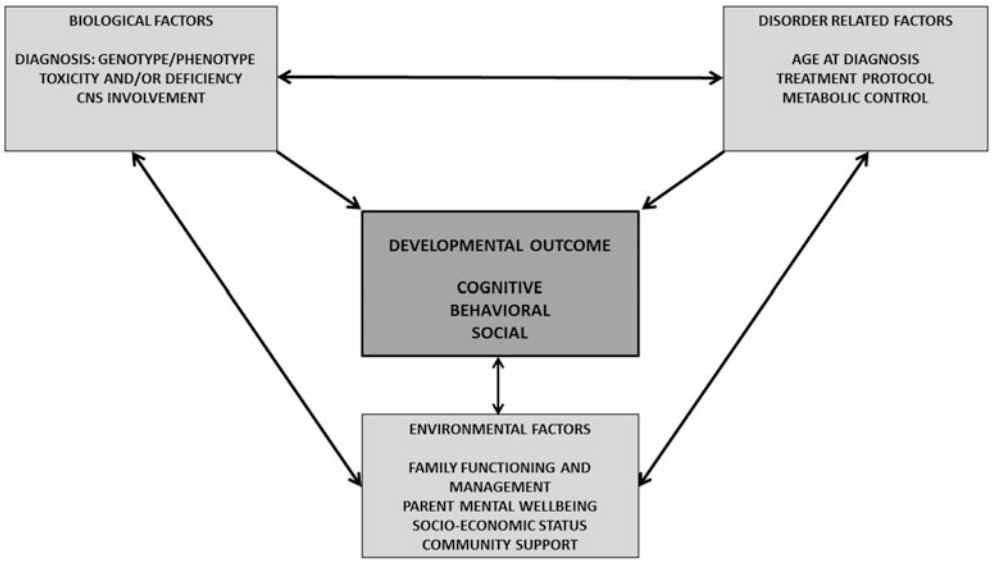

Fig. 1 Conceptual model of biological and environmental variables that influence developmental outcomes of children with IMD

(Saleem et al. 1998). Those with a high level of social support will feel less stressed and more equipped to manage the child's condition (Hatzmann et al. 2009; Torkelson and Trahms 2010).

Stress levels in parents of children with IMD are related to competence in managing the disorder, social isolation and emotional strain (Dellve et al. 2006). Parents of children with maple syrup urine disorder (MSUD) (Packman et al. 2012), glycogen storage disease type I (GSD) (Storch et al. 2008) and urea cycle disorders (Cederbaum et al. 2001; Packman et al. 2012) experience high levels of anxiety concerning the future, dietary management and fear of death. Whilst some parents struggle, others report positive feelings of bringing the family closer together and increased compassion (Cederbaum et al. 2001).

Parental stress and its impact on family function affect the development and well-being of healthy children (Fisak et al. 2012), as well as those with chronic illness (Thompson et al. 1999; Immelt 2006; Knafl et al. 2007). Parental stress affects glycaemic control in children with type I diabetes mellitus (Tsiouli et al. 2013) and behaviour and social development in children with cancer (Colletti et al. 2008; Hilliard et al. 2011) and with spina bifida (Friedman et al. 2004). Parents of children with PKU reported their families were less cohesive and adaptive compared with parents of healthy children (Kazak et al. 1988), yet no differences were found in GSD I (Storch et al. 2008). Parents of children with MSUD reported that the burden of responsibility of their child's condition impacted most on their family relationships (Packman et al. 2012). However, findings regarding the impact of family functioning on child outcomes are equivocal (Shulman et al. 1991; Grünert et al. 2013).

The 'double-hazard theory' proposes that children with brain insult or injury are not only susceptible to poor cognitive, psychological and behavioural outcomes due to the impact on the brain, but they are also more susceptible to their environment. Children may also have a certain amount of 'reserve', whereby family and social support can act as a buffer to enhance outcomes (Breslau 1990; Dennis 2000). Thus, it is possible that targeting problem environmental factors offer the opportunity to modify children's cognitive, behavioural and social outcomes.

Integrating the available literature, we present a biopsychosocial model to conceptualise the various factors that contribute to child outcomes in IMD (Fig. 1). The model incorporates biological (e.g., central nervous system involvement) and disorder-related factors (such as age at diagnosis), as well as environmental (family stress levels, ability to manage complex treatment regimens, etc.). We suggest that the contribution of these factors differs across disorders, with a key factor being timing of diagnosis (e.g., newborn screening versus clinical diagnosis).

In the present study, we explored the impact of the environmental factors, specifically the level of coping and well-being of parents of children with IMD and their family management. We also explored whether parent functioning was related to cognitive, social and behavioural outcomes in their children.

Method

Participants

Families were identified via Victorian Inborn Errors of Metabolism (VICIEM), an in-house metabolic databank. Inclusion criteria were parents of children (1) with

Table 1 Diagnosis of children in the sample (*n*)

Inherited metabolic disorder diagnosis	*n*
Newborn screening	
Methylmalonic aciduria (MMA) mut°	1
Isovaleric aciduria (IVA)	1
Propionic aciduria (PA)	1
Glutaric aciduria type I (GA I)	6
Maple syrup urine disease (MSUD)	1
Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	6
Clinically diagnosed	
Glycogen storage disease (GSD) type Ia and Ib	2
Glycogen storage disease (GSD) type III	1
Isovaleric aciduria	1
Ornithine transcarbamylase (OTC) deficiency	2
Total	22

mut°: this is the mutation type mut° (as opposed to mut-)

confirmed glutaric aciduria type I, methylmalonic aciduria, propionic aciduria, isovaleric aciduria, glycogen storage disease, maple syrup urine disease, ornithine transcarbamylase or very long-chain acyl-CoA dehydrogenase deficiency via newborn screening or diagnosed clinically (Table 1) and between 3 and 16 years of age; (2) not diagnosed with any other medical condition that could interfere with the interpretation of results; and (3) patients at Royal Children's Hospital (RCH) in Melbourne, Australia.

Measures

Parent Mental Well-Being

Kessler 10 Psychological Distress Scale (Kessler et al. 2002) Kessler 10 is a 10-item measure of parent anxiety and depression during the past 4 weeks. Respondents rate each item based on 5-point scale where 1 indicates none of the time, and 5 indicates all of the time. Scores were categorised based on standard cut-off values: low (10–15), moderate (16–21), high (22–30) and very high (31–50) psychological distress. It has been shown to be superior as a screener for mental disorders, good construct validity (Andrews and Slade 2001; Slade et al. 2011) and excellent reliability of 0.94 (Donker et al. 2010).

Parent Coping and Support

Parent Experience of Childhood Illness (PECI) Short Form (Bonner et al. 2006) The PECI is designed to assess emotional adjustment, coping and perceived level of support available, in the primary caregiver of children who have a chronic illness. It contains 25 questions and four scales: guilt and worry, unresolved sorrow and anger, long-term uncertainty and emotional resources. Higher scores indicate greater distress or poorer coping except for on emotional resources, which indicates a higher level of support. It demonstrates acceptable reliability between 0.72 and 0.89 (Bonner et al. 2006).

Family Functioning and Management

Family Management Measure (FaMM) (Knafl et al. 2011) The FaMM is constructed to measure family functioning and management in families who have a child with a chronic illness. It contains six subscales with 45 questions: child's daily life, condition management ability, condition management effort, family life difficulty, view of condition impact and parent mutuality. For the current study, we excluded the variables 'child's daily life' and 'view of condition impact' as we were primarily concerned with the parents' view of only their family and to reduce variables. The FaMM has good internal consistency ranging from 0.72 to 0.90 for mothers (Knafl et al. 2011) and has been validated in Australian samples (Hutton et al. 2012).

Behaviour, Emotional and Social Functioning

Strengths and Difficulties Questionnaire (SDQ) (Goodman 2001) The SDQ is a widely used measure of behaviour, emotional and social functioning that is completed by a parent regarding their child aged between 3 and 16 years. It assesses emotional symptoms, conduct problems, hyperactivity, peer problems and prosocial behaviour. Higher scores indicate more problematic behaviours except for prosocial behaviour where higher scores indicate positive behaviours. The parent form contains 25 items, and scores for each subscale are categorised into normal, borderline or abnormal according to Australian norms (Mellor 2005).

Intelligence

Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler 1999) and Wechsler Preschool and Primary Scale of Intelligence Third Edition (WPPSI-III) (Wechsler 2002) Intelligence was assessed using the Wechsler scales for the appropriate age range ($M = 100$, SD = 15).

Demographic Questionnaire, Socioeconomic Status and Background Medical History A questionnaire was administered to gather information about family, developmental and medical history. Socioeconomic status (SES) was assessed using deciles (1–10) from the Socio-Economic Index of Areas (SEIFA) 2011 data (ABS 2013b). These are calculated using census data from a number of

variables based on the population demographics of the area postcode, with 1 representing the lowest 10% of areas.

Statistical Analysis Exploratory analyses were conducted using Statistical Package for Social Sciences (SPSS) for Windows (version 18). Due to the small sample size and nonnormal distributions, exploratory nonparametric statistical analysis was utilised. It should be emphasised that this analysis was exploratory, and therefore correlations do not represent causal relationships. Spearman correlation coefficients (r_s) are reported for the relationship between parent coping and child outcomes. As both the PECI and FaMM are measures intended for parents of children with chronic illness, no normative data are available. For comparison, PECI scores are presented with results from parents of children with brain tumour from the test authors (Bonner et al. 2006). FaMM scores are presented with the results from parents of children with diabetes, also from the authors of the test (Rearick et al. 2011).

Procedure

The study protocol was approved by the Human Research Ethics Committee at the RCH (HREC #32218A). Parents were sent information letters, and once informed consent was received, they were invited to complete the study questionnaire. Most parents were part of a larger study of neurodevelopmental outcomes where children completed a full neuropsychological assessment, and some parents only completed the questionnaire component ($n = 4$).

Results

Thirty-nine patients were identified through the VCIEM database. Seven children were too young and ten families declined or did not return study questionnaires. There were 16/22 male patients (72%). Only one parent per family responded to the questionnaire (total $n = 22$; age range 28–50 years). SES ranged from 1 to 10 ($m = 6.60$, SD = 2.70). The average number of hospitalisations was 10.41 (SD = 12.62). Based on respondents' answers, 18/22 (82%) children resided with both parents, and four (18%) resided with their mother only (three separated from father, one deceased), reflecting proportions similar to those reported in the Australian population (ABS 2013a). Only one child, with GA I and diagnosed through newborn screening, was classified as intellectually disabled (<70).

Parent Mental Well-Being

Responses on the Psychological Distress Scale ($M = 17.05$, SD = 8.24) showed 16/22 parents were well adjusted, two parents were in the moderate risk range, one was in the high-risk range, and three were experiencing very high-risk levels of distress (children were diagnosed with GSD, MMA, VLCAD and OTC deficiency). Only one parent reported a history of previous depression. Of the four mothers that reported the highest distress, three had children diagnosed clinically.

Parent Coping and Family Management

Overall, parents of children with IMD showed better coping than parents of children with a brain tumour (Table 2). Whilst there were a number of parents with higher scores in comparison to the paediatric brain tumour group, three parents reported levels well above the mean, indicating that, whilst most parents were coping well, some parents were experiencing high levels of negative emotions associated with the IMD.

Scores on the FaMM were compared to those from parents of children with diabetes (Rearick et al. 2011) and were found to be similar (Table 2).

Child Outcomes

Children ($n = 18$) had IQ scores mostly within the average range ($M = 100.67$, SD = 17.53). There was no relationship found between child IQ and any of the parent distress, parent coping or family management variables. There was also no significant correlation between SES and child outcomes, although the relationship with peer problems approached significance ($p = 0.055$).

Child Behaviour and Emotional Problems (SDQ)

Results from the SDQ (Table 3) indicate that a proportion of children were displaying behaviour problems within the 'abnormal' range, which is considered to be of clinical significance (Goodman 2001; Mellor 2005). Three children of the four mothers who reported the highest distress had emotional symptoms in the abnormal range (one diagnosed through newborn screening, two diagnosed clinically).

Child's emotional symptoms such as depression and anxiety were positively correlated with parent psychological distress ($r_s = 0.47$, $p = 0.03$) and negatively correlated with parent emotional resources ($r_s = -0.42$, $p = 0.05$).

Child's conduct problems were positively correlated with condition management effort ($r_s = 0.48$, $p = 0.02$) and family life difficulty ($r_s = 0.43$, $p = 0.05$). Child's hyperactivity was positively correlated with condition management effort ($r_s = 0.61$, $p = 0.003$) and family life difficulty ($r_s = 0.61$, $p = 0.002$) and negatively correlated with condition management ability ($r_s = -0.46$, $p = 0.03$) and emotional resources ($r_s = -0.50$, $p = 0.02$).

Table 2 Parent mean scores of self-reported coping and emotional resources (PECI) and family management mean scores (FaMM)

	Possible score range	Paediatric brain tumour group ($n = 148$)[a] M (SD)	Metabolic disorders group ($n = 22$) M (SD)	Parents scoring above the mean of paediatric brain tumour group
PECI				
Guilt and worry	0–3.82	1.72 (0.77)	1.26 (0.81)	5
Unresolved sorrow and anger	0–3.63	1.51 (0.82)	0.97 (0.81)	3
Long-term uncertainty	0–4.00	1.97 (0.87)	1.20 (0.80)	1
Emotional resources	80–4.00	2.70 (0.66)	3.05 (0.63)	7
		Diabetes group ($n = 11$)[a]	Metabolic disorder group ($n = 22$)	Parents scoring above the mean of diabetes group
FaMM				
Condition management ability	12–60	50.55	52.36	7
Condition management effort	4–20	13.82	10.14	4
Family life difficulty	14–70	28.27	26.14	6
Parent mutuality[b]	8–40	31.27	31.00	9

Note: $n = 22$ parents/families, only one parent completed questionnaires per family
[a] Paediatric brain tumour results from original test authors (Bonner et al. 2006), Diabetes group results from (Rearick et al. 2011) no SD available
[b] $n = 18$ coupled parents only

Table 3 Child behavioural, emotional and social functioning ($n = 22$)

Scales	Normal (n %)	Borderline (n %)	Abnormal (n %)
Emotional symptoms	17 (77)	0 (0)	5 (23)
Conduct problems	16 (72)	3 (14)	3 (14)
Hyperactivity	14 (64)	2 (9)	6 (27)
Peer problems	15 (68)	1 (5)	6 (27)
Prosocial behaviour	18 (82)	3 (14)	1 (4)

Child Social Skills

Peer problems were not correlated with parent coping or family management variables. Prosocial behaviours on the SDQ were negatively correlated with condition management effort ($r_s = -0.48$, $p = 0.02$), suggesting that parents who indicated lower levels of effort required to manage the IMD rated their children as having higher levels of prosocial behaviours, such as sharing with others and considering others' feelings.

Discussion

The aim of the current study was to investigate coping and family management in a cohort of parents of children with IMD by exploring the relationship between parent functioning and child's cognitive, social and behavioural functioning. The results suggest that most parents were coping and managing well. Parent coping and family management were related to child behavioural and emotional outcomes and prosocial behaviours. However, no such relationship was found with cognitive outcomes.

Most parents of children with IMD in our cohort did not demonstrate elevated psychological distress according to Australian normative data, yet in a small number of parents, levels of psychological distress fell in the 'at risk' range and three parents fell in the clinically significant range (>30). Three of the six parents of children diagnosed clinically had high psychological distress scores. The interaction between the child's disorder factors, such as the age at diagnosis, potentially impacts upon parent stress; however, the number of families is too small to draw robust conclusions. The difference in parental stress between those with children diagnosed clinically and those diagnosed by newborn screening has been alluded to previously (Waisbren et al. 2003).

Some parents reported higher levels of coping in comparison with parents of children with a brain tumour (Bonner et al. 2006). This could be due to the fact that the latter may enable less time for adjustment in comparison to IMD, despite the long-term uncertainty in both groups of disorders (Bonner et al. 2008). Family management scores were comparable to those in a cohort of parents with children diagnosed with diabetes (Rearick et al. 2011) who, like children with IMD, require lifelong management, dietary changes and a high level of parental responsibility. This is despite the fact that diabetes is more prevalent, and

there is greater public understanding and knowledge about it in comparison with IMD. We speculate that coping and family adjustment in IMD may vary over time, with more distress at diagnosis and during periods of health decline in the child. It is possible that this may also vary between parents with children diagnosed clinically and newborn screening.

It is well documented that IMD present an emotional burden to parents (Cederbaum et al. 2001; Packman et al. 2007) and that parents who may be struggling to cope potentially create greater anxiety and worry in their children (Mullins et al. 2007). In the current study, poorer parent coping was associated with more emotional symptoms in children, and poorer family management was associated with more externalising behaviours such as conduct problems and hyperactivity in children. The link between family management and externalising behaviours was the strongest in our study, consistent with previous reports in parents of children with MSUD (Packman et al. 2007). Similarly, in families of children with spina bifida, family functioning predicted child externalising behaviour, whereas parental functioning predicted child internalising symptoms (Friedman et al. 2004).

This significant relationship suggests parent coping and family management are particularly salient to child emotional and behavioural outcomes, in accordance with our model. As there are no other studies that have investigated this relationship, results should be considered as preliminary and interpreted with caution as correlations are exploratory in nature and do not indicate causal relationships. Further research should aim to clarify the strength of this relationship and whether it may be reciprocal as suggested by findings in other populations such as traumatic brain injury (Taylor et al. 2001). It is important to note the potential protective value of parent emotional resources on child outcomes, as is apparent from these results.

Prosocial behaviours were related to family management, in support of our model. Previous reports of social outcomes in children and adults with an IMD suggested that they may be vulnerable to social problems (Storch et al. 2008; Packman et al. 2012; Grünert et al. 2013). The role of parents and family functioning in relation to child social outcomes is well established (Yeates et al. 2007). For example, parental stress is related to child's social adjustment (Colletti et al. 2008). Families who are coping better with the disorder may have more opportunity for family social outings, and children therefore have more social experiences.

No relationship was found between child cognitive functioning and any of the parent variables. This is consistent with recent research on children with propionic aciduria (Grünert et al. 2013). It is possible that in some disorders the biological-pathological factor is the strongest, regardless of the mode of diagnosis and parental management. There was also no relationship between SES and child outcomes, conflicting previous research that found a relationship between SES and depression in PKU children (Wu et al. 2011), possibly because of the difference between countries or due to different measures or age groups.

The main limitation of the study was a small sample size that affects power and statistical analysis. Larger sample sizes and longitudinal data would enable further statistical validation and exploration of predictive factors. A major limitation in this, and other studies, is the use of parent self-report questionnaires, which can be unreliable as parents may be biased in their responses, at times underestimating their own stress levels (Read 2003) and overestimating their child's abilities (Gramer et al. 2013). Future studies should include additional respondents (e.g., teacher, self-report) and compare responses from mothers and fathers.

Conclusion

Our results suggest that whilst most parents of children diagnosed with an IMD may cope well and their families are managing, there are a small number of parents who may not adapt as easily and this may impact on the child's behaviour and social outcomes, as proposed by our model. Questionnaires such as the PECI and FaMM may be utilised to screen parents within the clinical setting. This study highlights the need for a multidisciplinary team for supporting families and to consider biopsychosocial factors.

Acknowledgements This work was supported by the Victorian Government's Operational Infrastructure Support Program.

Take-Home Message

Most parents report coping well with having a child with IMD. However, a small subset may not adapt as easily, potentially impacting on child behavioural outcomes.

Compliance with Ethics Guidelines

Conflict of Interest

Amy Brown, Louise Crowe, Avihu Boneh and Vicki Anderson declare no conflict of interest.

All procedures followed were in accordance with the ethical standards of responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as advised in 2000 (5).

Informed consent was obtained from all patients for being included in the study. Ethics approval was granted from the RCH Human Research Ethics Committee (HREC #32218A).

Details of the Contributions of Individual Authors

Amy Brown recruited patient subjects, performed neuropsychological assessments, devised a study protocol, collected data and wrote and edited the manuscript.

Louise Crowe participated in discussions about the manuscript and assisted in reviewing and editing the manuscript.

Avihu Boneh participated in devising a study protocol, discussions about the manuscript and reviewing and editing the manuscript.

Vicki Anderson participated in devising a study protocol, discussions about the manuscript and reviewing and editing the manuscript.

Details of Previous Publication

- Abstract originating from this manuscript has been accepted for poster presentation at the SSIEM Annual Symposium, Lyon, France, September 2015.
- Cognitive and behaviour scores of children with glutaric aciduria type I and very long-chain acyl-CoA dehydrogenase deficiency are reported in the following articles:

– Brown A, Crowe L, Beauchamp MH, Anderson V, Boneh A (2014) Neurodevelopmental profiles of children with glutaric aciduria type I diagnosed by newborn screening: a follow-up case series. JIMD Reports 18:125–134
– Brown A, Crowe L, Andresen BS, Anderson V, Boneh A (2014) Neurodevelopmental profiles of children with very long-chain acyl-CoA dehydrogenase deficiency diagnosed by newborn screening. Mol Genet Metab 113(4):278–282

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JIMD Reports
DOI 10.1007/8904_2016_549

RESEARCH REPORT

Sleep Disturbance, Obstructive Sleep Apnoea and Abnormal Periodic Leg Movements: Very Common Problems in Fabry Disease

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Received: 22 September 2015 / Revised: 24 January 2016 / Accepted: 16 February 2016 / Published online: 24 March 2016

Abstract *Objectives*: To assess the prevalence of sleep disorder(s) in males with Fabry disease and explore possible association with disease phenotype.

Background: Fabry disease, an X-linked lysosomal storage disease caused by deficiency in α-galactosidase, results in intracellular accumulation of globotriaosylceramide. It causes organ dysfunction, most significantly affecting renal, cerebrovascular and cardiovascular systems. Respiratory involvement may include obstructive lung disease, reduced diffusing capacity and thickened soft and hard palates. Patients commonly develop small-fibre sensory peripheral neuropathy manifested by acroparaesthesia and pain crises. Combined with self-reported sleep disturbance and snoring, these features suggest an increased risk of sleep disorders.

Methods: In-laboratory polysomnography (PSG) studies and sleep inventory assessments, including Epworth Sleepiness Scale (ESS), were performed in a cohort of male Fabry patients. PSGs were reviewed by a sleep physician. Sleep-disordered breathing and periodic leg movements were targeted for analysis. Associations with renal, cardiovascular and cerebrovascular function were sought.

Results: Twenty males underwent overnight PSG. Patient baseline characteristics included age 43.9 ± 10.7 years, BMI 24.3 ± 3.8 kg/m^2, neck circumference 39.7 ± 3.3 cm and ESS 9.8 ± 5.1 (7/20, abnormal ESS >10). Abnormal periodic leg movement index (PLMI) was present in 95% (mean frequency 42.4 ± 28.5/min) and sleep-disordered breathing in 50% patients. Periodic leg movements were associated with pain and depression but not with increased cortical arousal.

Conclusions: Sleep-disordered breathing and abnormal PLMI are highly prevalent in patients with FD. The presence of abnormal PLMI alone appears to have minimal impact on sleep disturbance, but is associated with depression and analgesic requirement.

Abbreviations

AHI	Apnoea–hypopnea index
EEG	Electroencephalogram
EMG	Electromyogram
ERT	Enzyme replacement therapy
FD	Fabry disease
Gb3	Globotriaosylceramide
OSA	Obstructive sleep apnoea
PLMI	Periodic leg movement index
PSG	Polysomnography

Communicated by: Markus Ries, MD, PhD, MHSc, FCP

Competing interests: None declared

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Introduction

Fabry disease (FD) is an X-linked storage disorder caused by a mutation in the gene encoding the lysosomal enzyme α-galactosidase A (OMIM 300644) (Desnick et al. 2003). It leads to impaired glycosphingolipid metabolism and resultant intracellular accumulation of globotriaosylceramide

(Gb3). Metabolites of Gb3 induce inflammation, hypertrophy and fibrosis, especially of vascular endothelium and smooth muscle cells (von Scheidt et al. 1991). Clinical manifestations are widespread with the heart, kidneys, cerebrovascular system, peripheral nerves and skin most severely affected (Mehta et al. 2009; Zarate and Hopkin 2008).

Respiratory outcomes have been infrequently reported in Fabry disease, probably due to both reduced life expectancy, especially in males, and the fact that cardiovascular, renal and cerebrovascular complications, being prevalent and life threatening, dominate clinical care. Symptoms include dyspnoea in up to 69% of men and 65% of women (Mehta et al. 2004), cough, wheeze, reduced exercise tolerance (Lobo et al. 2008; Germain 2010) and fatigue (Lobo et al. 2008; Franzen et al. 2013). Obstructive airway disease has been reported in up to 26% of women and 61% of men (Rosenberg et al. 1980; Germain 2010; Franzen et al. 2013). In men, a significant decrease in all spirometric variables, including a reduced diffusion capacity, has been observed compared with unaffected controls (Magage et al. 2007), while women have decreased percent fixed vital capacity. The primary mechanism is likely to be Gb3 accumulation within the endothelial and bronchial smooth muscle cells resulting in hyperplasia of the pulmonary small airways combined with impaired smooth muscle relaxation (Magage et al. 2007; Raiman and Clarke 2010; Franzen et al. 2013).

Sleep disturbance is a frequent clinical finding; up to 68% Fabry males have excessive daytime sleepiness (Duning et al. 2009). Possible causes include central sleep apnoea (CSA) and obstructive sleep apnoea (OSA), respectively, seen in 22% and 17% of patients (Duning et al. 2013), and restless legs secondary to painful peripheral neuropathy, reported in 36% patients (Dominguez et al. 2007). In comparison 2–4% of the adult normal population have OSA (Epstein et al. 2009), while 5–15% are affected by restless legs (Earley and Silber 2014; Becker and Novak 2014; Winkelman et al. 2013). Furthermore, 2.7% of the population meet criteria for clinically significant restless leg syndrome (Winkelman et al. 2013). Enzyme replacement therapy in Fabry disease has been shown to stabilise or alleviate bronchial obstruction and sleep apnoea (Franzen et al. 2013; Wang et al. 2008; Kim et al. 2007) and reduce neuropathic pain (Dominguez et al. 2007).

We aimed to explore the prevalence and possible causes of sleep disturbance in a cohort of Fabry patients using polysomnography. Known associated risk factors including cardiovascular disease, cerebrovascular disease and iron deficiency were also assessed.

Methods

Study Population

All male Fabry patients attending the Royal Melbourne Hospital Fabry clinic were invited to undergo overnight polysomnography (PSG). Inclusion criteria were confirmed genetic diagnosis of Fabry disease and ability to tolerate overnight PSG. Patients were not required to meet specific respiratory or sleep disturbance criteria. Informed consent was obtained for data analysis. Patient characteristics of height, weight, body mass index and smoking status were collected prior to the study. Disease severity was determined by validated health surveys and quality-of-life scores. Daytime sleepiness was determined by the Epworth Sleepiness Scale (ESS) (Johns 1992) with an abnormal score being >10. Symptoms of restless legs, defined by the International RLS Study Group (2012), were assessed at the time of recruitment.

Polysomnography

Patients underwent overnight PSG at the Department of Respiratory and Sleep Medicine at Royal Melbourne Hospital, conducted by qualified sleep scientists and reported by a sleep physician. Measurements were performed according to the American Academy of Sleep Medicine guidelines (AASM, Berry et al. 2012). A 17-channel PSG recorded readings from electrocardiogram, electroencephalogram (EEG), electrooculogram, chin and lower leg (anterior tibialis) electromyograms (EMG), chest and abdominal inductance respiratory belts, pulse oximetry, body position, airflow monitoring via oronasal thermistor and nasal cannula and a snore microphone positioned on the neck.

Sleep stages and physiological events were scored according to the 2012 AASM Rules (Berry et al. 2012), with apnoea defined as a $\geq$ 90% drop in oronasal airflow for $\geq$10 s and hypopnea defined as a $\geq$30% drop in nasal airflow amplitude lasting $\geq$10 s, leading to either a $\geq$3% oxygen desaturation or a cortical arousal. Apnoeas were scored as obstructive events (apnoeic event with increased or ongoing respiratory effort throughout the period of airflow cessation) or central events (apnoeic event with absent respiratory effort throughout the period of airflow cessation). The apnoea–hypopnea index (AHI) defined as the average frequency of events per hour of sleep, with an AHI of 5–14, 15–29 and $\geq$30 defined as mild, moderate and severe, respectively.

Periodic leg movements during sleep are defined as rhythmical extensions of big toe and dorsiflexion at the

ankle, knee and hip as in retraction response. Leg movements were scored on each leg channel when they reached an amplitude $\geq$8 μV and duration of 0.5–10 s. A series of periodic movements was defined by four or more leg movements, separated by 5–90 s. Normal periodic leg movement index (PLMI) is defined as <5/h (Somers et al. 2008). Cortical arousals were defined as an abrupt shift in EEG frequency (excluding spindles) lasting more than 3 s. During REM, a concurrent increase in chin EMG amplitude lasting at least 1 s was also required. Leg movements and arousals are associated when a period of <0.5 s occurs between the end of one event and the onset of another, irrespective of the order in which they appear.

Cardiac Function

Transthoracic Echocardiography

Transthoracic echocardiography is routinely performed annually in adult male Fabry patients. Measurements were performed according to American Society of Echocardiography recommendations (Lang et al. 2005) and included interventricular septal and posterior wall diameters and left atrial diameter. Standard assessment of diastolic function was performed, including tissue Doppler imaging, to determine estimated left ventricular filling pressure. Heart failure was defined as presence of systolic and/or diastolic dysfunction. Diastolic dysfunction is defined as a left ventricular filling pressure (E/Ea) >15 or deceleration time >220 ms.

Magnetic Resonance Imaging

Presence of cardiac fibrosis was determined by late gadolinium enhancement on cardiac magnetic resonance imaging (MRI) after intravenous injection of gadobenate dimeglumine 0.1 mmol/kg.

Renal Function

Glomerular filtration rate was measured yearly by renal clearance of radionuclide ^{51}Cr-EDTA, normalised to body surface area.

Cerebrovascular Disease

Multiplanar, multisequence images were obtained through the brain of each patient using 3-Tesla system and included time of flight magnetic resonance angiography. The presence of white matter lesions, above the age appropriate level, infarct(s) and/or dolichoectasia was determined as markers of cerebrovascular disease.

Metabolic Studies

Calcium, magnesium, 25OH vitamin D and iron levels, including total iron and ferritin, were measured prior to each PSG. Anaemia was defined as haemoglobin <130 g/l, iron <10 μmol/l, ferritin <20 μg/l or transferrin saturation <20%.

Health and Quality-of-Life Scores

Disease severity index was measured according to validated scoring systems:

1. SF-36 Health Survey – The 36-item short-form health status questionnaire measured the level of disability at the time of each PSG.
2. EQ5D Generic QOL – Five dimension simple quality-of-life status questionnaire recorded at the time of each PSG.

A diagnosis of depression was made clinically, based on subjective symptoms and response to antidepressant agents.

Statistical Analysis

Statistical analysis and graphical presentation utilised GraphPad Prism v6.0c for Mac OSX (1994–2013 GraphPad Software Inc). Descriptive statistics were presented as median with interquartile range or mean and (standard deviation). Shapiro–Wilk test assessed continuous variables for normality prior to data analysis. Patient groups were compared using Mann–Whitney *U* tests. Wilcoxon rank or Fishers exact tests were used to compare the frequencies of recorded parameters within patient groups. Spearman's matrix was used to determine correlations between continuous variables. A p value <0.05 was considered statistically significant.

Results

Study Population

Thirty-two male patients with genetically confirmed Fabry disease who attended the outpatient clinic between June 2008 and June 2015 were invited to undergo nocturnal polysomnography. PSG studies were undertaken in 21 of these, with one study terminated early due to insomnia. Of the remaining 11 patients, four were international candidates unable to participate, four declined investigation and three dialysis patients were unable to attend. Patient characteristics at the time of PSG are shown in Table 1.

Table 1 Parameters of patients with and without obstructive sleep apnoea (OSA)

Variable	OSA $N = 8$	No OSA $N = 12$	p
Age	44.9 ± 11.9	42.5 ± 10.5	0.65[a]
BMI	24.3 ± 5	24 ± 2.8	0.98[a]
Neck circumference (cm)	40 (37.1–45.8)	39 (37.5–40.9)	0.51[a]
Mallampati score	3 (1–3)	3 (2–4)	0.39[a]
ESS	10.5 (6–18)	9 (4–13)	0.34[a]
DT (ms)	207.7 (183–235)	197 (182–238)	0.84[a]
IVWT (mm)	10.5 (9.3–17.3)	14 (10–15.8)	0.099[a]
PWT (mm)	10 (9–13.5)	13 (10–16)	0.16[a]
LVMI (g/m^2)	110 (84–153)	125 (96–198)	0.26[a]
E/Ea	8 (6–10)	12 (9–15)	0.08[a]
LAD (mm)	4 (3.4–4.8)	3.9 (3.3–4.6)	0.63[a]
GFR (ml/min/1.73 m^2)	86.5 (66.3–100)	82.5 (42.3–87.3)	0.33[a]
Cerebrovascular disease (n)	4 (50%)	5 (45.5%)	0.85[b]

BMI body mass index, *DT* ventricular deceleration time, *IVWT* interventricular wall thickness, *PWT* posterior wall thickness, *E/Ea* estimated left ventricular filling pressure, *LAD* left atrial diameter, *GFR* glomerular filtration rate
[a] Mann–Whitney *U*-test
[b] Fishers Exact Test

Periodic leg movements were not available in one PSG recording due to a technical difficulty.

The mean age of the 20 males completing the study was 43.9 ± 10.7 years (range 23–71 years). Body mass index was 24.3 ± 3.3 kg/m^2 (range 17.1–31.6) and neck size 39.7 ± 3.3 cm (range 33–46). Enzyme replacement therapy was being used by 16 (80%) of patients. Sleepiness scores by ESS were 9.8 ± 5.1, with 7/20 having abnormal results.

Polysomnography

Sleep-Disordered Breathing

Sleep-disordered breathing with AHI $\geq$ 5 and $\geq$15 events/h was, respectively, present in 10 (50%) and 5 (25%) of the 20 patients studied. The severity of OSA was mild in three, moderate in five and severe in two cases. No obvious demographic differences were found between patients with sleep-disordered breathing and those without. Age, BMI, neck size, and Mallampati scores were not statistically different in patients with OSA (see Table 1).

Abnormal PLMI and Restless Legs

Fifteen of the 20 patients (75%) assessed described symptoms consistent with restless leg syndrome, with none taking preventative medications. Nineteen patients had periodic leg movement syndrome having recorded abnormal PLMI >5/h. Only two males had cortical arousals that were attributable to abnormal PLMI. There were no obvious demographic differences associated with increasing PLMI (see Table 2). Increased PLMI was associated with depression (Mann–Whitney $p = 0.0166$), with increased neuropathic pain, as measured by anti-epileptic drug requirements (Mann–Whitney, $p = 0.0412$), and with markers of cardiomyopathy – left ventricular mass index and left atrial diameter (Spearman's rho = 0.53, df 16, $p = 0.035$ and rho = 0.66, $p = 0.006$, respectively). Ferritin and PLMI were negatively correlated (Spearman's rho = −0.58, df 19, $p = 0.01$), but no other metabolic parameters nor the presence of cerebrovascular disease correlated with increased PLMI.

Cardiac Function

Mean interventricular and posterior wall thicknesses for the entire cohort were 13.6 ± 4.7 and 12.2 ± 3 mm, respectively. Deceleration time and E/Ea, markers of diastolic function, were 226 ± 92 and 11.2 ± 4.8 ms, respectively. There were no differences in any cardiac parameters or diastolic dysfunction between those with or without OSA. Two patients had systolic dysfunction but neither had OSA. Neither the presence of heart failure (diastolic or systolic

Table 2 Correlation of PLMI scores with presence of risk factors for secondary restless legs

	PLMI score factor present	PLMI score factor not present	p^a
Anti-epileptic drug use	54.6 (25.1–73)[a]	36.5 (15.4–41.1)	0.041
Depression	62.9 (40.7–77)	27 (12–42)	0.017
Cardiac dysfunction	44.8 (40.3–70)	27 (12–42.1)	0.051
IVWT or PWT >12 mm	42.1 (27–67)	27.9 (12.2–57.5)	0.201
Cardiac fibrosis (MRI)	56.8 (34.6–83.4)	31.8 (12.2–42.8)	0.059
Cerebrovascular disease	46.5 (26.5–75)	38.5 (11.5–42.3)	0.094
Anaemia	27 (19.2–67)	42.1 (24–58.7)	0.624
25OH-vitamin D <55 nmol/l	42.1 (24.9–66.8)	39.7 (15.6–55)	0.486

Anti-epileptic drugs were prescribed for neuropathic analgesia with no patient having a seizure history. Depression diagnosed with clinical symptoms requiring treatment with anti-depressive agents

IVWT, interventricular wall thickness; *PWT* posterior wall thickness; cerebrovascular disease – cerebrovascular accident or dolichoectasia; anaemia – haemoglobin <130 g/l, transferrin saturation <20% or iron <10 μmol/l

[a] Median and interquartile range

[b] Mann–Whitney *U*-test

dysfunction), cardiac fibrosis measured by MRI nor cerebrovascular disease was associated with increased OSA.

Renal Function

Overall renal function (as measured by renal clearance of radionuclide ^{51}Cr-EDTA) was 75.7 ± 26.3 ml/min/1.73 m^2 and was not statistically different between patients with or without OSA (83.9 ± 22.4 versus 70.3 ± 28.2 ml/min/1.73 m^2, $p = 0.26$).

Cerebrovascular Disease

Nine (45%) of the studied males patients had evidence of cerebrovascular disease on brain MRI. Seven of these patients had established infarcts, with white matter lesions greater than expected for age. Four patients had dolichoectasia, two of these also having had infarcts. There was no association of cerebrovascular disease, defined by the presence of white matter lesion, dolichoectasia or established infarcts, with either OSA (Fisher exact $p = 0.85$) or increased PLMI (Mann–Whitney, $p = 0.094$). No patient experienced seizures or had been diagnosed with epilepsy.

Metabolic Studies

Calcium and magnesium were within normal ranges. Three patients were anaemic with a haemoglobin <130 g/l and another four were iron deficient (either iron <10 μmol/l or transferrin saturation <20%). Ferritin level was negatively correlated with increased PLMI in this cohort. Eleven patients had suboptimal vitamin D with level in range 26–54 nmol/l and three of these were deficient with level < 25 nmol/l, but no association with increased PLMI was found.

Health and Quality-of-Life Scores

The mean and median SF-36 scores of this male cohort, across all domains, were lower than reported normative controls, but there was no association between PLMI and any domain.

EQ5D generic quality-of-life average score was 62 ± 17, with no association between EQ5D score and PLMI. Ten patients were being treated with antidepressant agents for clinical diagnoses of depression.

Discussion

We have identified very high prevalence of sleep-disordered breathing, OSA (50%) and abnormal periodic leg movements (94.7%), in our cohort of male Fabry patients. Our patients had wide phenotypic variation, but significant disease severity was well represented, with 59% having cardiomyopathy and 37% cerebrovascular disease. Limited data has previously been available regarding the severity of sleep disorders in Fabry disease. Duning et al. 2013, in a mixed-gender cohort of 23 Fabry patients, reported an incidence of OSA (17%) and CSA (23%). However, this group had only mild to moderate disease with no cerebrovascular events and a low incidence of cardiomyopathy (22%). We found no cases of CSA in our male cohort despite greater disease severity. The single published study of restless legs in Fabry disease (Dominguez et al. 2007)

reported an overall incidence of 36% in a mixed cohort of 11 Fabry patients. In their study only 50% of males were affected, but these were significantly younger (age range 19–32) than our patients.

None of the traditional risk factors for OSA, including BMI, neck size and Mallampati score, explained the high incidence of obstructive sleep apnoea in our cohort. Indeed despite the high prevalence of both heart failure and hypertrophic cardiomyopathy, no association with OSA was present for any cardiovascular parameter. In addition there was no association of OSA with cerebrovascular disease, impaired renal function or vitamin D deficiency. Likely contributors to the high incidence of OSA in Fabry disease are facial dysmorphology (Cox-Brinkman et al. 2007; Ries et al. 2006) including prominent nasal bridge and pseudo-acromegalic features with prognathism (Hogarth et al. 2012) and possible thickening of upper airway. While pharyngeal diameters in our cohort were within the normal range, and Mallampati scores normal, the upper airways may well be less compliant, possibly contributing to snoring.

Abnormal PLMI, or periodic leg movement syndrome, and restless leg syndrome are common afflictions affecting 5–15% of the general population (Earley and Silber 2014; Becker and Novak 2014; Winkelman et al. 2013) and associated with insomnia in 50–85% of people (Becker and Novak 2014). We identified only two patients having cortical arousals secondary to periodic leg movement that were greater than expected age norms. Increased PLMI within our cohort could not be explained by secondary factors including anaemia, iron deficiency, cerebrovascular or cardiovascular disease. Increased PLMI was significantly associated with left atrial diameter and left ventricular mass and approached significance with cardiac dysfunction. These factors probably reflect the overall burden of disease. Ferritin itself was inversely correlated with PLMI in this study. PLMI was also significantly higher in patients with OSA, as seen in previous studies (Baran et al. 2003). It is possible that OSA itself increases the risk of developing abnormal PLMI through increased catecholamine or other hormone release.

Abnormal PLMI is usually multifactorial and is frequently found in chronic pain conditions and peripheral neuropathies. However, PLMI scores do not correlate well with pain scores (Winkelman et al. 2013), perhaps reflecting the difficulties involved in scoring pain. Depression and anxiety are both highly prevalent in Fabry disease, ranging from 15% to 62% people, and are strongly associated with neuropathic pain (Bolsover et al. 2014). Within our cohort, associations between higher PLMI and both depression and increased use of neuropathic analgesics were present, but did not extend to quality-of-life scores. Sequelae of elevated PLMI and restless legs include greater risks of depression, cerebrovascular disease and cardiovascular disease (Ferini-Strambi et al. 2014). Depression was highly prevalent in our study group, and the strong association with increased PLMI is likely to reflect the overall burden of disease and presence of peripheral nerve injury. Presence of cardiovascular and cerebrovascular disease did not correlate with either PLMI or OSA, but our small cohort size may limit this observation.

There are inherent limitations in studying Fabry patient cohorts. Firstly, phenotype is heterogeneous even within a small patient group. Secondly reported neuropathic pain is subjective, with frequent exacerbations resulting in variable neuralgic requirements. No data on nerve conduction studies or nerve biopsy data were available to further determine neuropathic involvement in individual patients.

Male Fabry patients carry a high burden of multi-organ disease in addition to significant pain and increased risk of depression. The very high prevalences of abnormal PLMI, OSA and restless legs that we observed are likely to further impact on patients' well-being, and treatment may improve quality of life. The impact of sleep disturbance on individual partners of the studied Fabry patients was not assessed. Extrapolation from the general community, however, would suggest a negative influence. This may add further strain to relationships dealing with a chronic and progressive disease.

Future investigation with repeated PSG may determine the level of response but is limited by availability. As for many manifestations of Fabry disease, it will be instructive to follow changes in abnormal PLMI and OSA as disease-specific treatment evolves and is instituted earlier.

Acknowledgements The authors thank Alexander Gorelik for invaluable assistance with statistics, Andrew Perkins for assistance with method preparation and performing sleep studies and Elizabeth Centra and Donna North for assistance with patient support.

Take-Home Message

Sleep-disordered breathing and abnormal periodic leg movements are highly prevalent in male Fabry patients and significantly impact on patient well-being.

Compliance with Ethics Guidelines

Details of Contributions of Individual Authors

Andrew Talbot was primarily responsible for planning the study, patient recruitment and primary data interpretation, including statistical analysis and original manuscript preparation.

Gary Hammerschlag conducted patient data collection, initial sleep study analysis and data interpretation including manuscript editing.

Jeremy Goldin performed sleep study interpretation, data collection and manuscript editing.

Kathy Nicholls contributed to patient recruitment and consent for study, data interpretation and original manuscript preparation.

Conflict of Interest: Nil Direct

Andrew Talbot has received research support, speaker honoraria and travel assistance from Shire Corporation and Sanofi Corporation, speaker honoraria and travel assistance from Dainippon Sumitomo Pharma Co and research support from Amicus Therapeutics and Protalix Biotherapeutics.

Kathy Nicholls has received research support, speaker honoraria and travel assistance from Shire Corporation and Sanofi Corporation and research support from Amicus Therapeutics and Protalix Biotherapeutics.

Gary Hammerschlag declares that he has no conflict of interest relevant to this project.

Jeremy Goldin declares that he has no conflict of interest relevant to this project.

Informed Consent

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

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

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JIMD Reports
DOI 10.1007/8904_2016_552

CASE REPORT

Spurious Elevation of Multiple Urine Amino Acids by Ion-Exchange Chromatography in Patients with Prolidase Deficiency

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Received: 13 January 2016 / Revised: 29 February 2016 / Accepted: 02 March 2016 / Published online: 12 April 2016

Abstract The enzyme prolidase cleaves dipeptides where the C-terminal amino acid corresponds to proline or hydroxyproline. As a consequence, a deficiency of this enzyme leads to accumulation of these dipeptides, which correspondingly are found to be elevated in urine. In fact, the absence of dipeptiduria is sufficient to rule out a diagnosis of prolidase deficiency. However, given the fact that these dipeptides elute at the same position as more common amino acids, the analyzer's software will instead call an elevation of these corresponding amino acids. Thus, an elevation of glycylproline, aspartylproline, glutamylproline, threonylproline and serylproline, valylproline, leucylproline, isoleucylproline, alanylproline, phenylalanylproline, and lysylproline will instead be interpreted as an elevation of leucine, citrulline, methionine, isoleucine, beta-aminoisobutyric acid, gamma-aminobutyric acid, ethanolamine, tyrosine, histidine, and anserine/carnosine, respectively. This particular profile of elevated amino acids, however, can easily be overlooked. We hope that the recognition of this characteristic pattern of falsely elevated urinary amino acids will aid in the recognition of prolidase deficiency.

Communicated by: Ina Knerr, MD

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2016_552) contains supplementary material, which is available to authorized users.

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Introduction

Prolidase deficiency is characterized by skin lesions (typically lower extremity ulcers), recurrent infections (particularly of the skin and respiratory tract), chronic lung disease, dysmorphic facial features, variable intellectual disability, short stature, mild anemia and thrombocytopenia, hypergammaglobulinemia, elevation of liver enzymes, and low complement levels. Only approximately 90 patients have been reported in the medical literature, although many cases probably remain undiagnosed (Ferreira and Wang 2015).

Prolidase cleaves dipeptides where the C-terminal amino acid is proline or hydroxyproline. Such amino acids are known as imidodipeptides, and they can be found in massive amounts in urine of patients with prolidase deficiency. The absence of imidodipeptiduria is sufficient to rule out a diagnosis of prolidase deficiency (Freij and Der Kaloustian 1986). However, the authors are aware of a few instances when the peaks produced by imidodipeptides in urine amino acid analysis were missed. Sometimes, they have been interpreted as non-specific ninhydrin-positive interfering compounds. Other times, the imidodipeptides, due to coelution, are reported as elevations of multiple amino acids in a non-specific pattern. The imidodipeptides have been known to obscure the normal peaks for methionine, isoleucine, leucine, and tyrosine (Buist et al. 1972; Wysocki et al. 1988).

We present the urine amino acid profiles of two patients with prolidase deficiency, and call attention to the fact that this rare condition leads to a characteristic appearance of unusual peaks that elute at the same position as other amino acids, and can thus lead to the erroneous interpretation of elevated leucine, isoleucine, methionine, citrulline, beta-aminoisobutyric acid, gamma-aminobutyric acid, tyrosine, and ethanolamine in urine.

Methods

The manuscript is a retrospective case series that does not require ethics committee approval at our institution. No identifiable patient information is provided in the manuscript.

A urine sample was obtained from a 24-year-old woman with chronic lower extremity ulcers of unknown etiology since 8 years of age (patient 1). Urine amino acids were quantitated by cation-exchange chromatography with post-column ninhydrin derivatization at a reference laboratory (Mayo Clinic's Biochemical Genetics Laboratory) using a Biochrom amino acid analyzer. Subsequently, we obtained urine samples from a patient with known, molecularly confirmed prolidase deficiency at 5 months and 16 months of age (patient 2), and measured amino acid concentrations by cation-exchange chromatography on a Biochrom 30 analyzer in our laboratory. A running time of 2 h was needed for one complete run of the profile. UV detection was obtained at two wavelengths, 570 nm and 440 nm, according to the type of the amino acids concerned. The free amino groups react with ninhydrin to produce an intense blue or purple color – Ruhemann's purple – which is best detected at 570 nm. The imino group of proline and hydroxyproline, on the other hand, produces a yellow color upon reacting with ninhydrin, that is best read at a wavelength of 440 nm. For patient 1, the internal standards used were glucosaminic acid (GSA) and aminoethylcysteine (AED), while for patient 2 the internal standards were *S*-2 aminoethyl-L-cysteine hydrochloride (ALCH) and D-glucosaminic acid (GLAM). The urine samples were not exposed to acid hydrolysis. Quantification of amino acids was performed using EZChrom Elite software after a calibration curve was obtained.

Results

Multiple amino acids were reported as being elevated in patient 1 (see Fig. 1a and Table 1). The calibration sample is shown in Fig. 1b. This profile was interpreted as an increased excretion of a few unrelated amino acids in a pattern not characteristic for any specific disorder. However, given the clinical suspicion of prolidase deficiency, sequencing for *PEPD* was obtained, and a homozygous canonical splice site mutation was found.

In patient 2, an elevation of similar amino acids was provided on the report (see Supplementary Fig. 1 and Table 1). A control urine amino acid sample processed in the same analyzer is shown in Supplementary Fig. 2.

Discussion

The imidodipeptides that accumulate in patients with prolidase deficiency coelute with other amino acids. The presence of these broad, unusual imidodipeptide peaks is thus reported instead as an elevated concentration of various urine amino acids, as seen in our patients. We are indeed aware of several instances where the laboratory has reported a non-specific elevation of various amino acids in a pattern not characteristic for any known inborn error of metabolism, as occurred in patient 1.

We inferred the relative position of the different imidodipeptide peaks (Table 2) from the previously published literature (Buist et al. 1972; Duran 2008; Goodman et al. 1968; Lou and Hamilton 1979; Nusgens and Lapiere 1973; Powell et al. 1974). Despite coelution of the imidodipeptides with several amino acids, the 570/440 absorbance ratio, a constant for each amino acid, was in each case much lower than expected. This is due to increased heights of the peaks at 440 nm, in turn secondary to the fact that the imidodipeptides contain proline, which reacts with ninhydrin to form a yellow compound with absorption maximum at 440 nm. A simple visual inspection of the 440 nm channel in affected patients (Fig. 1a and Supplementary Fig. 1) as compared to controls (Fig. 1b and Supplementary Fig. 2) can attest to the increased height of these peaks – leading to the aforementioned decreased 570/440 ratio.

This peculiar pattern of urine amino acid elevation is noted on unhydrolyzed samples. When the urine samples are exposed to an equal volume of 6N hydrochloric acid and subsequently hydrolyzed by heating at 100°C for 20–24 h, then the unusual peaks will disappear, giving rise instead to a marked increase in proline, hydroxyproline, and glycine (Ferreira and Wang 2015).

Although not performed, we would expect similar profiles to the patients presented here (Fig. 1a and Supplementary Fig. 1) on any of the dedicated amino acid analyzers such as Beckman and Hitachi instruments given the similarities of the methodologies to the Biochrom.

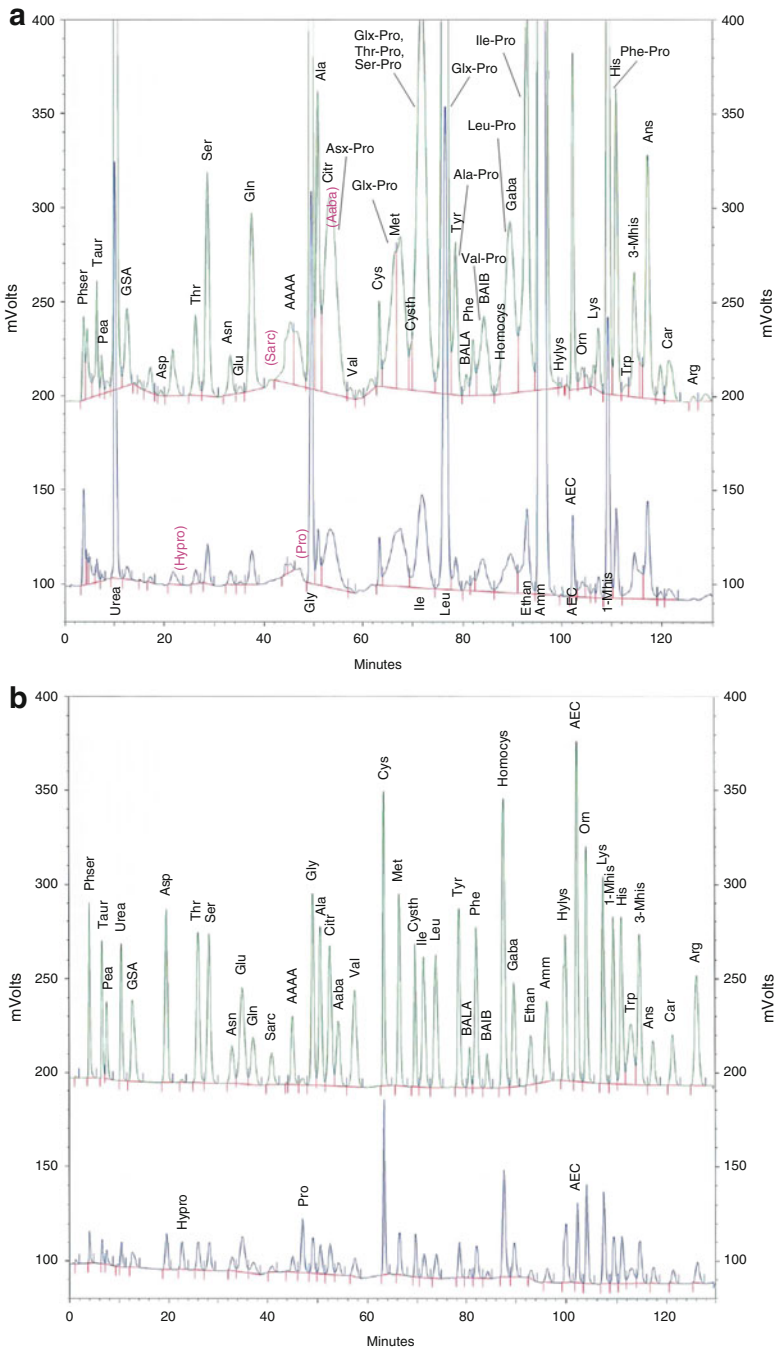

Fig. 1 (**a**) Urine amino acid chromatogram from patient 1. (**b**) Calibration sample. The 570 nm channel appears on top in *green*, and the 440 nm channel at the bottom of each figure, in *blue*

Conversely, we would not expect to see the imidodipeptide peaks using LC-MS or LC-MS/MS methods as the multiple reaction monitoring (MRM) pairs for these compounds are not typically incorporated into these methods and had the urine been analyzed this way, it likely would have been interpreted as normal pattern of urine amino acids.

It is valuable to note that the order of the tallest peaks, even in the same patient, varied over time. However, the tallest peaks tend to be those coeluting with leucine, isoleucine, methionine, beta-aminoisobutyric acid, and citrulline, followed by smaller peaks coeluting with gamma-aminobutyric acid, tyrosine, and ethanolamine.

Table 1 Reported concentration of urine amino acids (in nmol/mg creatinine)

Amino acid	Patient 1	Reference range	Patient 2 (5 months)	Patient 2 (16 months)	Reference range
Citrulline	**1,084**	8–50	**5,281**	**3,411**	0–124
Methionine	**411**	38–210	**7,024**	**4,736**	0–644
Isoleucine	**1,896**	16–180	**12,131**	**5,144**	0–259
Leucine	**3,516**	30–150	**10,055**	**5,998**	18–220
Tyrosine	278	90–290	**2,477**	**1,363**	0–934
BAIBA	**1,482**	10–510	**6,581**	**8,855**	46–680
GABA	NR	–	**2,691**	**3,273**	0–166
Ethanolamine	NR	–	**1,789**	**2,913**	0–1,448

Values in boldface are elevated with respect to the reference range
BAIBA beta-aminoisobutyric acid, *GABA* gamma-aminobutyric acid, *NR* not reported

Table 2 Urinary dipeptides and their corresponding coeluting peaks

Dipeptide	Neighboring peak
Asx-Pro (Asp-Pro or Asn-Pro)	Citrulline
Glx-Pro (Glu-Pro or Gln-Pro)	Methionine
Glx-Pro, Thr-Pro, and Ser-Pro	Isoleucine
Gly-Pro	Leucine
Ala-Pro	Tyrosine
Val-Pro	β-aminoisobutyric acid (BAIBA)
Leu-Pro	Gamma-aminobutyric acid (GABA)
Ile-Pro	Ethanolamine/ammonia
Phe-Pro	Histidine/1-methylhistidine
Lys-Pro	Anserine/carnosine

This is in keeping with the known excretion pattern seen in patients with prolidase deficiency, where the more abundant imidodipeptides are Gly-Pro, Asx-Pro, and Glx-Pro (Hechtman 2014).

Conclusion

Prolidase deficiency leads to an increased excretion of urinary imidodipeptides that appear as broad, unusual peaks in the chromatogram. These peaks elute at the same position as other amino acids, leading to an incorrectly increased quantitation of urinary leucine, isoleucine, methionine, beta-aminoisobutyric acid, and citrulline – and to a lesser extent also of gamma-aminobutyric acid, tyrosine, and ethanolamine. We hope that our report will help elicit suspicion of this rare inborn error of metabolism.

A Concise One Sentence Take-Home Message

Prolidase deficiency can lead to a particular pattern of spurious elevation of several urine amino acids, given their coelution with uncleaved imidodipeptides.

Compliance with Ethics Guidelines

Conflict of Interest

– Carlos R. Ferreira and Kristina Cusmano-Ozog declare that they have no conflict of interest.

- No identifying information about patients is included in the article.
- This article does not contain any studies with animal subjects performed by the any of the authors.
- Writing, preparation, and critical review of the manuscript: CRF and KCO.

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JIMD Reports
DOI 10.1007/8904_2016_554

RESEARCH REPORT

Quick Diagnosis of Alkaptonuria by Homogentisic Acid Determination in Urine Paper Spots

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Received: 24 September 2015 / Revised: 07 March 2016 / Accepted: 08 March 2016 / Published online: 14 April 2016

Abstract *Objectives*: Two methods are described for homogentisic acid (HGA) determination in dried urine spots (DUS) on paper from Alkaptonuria (AKU) patients, devised for quick early diagnosis. AKU is a rare autosomal recessive disorder caused by deficiency of homogentisate 1,2-dioxygenase, yielding in accumulation of HGA. Its massive excretion causes urine darkening by exposure to air or alkalinization, and is a diagnostic marker. The deposition of polymers produced after HGA oxidation within the connective tissues causes ochronotic arthritis, a degenerative joint disease manifesting in adulthood and only rarely in childhood. No early diagnosis is usually accomplished, awareness following symptom development.

Design and methods: Two methods were designed for HGA determination in DUS: (1) a rapid semi-quantitative reliable method based on colour development in alkali and quantification by comparison with dried paper spots from HGA solutions of known concentration and (2) a quantitative and sensitive HPLC-linked method, previously devised for purine and pyrimidine analysis in urine and plasma.

Results: Colour intensity developed by DUS after alkali addition was proportional to HGA concentration, and calculated amounts were in good agreement with quantitative analysis performed by RP-HPLC on DUS and on urines as such.

Conclusions: DUS, often used for different diagnostic purpose, are easily prepared and safely delivered. The simple and quick colour method proposed provides reliable HGA assessment and is fit for large screening. HGA concentration determined in 10 AKU patient DUS by both methods 1 and 2 was in agreement with direct urine assay and in the range reported by literature.

A reliable HGA quantification based on colour development in paper urine spots is validated by HPLC-linked HGA quantification, and proposed as a quick diagnostic tool for AKU patients.

Introduction

Alkaptonuria (AKU, OMIM: 203500) is a rare disease, associated with inherited recessive mutations in *HGD* gene leading to homogentisate 1,2-dioxygenase (HGD, E. C.1.13.11.5) deficiency. As a result homogentisic acid (HGA), deriving from tyrosine and phenylalanine, accumulates and is massively excreted in urine, causing its darkening after air exposure. Brown coloured urine is considered a pathognomonic sign of AKU. The deposition of an "ochronotic" pigment produced after circulating HGA oxidation within the articular connective tissues causes ochronotic arthritis, a degenerative joint disease (Ranganath et al. 2013; Millucci et al. 2015); deposition may also occur in cardiac valves, leading to cardiovascular pathologies. Currently, there is no therapy for AKU, but a clinical trial with nitisinone is in progress (Ranganath et al. 2016).

AKU diagnosis may be delayed until the ochronotic arthropathy manifests, usually in adulthood, and very few data are available about childhood and the earlier stages of the disease. Dark colour development in urines can be delayed depending on urine acidity, and misdiagnosis can

Communicated by: Jörn Oliver Sass

Competing interests: None declared

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occur. No clear relationship between circulating HGA amount and symptom severity, age, sex, etc., has been described. Early identification of AKU patients would allow epidemiologic studies and important correlations with physiopathologic parameters.

Dried urine spots (DUS) are used in the diagnosis of inherited disorders, included AKU as a sporadic finding, usually as a qualitative diagnostic tool (Bradley 1975; Barbas et al. 2002).

Dark brown colour is well known to develop by adding alkali (e.g. sodium hydroxide) to either HGA solutions or AKU urines (Bradley 1975; Mathieu et al. 1997; Zibolen et al. 2000; Koska and Srsen 1977; Zhao et al. 2009; Pecker et al. 2008).

In this paper two methods for HGA determination in DUS are presented: method 1, a rapid semi-quantitative method based on colour development in alkali, to be used for the early diagnosis of AKU, and method 2, a quantitative reliable HPLC-linked method. The novelty in the proposed method 1 is HGA quantification by a simple reference colour scale quickly providing reliable results. The advantage in method 2 is the accurate HGA quantification in single dried urine spots. Results from 10 AKU patients are presented.

Materials and methods

Chemicals for HPLC separation were of the highest quality available and reagents of analytical grade were purchased from SIGMA (St. Louis MO, USA). The filter paper used to spot urine was S&S® 2992™, Schleicher & Schuell.

Patients

Adult patients (five females and five males ranging 42–69 years, 54.7 ± 10.5 mean ± sd) presenting different degrees of ochronotic arthropathy were studied after the clinical diagnosis of AKU was established. None of the patients was under specific treatment. Age-matched controls of both sexes with no arthropathies or metabolic disorders were also analysed ($n = 28$). Urine samples (single specimen or 24 h collection) were obtained from patients and controls and dried urine spots were prepared.

The whole study was conducted following the approval of Siena University Hospital Ethics Committee. Patients gave a written informed consent prior to inclusion in the study. The informed consent conformed to the standards set by the latest revision of the Declaration of Helsinki containing few substantive changes relative to the April 2013 iteration.

Dried Urine Spot (DUS) Preparation

Urine samples from 10 AKU patients were both analysed as such and used for DUS preparation by dropping 30 µL on filter paper S&S® 2992™ Schleicher & Schuell. Spots were allowed to dry in darkness for at least 3–4 h at room temperature. Spots of HGA solutions from 1.4 to 46 mmol/L or from 2.5 to 40 mmol/L were prepared following the same procedure. DUS and spots of HGA solutions were used both for colour development (method 1) or eluted for HPLC analysis (method 2, described in Sects. 2.4 and 2.5).

Semi-Quantitative Colour-Based Method for DUS (Method 1)

Brown colour was developed dropping 10 µl of 1 mol/L NaOH on dried spots of both patient urine and HGA solutions. Good colour development was also obtained adding 1 mol/L KOH, K_2CO_3, but not NH_4OH (data not shown); NaOH was used routinely.

Semi-quantitative evaluation of urine HGA was achieved by comparison of colour intensity developed by DUS with that developed by HGA solution spots.

HPLC Analysis

A previously described HPLC method, initially devised to detect purines, pyrimidines, and other metabolites in plasma and urines (Micheli et al. 1999), was used to measure HGA in DUS, in HGA solution spots and in urines.

HPLC apparatus was a System Gold module 125S with a mod. 168 diode array detector module. The column was Phenomenex C18 RP (75 mm × 4.6 mm × 3 µm) equipped with Phenomenex Security guard column (4 × 3 mm). Analysis was performed by gradient elution using 10 mmol/L potassium phosphate, pH 5.5 (Eluant A) and methanol (Eluant B). Sample injection volume was 50 µL. The HPLC procedures were performed at room temperature, with 1 mL/min flow rate; absorbance was monitored at 260 and 280 nm. The elution pattern was as follows: isocratic phase at 100% A for 4 min, then to 21% B in 4 min, then immediately to 30% B and back to initial conditions after 3 min; initial conditions were restored in 7 min, total run time was 18 min. Peak identities were confirmed by retention time, coelution with added standards, 280/260 nm absorbance ratios and UV/Vis spectra. All standard solutions (creatinine, uric acid, L-tyrosine, hypoxanthine, xanthine, phenylalanine, tryptophan and HGA) were prepared in deionized water and stored at -20°C. HGA 40–50 mmol/L stock solutions were aliquoted and stored at

−20°C; suitable dilutions were obtained when needed after quick defreezing. Concentration/peak area linear plots were developed for quantification. A minimum of six calibration points, from 0.02 to 0.8 mmol/L, were used for HGA quantification. Linearity was checked using standard curves fitted by linear regression, and performance of fitted curves is presented as the coefficient of determination (r^2).

Accuracy was determined as closeness to the nominal spiked concentration in control urine with $n = 6$. Precision was determined with $n = 6$ and expressed as coefficient of variation (CV). The limit of quantification was the lowest concentration allowing a CV<20%.

Quantitative HPLC-Linked Method for DUS (Method 2) and Urine Analysis

Punches from DUS or HGA solution spots (7-mm-diameter) were transferred to Eppendorf tubes and eluted for 30 min with 250 µL of 10 mmol/L potassium phosphate buffer, pH 5.5, with occasional gentle shaking. Spot eluates were analysed by RP-HPLC as described above. HGA content in DUS was quantified on the basis of concentration/peak area linear plots obtained from eluted spots of HGA solutions of known concentration (prepared as described in Sect. 2.2). Stability of HGA in DUS was checked over 2 weeks storage.

Urine samples from all patients were also directly analysed by the above RP-HPLC method, after heating at 56°C for 30 min and diluting with 30 volumes of 10 mmol/L potassium phosphate buffer, pH 5.5. Urine HGA content was expressed as mmol/L, or mmol/mol creatinine, or, when possible, mmol/24 h.

HGA recovery during 24 h urine storage at 4°C was checked by adding a known amount (final 10 mmol/L) to control urines; storage was conducted both in the absence and presence of acid (final 0.25 mol/L HCl or H_2SO_4).

Results

Colour-Based Semi-Quantitative Method (Method 1)

Both HGA solutions and their dried spots developed brown colour after addition of NaOH. Colour intensity was proportional to HGA concentration from 1.4 (the lowest developing appreciable colour) to 46 mmol/L (Fig. 1a, from 1 to 6). An example of the brown colour developed after alkali addition by five AKU patient DUS is shown in Fig. 1b (1–5); no colour was developed by control (number 6). Concentration values were estimated by comparing colour intensity with HGA solution spots and were expressed as the range between the two standard spots comparable to patient DUS. For each of the ten patients examined, comparison of colour-based values with those determined by Method 2 in eluted spots and by direct HPLC analysis of urines supported the reliability of semi-quantitative evaluation (Table 1).

The developed colour was stable for at least 3 months; afterwards, slight intensity decrease was observed to occur in both DUS and HGA solution spots, still allowing HGA estimation by comparison.

HPLC Analysis: Method Validation

The described HPLC method, initially devised to measure purines, pyrimidines and other metabolites in plasma and urines (Micheli et al. 1999), allows the identification and quantification of several compounds, including creatinine, uric acid, tyrosine, hypoxanthine, xanthine, HGA, phenylalanine and tryptophan (Fig.2a and b).

Concentration/peak area plots developed for HGA quantification exhibited a good linear fit over the range examined (20–800 µmol/L) with $r^2 = 0.999$.

Accuracy was determined in urine matrix. Percentage recovery of a nominal amount of HGA (8–10 mmol/L) spiked into matrix ($n = 6$) was 94.3–110.8%. Imprecision was determined with $n = 6$ using spiked urines (8–10 mmol/L HGA); %CV was 8.7%.

The limit of quantification was 5 µmol/L, the lowest concentration allowing a CV<20%.

HGA recovery during 24 h urine storage at 4°C (checked as described in Sect. 2.5) was 94.3–107.8% in the absence and 102.3–110.8% in the presence of acid.

HPLC Analysis of Urines and DUS (Method 2)

HGA in DUS and in urines was quantified as described under methods (see Sects. 2.4 and 2.5). Concentration/peak area plots obtained from eluted spots of five HGA solutions of known concentration exhibited a good linear fit over the range examined (2.8–23 mmol/L), with $r^2 = 0.995$.

HGA amount measured in DUS from nine patients (14.0 ± 5.2 mmol/L) was in good agreement with that directly measured in their urines (14.7 ± 4.1 mmol/L) with 76.5–117.0% recovery. Reproducibility of results in DUS analysis was tested by repeating the whole procedure on six separate DUS from one patient's urine (CV = 6.9%). Reproducibility test was repeated after 2 week storage of DUS, with no appreciable loss compared to fresh spots (recovery 93.7–105.6%; CV = 8.7%). These data and values expressed as mmol/mol creatinine (1,741 ± 541) or mmol/24 h (17.0 ± 7.4) were in agreement with those reported in the literature (Bory et al. 1989; Mannoni et al. 2004; Hughes et al. 2014; Ranganath et al. 2016). No appreciable HGA could be detected in DUS or urine samples from controls.

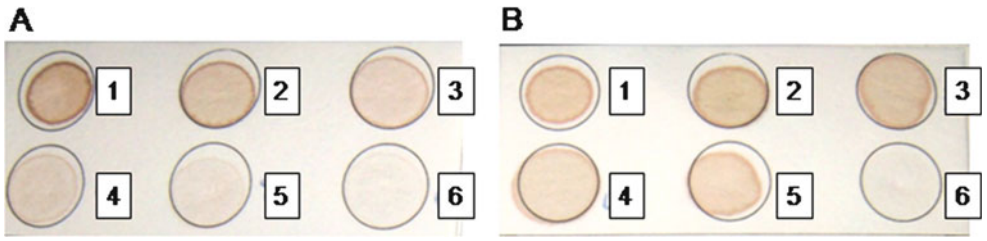

Fig. 1 Colour development in alkali – (**a**) HGA solution spots (from 1 to 6: 46, 23,11.5, 5.8, 2.8, 1.4 mmol/L); (**b**) AKU patient urine spots (from 1 to 5); control urine spot (6). NaOH (1 mol/L 10 μl) was dropped on all spots

Table 1 HGA concentration (mmol/L) measured in DUS and urine of AKU patients. DUS: method 1, colour development (estimated range); method 2: HPLC-linked detection. Urine: direct HPLC-linked detection

Patient	Age	Sex	DUS method 1	DUS method 2	Urine
1	69	F	11.5–23	18.7	15.9
2	50	F	10–20	15.4	16.9
3	41	F	10–20	11.9	13.2
4	48	F	11.5–23	11.9	16.9
5	67	F	10–20	17.0	16.7
6	42	M	11.5–23	19.6	16.0
7	57	M	11.5–23	na	12.6
8	53	M	5–10	4.0	5.3
9	65	M	10–20	18.4	20.3
10	64	M	10–20	9.2	11.4

na not analysed

Results obtained by methods 1 and 2 in DUS, and by direct urine analysis by HPLC are summarized in Table 1.

There was no significant difference in urine HGA concentration between female and male patients (*t* test analysis), nor was there any significant correlation with age in this cohort of patients.

Discussion and Conclusions

Early diagnosis of AKU is not frequently accomplished and at our knowledge only few data are available about HGA concentration in biological fluids of AKU patients before ochronotic symptoms occur. Availability of simple diagnostic methods would allow increased awareness of the disease before irreversible ochronotic arthropathy occurs. Early therapeutic trials to prevent such serious consequences would also be possible.

Biochemical diagnosis is based on the detection of HGA in body fluids, mainly urine, by different analytical methods such as high performance liquid chromatography (Bory et al. 1989), gas chromatography, capillary electrophoresis, mass spectrometry (Hughes et al. 2014), enzymatic and spectrophotometric methods (Tokuhara et al. 2014). Such sensitive methods can only be performed in specialized laboratories with appropriate equipments. In this paper we present two methods developed for the analysis of dried urine spots. Method 1 is a simple method based on colour development by alkali addition on paper dried urine spots (DUS), suitable for HGA approximate quantification. This method does not require any complex equipment and can be used by practitioners or parents, quickly providing a rough evaluation of HGA presence in urines, indicating the range of HGA excretion. Industrial production of alkali pre-soaked papers would render such operation even easier and suitable for the early diagnosis of alkaptonuria. Similar procedures have been reported by other authors as “dipstick test” with a mere qualitative purpose (“yes-or-no” colour development) (Barbas et al. 2002; Zibolen et al. 2000; Koska and Srsen 1977). The novelty in our system is that colour-based quantification on paper DUS can be achieved by comparison with spots of HGA solutions of known concentration, sensitivity reaching 1.4 mmol/L. Such value, the lowest HGA concentration displaying appreciable colour development in dried spots, is tenfold lower than usually found in adult patients; nevertheless such sensitivity might be useful for early detection, e.g., in children. Larger screening in all members of AKU affected families, and early identification of the disease might be achieved by this method, and only selected samples should be conveyed to laboratories for accurate quantification.

Method 2 described in this paper allows the quantification of HGA in DUS by our HPLC-linked method, with results in good agreement with direct urine analysis. Such HPLC-linked method with UV detection is sensitive and reliable and can be performed in many laboratories.

The use of HPLC-linked methods for HGA analysis in body fluids is not new (Bory et al. 1989); the novelty of the proposed method lies in its utilization for DUS, which are easy to collect and deliver, thus representing a low-cost reliable analytical tool for AKU early diagnosis.

Both urine and serum HGA concentration are of paramount importance in the follow up of the disease and in monitoring the effect of drugs. Due to its sensitivity (lower limit 5 μmol/L HGA) the described HPLC method can be applied to any body fluid with low HGA content

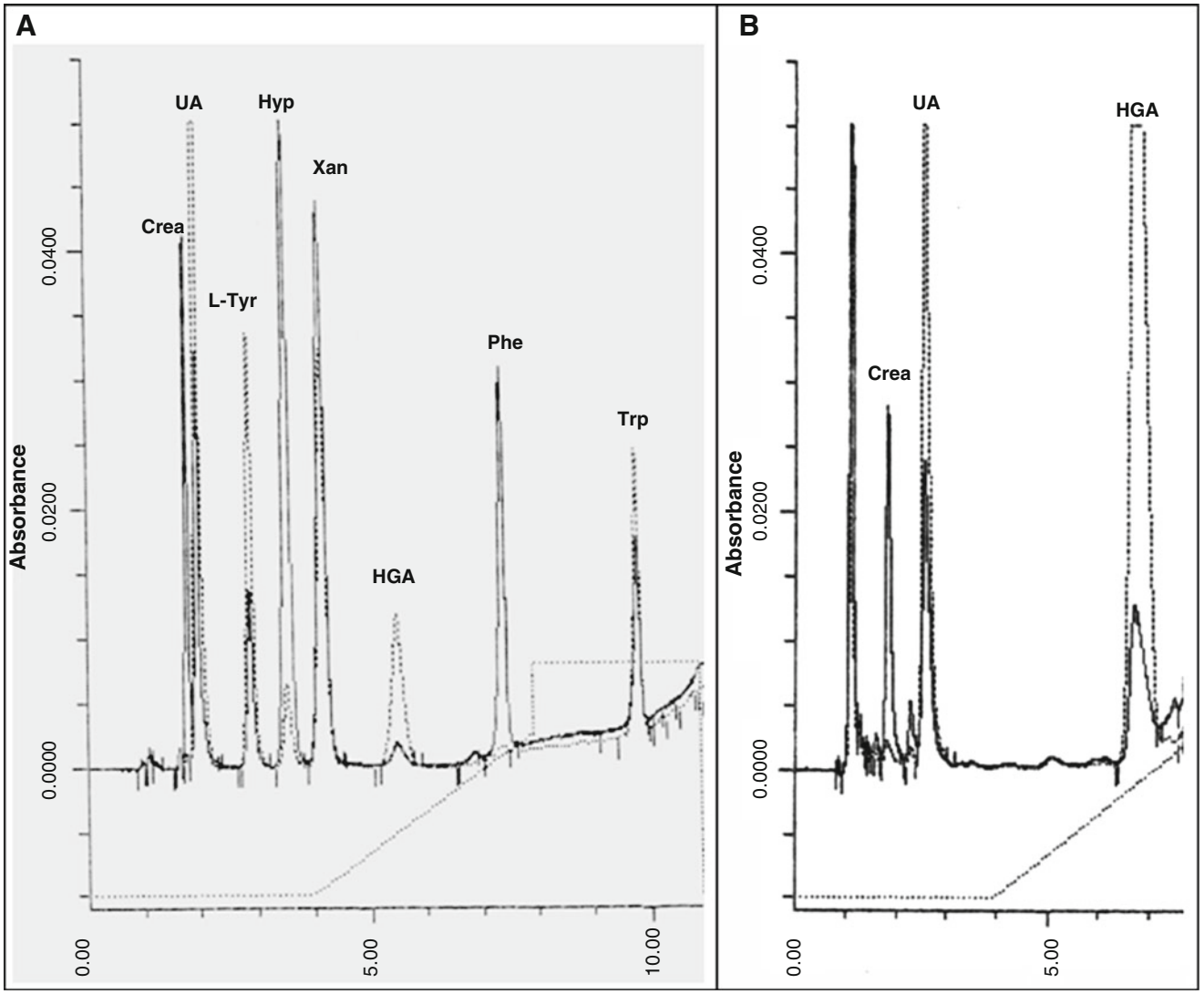

Fig. 2 HPLC profiles of absorbance (*dotted line* 280 nm, *continuous line* 260 nm) – standard mixture of creatinine, uric acid, L-tyrosine, hypoxanthine, xanthine, HGA, phenylalanine and tryptophan (**a**); AKU patient urine (**b**)

such as plasma/serum, which are in the micromolar range (Bory et al. 1989; Hughes et al. 2015).

Acknowledgements This work has been supported by Telethon Italy grant GGP10058. The authors also thank Toscana Life Sciences Orphan_1 project, Fondazione Monte dei Paschi di Siena and aimAKU – Associazione Italiana Malati di Alcaptonuria (ORPHA263402).

Compliance with Ethics Guidelines

Conflict of Interest

Gabriella Jacomelli, Vanna Micheli, Giulia Bernardini, Lia Millucci and Annalisa Santucci declare that they have no conflict of interest.

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

Contribution of Authors

Gabriella Jacomelli: planning, conducting the experimental part, analyzing and reporting results.

Vanna Micheli: planning, analyzing and reporting results.

Giulia Bernardini : data analysis and reporting results.

Lia Millucci : contacting patients, managing samples and planning experiments.

Annalisa Santucci: planning and supervising.

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JIMD Reports
DOI 10.1007/8904_2016_557

CASE REPORT

Mitochondrial Complex III Deficiency with Ketoacidosis and Hyperglycemia Mimicking Neonatal Diabetes

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Received: 20 January 2016 / Revised: 10 March 2016 / Accepted: 15 March 2016 / Published online: 14 April 2016

Abstract Hyperglycemia is a rare presenting symptom of mitochondrial disorders. We report a case of a young girl who presented shortly after birth with ketoacidosis, hyperlactatemia, hyperammonemia, and insulin-responsive hyperglycemia. Initial metabolic work-up suggested mitochondrial dysfunction. Given our patient's unusual presentation, whole-exome sequencing (WES) was performed on the parent–offspring trio. The patient was homozygous for the c.643C>T (p.Leu215Phe) variant in *CYC1*, a nuclear gene which encodes cytochrome c_1, a subunit of respiratory chain complex III. Variants in this gene have only been previously reported in two patients with similar presentation, one of whom carries the same variant as our patient who is also of Sri Lankan origin.

Primary complex III deficiencies are rare and its phenotypes can vary significantly, even among patients with the same genotype.

Communicated by: Garry Brown

Competing interests: None declared

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Introduction

Complex III (CIII) forms the central part of the mitochondrial respiratory chain. CIII oxidizes coenzyme Q, reduces cytochrome *c*, and pumps protons from the mitochondrial matrix to the inter-membrane space through the Q-cycle mechanism. CIII is composed of 11 subunits. Cytochrome c_1, cytochrome *b*, and the Rieske protein are the catalytic subunits of CIII. Ten subunits are encoded by nuclear DNA (*CYC1* (OMIM #615453), *UQCRFS1*, *UQCRQ* (#615159), *UQCRC1*, *UQCRC2* (#615160), *UQCRH*, *UQCRB* (#615158), *UQCR10*, *UQCR11*, subunit IX) and cytochrome *b* (*MT-CYB*, OMIM #516020) is encoded by the mitochondrial DNA. There are also a number of associated proteins required for CIII's proper assembly and functioning (Fernández-Vizarra and Zeviani 2015; Gaignard et al. 2013).

Genetic defects that result in CIII-deficiency are among the rarest and most infrequently diagnosed mitochondrial disorders. Until recently only three of the genes causing CIII-deficiency were known (*BCS1L*, *MT-CYB*, and

UQCRB) but recent advances in DNA sequencing technology as well as the continued study of the CIII subunit orthologues in yeast have enabled the discovery of seven additional human genes causing CIII-deficiency (Fernández-Vizarra and Zeviani 2015). The diagnosis of disorders of CIII-deficiency is challenging as they comprise a group of rare conditions that have vastly different phenotypes. The advent of whole-exome sequencing has also facilitated the molecular diagnosis of these rare disorders (Kohda et al. 2016).

In 2013, Gaignard et al. reported two unrelated cases of CIII-deficiency caused by mutations in the gene encoding cytochrome c_1 (*CYC1*). Cytochrome c_1 is the heme-containing subunit of CIII and facilitates the transfer of electrons from the Rieske complex to cytochrome *C*. The two reported patients both presented with recurrent episodes of ketoacidosis and insulin-responsive hyperglycemia in the context of intercurrent illness (Gaignard et al. 2013). Both patients also responded well to fluid resuscitation and insulin administration.

Here we describe a case of hyperammonemia, hyperlactatemia, and insulin-responsive hyperglycemia. Exome sequencing revealed homozygous variants in *CYC1*. This is the third report of disease-causing variants in this gene and the first report of a patient presenting in the neonatal period.

Case Report

Our patient is the first child of healthy non-consanguineous individuals of Sri Lankan origin and she presented to our care on the first day of life. Her mother had no previous history of miscarriage. She received prenatal care throughout the pregnancy and there was no history of gestational diabetes. Due to severe intra-uterine growth restriction (IUGR) and oligohydramnios of unknown etiology, the baby was delivered via cesarean section at 34 weeks. Birth weight was 1.4 kg (3rd percentile). The patient required respiratory support at birth for decreased saturation on room air. Initial capillary blood gas demonstrated respiratory acidosis. Throughout the first day of life, the patient continued to be significantly tachypneic with increased anion gap metabolic acidosis, severe lactic acidemia, ketoacidosis, and impressive hyperglycemia (Fig. 1 and Fig. 2a). When she was transferred to our hospital at 17 h of life, she was also found to have an elevated ammonia level of 212 μmol/L (normal 0–55 μmol/L).

Acidosis was managed via sodium bicarbonate infusion and hyperammonemia was managed with carglumic acid and carnitine, with good response. Continuous intravenous insulin infusion was started at 25 h of life but hyperglycemia remained particularly difficult to manage despite the administration of extremely high doses of intravenous insulin; up to 0.45 units/kg/h (Fig. 2a, b). Over the next 48 h of life, insulin administration was weaned quickly because of rapid glucose normalization (Fig. 2b). The working diagnosis was transient neonatal diabetic ketoacidosis versus mitochondrial disease. The patient was not dysmorphic and physical exam was unremarkable aside from tachypnea.

Due to her low birth weight, the patient remained in hospital for a total of 6 weeks. At 3 weeks of life, the baby developed Klebsiella urinary tract infection and was managed with antibiotics. She remained stable and did not require any further insulin treatment or special diet. Her blood glucose remained between 5 and 7 mmol/L. The patient was subsequently discharged home on levocarnitine, vitamin D, and iron. At 8 months of age, the patient once again presented with a similar episode in the context of viral gastroenteritis. She was admitted to the intensive care unit for correction of severe hyperglycemia (highest glucose 28 mmol/L), hyperammonemia (up to 184 μmol/L), and ketoacidosis. She required continuous intravenous insulin infusion (0.1 U/kg/h). The hyperglycemia was quickly corrected and the patient did not require long-term insulin treatment. The patient is now 23 months of age and she suffers from feeding issues such as poor appetite and an aversion to solids. Her diet is supplemented by feeds via nasogastric tube. She also has moderate motor and language delay. She is following her growth curves, though all growth parameters are well below the 1st percentile for age (at 23 months: weight 7.37 kg, height 73.5 cm, and head circumference 45.9 cm).

During the initial metabolic decompensation, the patient underwent an extensive genetic and metabolic work-up, including an array comparative genomic hybridization (aCGH) which was read as normal. The initial metabolic investigation revealed an acylcarnitine profile with increased C2 and C4-OH (related to ketosis) and numerous non-specific elevations in keeping with mitochondrial dysfunction. The plasma amino acids (PAA) profile showed elevated levels of glutamine, alanine, proline, and glycine. The urine organic acids (UOA) profile on day of life 2 was highly suggestive of a mitochondrial disorder. It revealed a massive lactic acid peak as well as elevated acetoacetate, 3-hydroxybutyrate, pyruvate, fumarate, and malic acid. Abdominal ultrasound, skeletal survey, ophthalmologic and hearing exams were all within normal limits. An echocardiogram identified a small secundum atrial-septal defect. Interestingly, brain magnetic resonance imaging showed focal enlargement of the pituitary gland with interval absence of the normal T1 hyperintensity of the neurohypophysis. MR spectroscopy revealed no abnormal peaks. MRI scan was unchanged 1 year later.

Given the patient's unusual presentation, whole-exome sequencing (WES) was deemed the most viable diagnostic

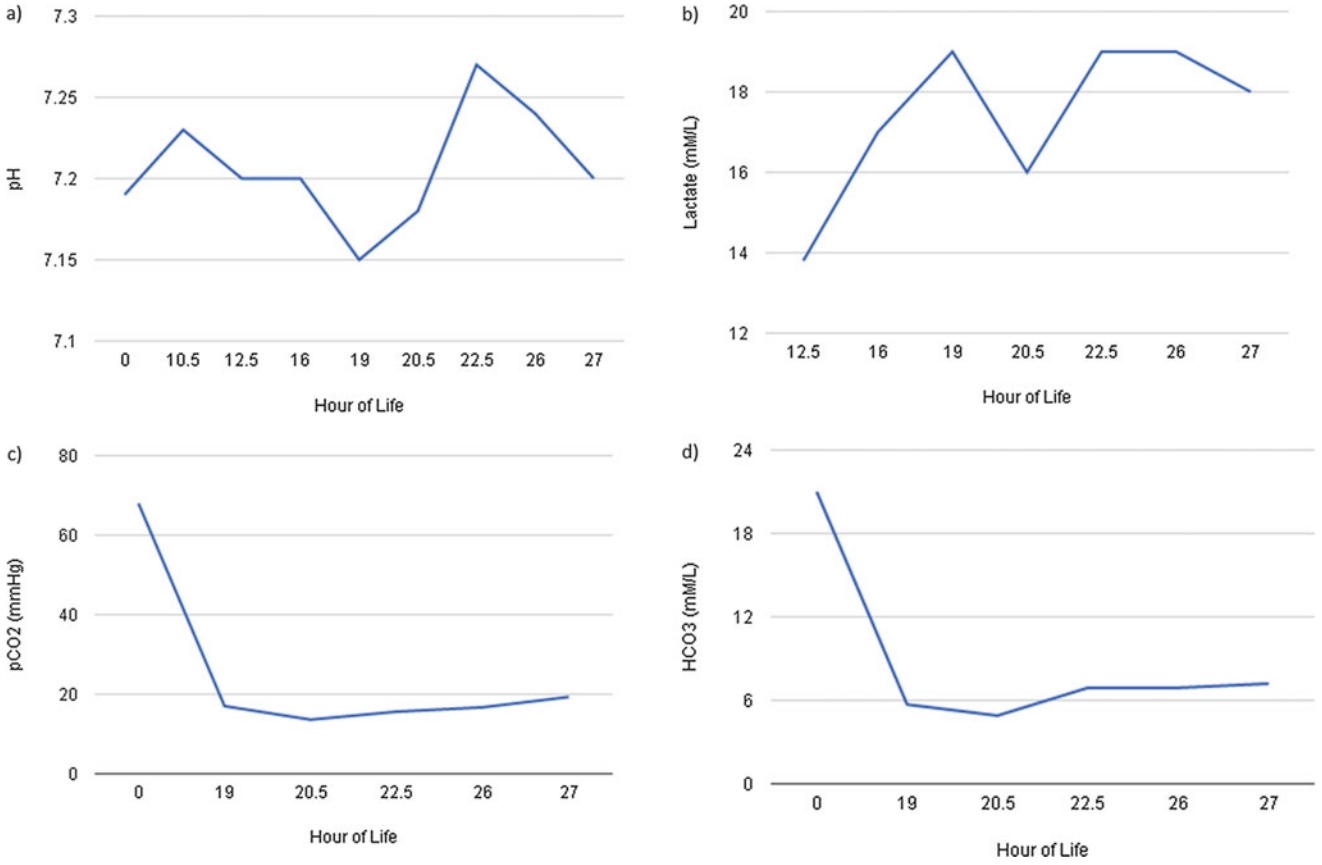

Fig. 1 Patient's (**a**) pH, (**b**) lactate, (**c**) pCO_2, and (**d**) HCO_3 levels during first 27 h of life

method. The family provided informed consent for participation in the TIDEX gene discovery project (UBC IRB approval H12-00067). WES was performed for the patient and her unaffected parents using the Agilent SureSelect kit and subsequent sequencing was performed on the Illumina HiSeq 2000 (Perkin-Elmer, USA). The sequencing reads (~28X coverage) were aligned to the human reference genome (version hg19) using the Bowtie21 aligner (Langmead and Salzberg 2012) and variants were called using SAMtools (Li et al. 2009). Allele frequencies were assessed in dbSNP142, the Exome Aggregation Consortium (ExAC) database, and an in-house database of more than 350 WES and whole-genome sequencing (WGS) results. Only rare variants ($\leq$1% in dbSNP) were selected for downstream analysis. SNPEff2 (Cingolani et al. 2012) was used to annotate variants and custom-made perl scripts were used to select for consequential variants (missense/nonsense and splice site variants). These variants were subsequently screened under a series of genetic models: homozygous, hemizygous, compound heterozygous, and de novo.

In total, we identified 36 candidate genes affected by rare variants. Of these 36 candidates, investigation of the functionality of the variants and subsequent review of the literature identified the homozygous missense variant g.145151518C>T (p.Leu215Phe; NM_001916) on chromosome 8 and affecting the *CYC1* gene (MIM 123980) as the most likely functional candidate. The presence of this variant was confirmed by Sanger re-sequencing (Fig. 3). This is a rare, previously reported pathogenic variant (Gaignard et al. 2013) observed with a frequency of 8.251e-06 in ExAC and not observed in dbSNP (version 142), NHLBI ESP, or our in-house genome database. The variant was predicted to be deleterious by all tested prediction software: a CADD score of 27.1 (Kircher et al. 2014); a SIFT prediction score of 0.001 (cutoff = 0.05) (Kumar et al. 2009); a PROVEAN prediction score of −4 (cutoff = −2.5) (Choi et al. 2012); and a Polyphen2 "probably damaging" score of 1.000 (Adzhubei et al. 2013). This variant has also been classified as pathogenic according to the recently published ACMG Standards and Guidelines (Richards et al. 2015). Furthermore, previously reported experimental results suggest that this variant primarily affects the structural integrity of Cytochrome c_1. Variations in this gene have only been previously reported in two other patients with similar presentation. One of these patients is also of Sri Lankan origin and she is homozygous for the same variant found in our patient (Gaignard et al. 2013).

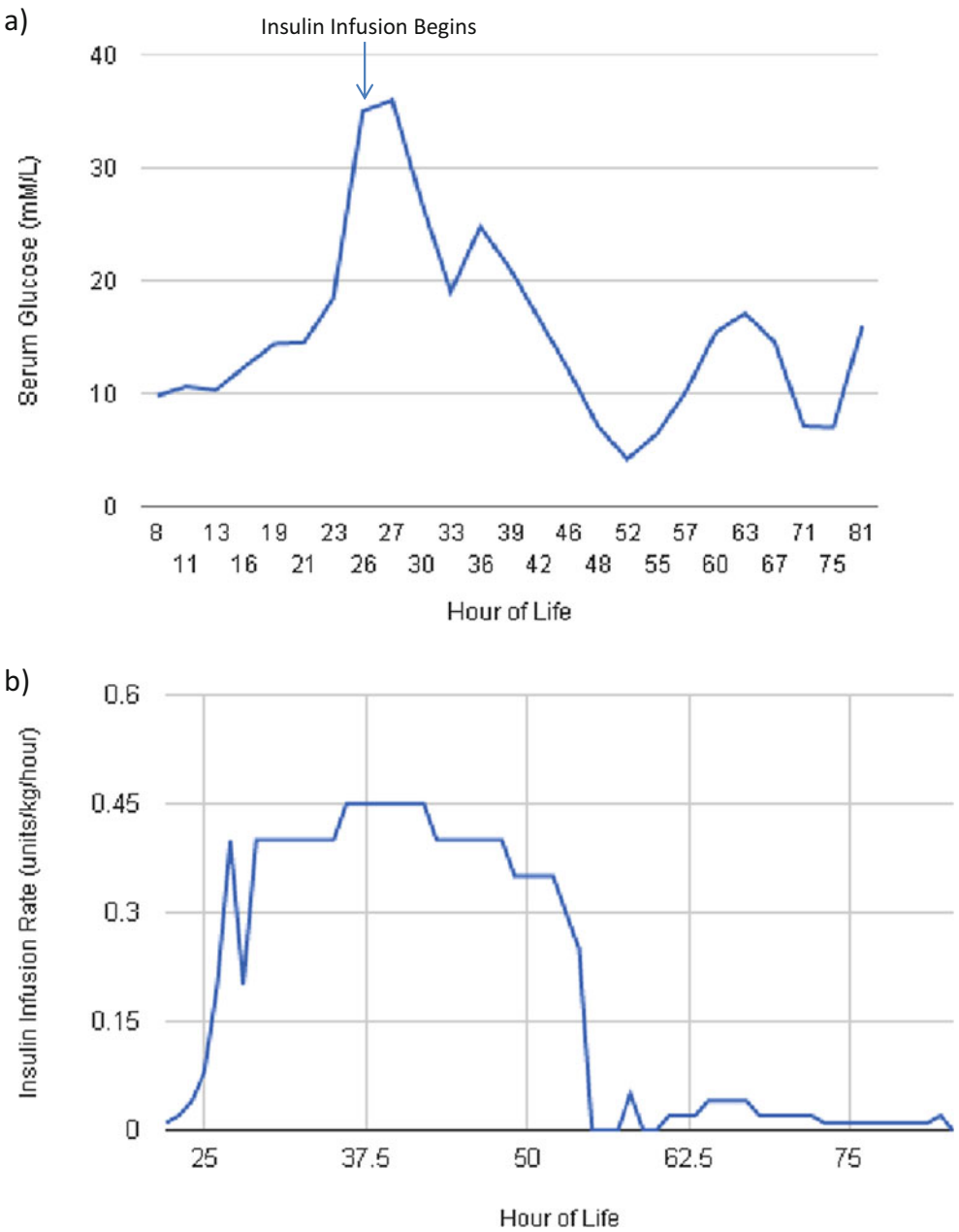

Fig. 2 Patient's (**a**) blood glucose levels versus hour of life and (**b**) corresponding insulin infusion rate versus hour of life. Insulin infusion began at approximately 25 h of life

Discussion

Our patient confirms episodic hyperlactatemia, hyperammonemia, and transient insulin-responsive hyperglycemia as the phenotype of CIII deficiency caused by variants in *CYC1*, which encodes the cytochrome c_1 subunit of mitochondrial complex III. However our patient is the first to present in the neonatal period. Our study is limited by the lack of available muscle for respiratory chain enzyme analyses, but it has previously been shown that the c.643C>T variant found in our patient results in reduced levels of cytochrome c_1 and CIII assembly subunits in the skeletal muscle and fibroblasts of affected patients. These effects are also reproduced when the variant is inserted at the orthologous position in the yeast *CYC1* ortholog. The variant is thought to affect the tertiary structure of the subunit, resulting in increased susceptibility to proteolysis and/or decreased ability to assemble with the other CIII subunits (Gaignard et al. 2013).

Our patient was found to have the same variant as a previously reported Sri Lankan patient, indicating that this variant may be a founder mutation in the Sri Lankan

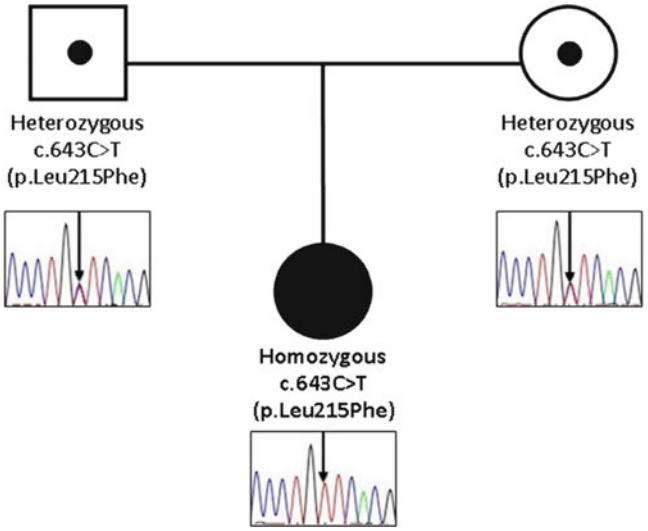

Fig. 3 Family pedigree and Sanger re-sequencing results for parent–patient trio

population. In contrast to the neonatal presentation of our patient, she presented later in childhood, at the age of 2.5 years, with dehydration, vomiting, liver failure, and neurological impairment eventually leading to coma. She was found to have lactic acidosis and hyperammonemia and, similarly to our patient, she improved dramatically with rehydration and insulin administration and did not require long-term insulin treatment. She also suffered numerous subsequent episodes of decompensation, precipitated by minor illness. Feeding issues were not described which contrasts with our patient (Gaignard et al. 2013). At the last reported follow-up at 18 years of age, she was found to have normal psychomotor development.

The other reported patient presented at the age of 5 months with a similar episode of metabolic ketoacidosis after a history of vomiting and febrile illness (Gaignard et al. 2013). He was homozygous for a c.288G>T (p.Trp96Cys) missense variant that was also shown experimentally to primarily affect the structural integrity of cytochrome c_1 (Gaignard et al. 2013). Like our patient, he has also suffered numerous episodes of lactic acidosis related to illness and has required insulin therapy during these episodes. This patient had an otherwise negative review of systems and normal development.

Our patient's early manifestations of IUGR, hyperglycemia, and insulin dependency resembled that of transient neonatal diabetes and pancreatic hypoplasia/agenesis (Naylor et al. 2011). However, in the case of transient neonatal diabetes, insulin dependency typically lasts for a few months postnatally and insulin-dependency is lifelong in pancreatic agenesis. Generally, the insulin dose required for these disorders is in the low-dose range: from 0.4 to 0.8 units/kg/day. Our patient required very high insulin infusion rates: up to 0.45 units/kg/h and unlike patients with transient neonatal diabetes and pancreatic agenesis, she was weaned off insulin quickly – within 48 hours. As previously mentioned, our patient had a normal physical exam at the time of presentation and pancreatic agenesis is usually seen in association with other abnormalities, such as hypothyroidism and immune dysregulation in IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) and biliary or duodenal atresia in Mitchell–Riley syndrome (Mitchell et al. 2004; Concepcion et al. 2014; Duclaux-Loras et al. 2015; Wildin et al. 2002).

As evidenced by our case, the age at presentation and phenotypes of complex III deficiencies can vary significantly, even among patients with the same genotype. This case also reiterates the clinical utility of WES, particularly in disorders with significant clinical and genetic heterogeneity. For the clinician, mutations in *CYC1* causing CIII-deficiency may be added to the differential diagnosis of neonatal transient hyperglycemia, lactic acidosis, and insulin dependency. Aggressive rehydration and insulin administration are the keys to recovery.

Acknowledgements We gratefully acknowledge the family for their participation in this study; Mrs. X. Han for Sanger sequencing; Mr. B. Sayson and Ms A. Ghani for consenting and data management; and Mrs. M. Higginson for DNA extraction, sample handling, and technical data (University of British Columbia, Vancouver, CA).

Take Home Message

By reading this third case report of a patient with mitochondrial complex III deficiency caused by mutations in the cytochrome C1 gene and the unique way in which

she presented, readers will learn the importance of keeping mitochondrial complex III deficiency in their differential diagnosis given the diverse presentation of patients.

Author Contributions

Natascia Anastasio, MD, MSc: Planning and drafting of article

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Compliance with Ethics Guidelines

Conflict of Interest

Natascia Anastasio, MajaTarailo-Graovac, Reem Al-Khalifah, Laurent Legault, Britt Drogemoller, Colin J.D. Ross, Wyeth W Wasserman, Clara van Karnebeek, and Daniela Buhas declare no conflict of interest.

Patient Consent

Parents provided informed consent for publication of this case report.

Ethical Standards

The authors declare that the experiments comply with the current laws of Canada, the country in which they were performed.

Funding

This work was supported by funding from the B.C. Children's Hospital Foundation as "1st Collaborative Area of Innovation" (www.tidebc.org), Genome BC (SOF-195 grant), the Canadian Institutes of Health Research (#301221 grant). Informatics infrastructure was supported by Genome BC and Genome Canada (ABC4DE Project). Clara van Karnebeek is recipient of the Michael Smith Foundation for Health Research Scholar Award.

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JIMD Reports
DOI 10.1007/8904_2016_558

RESEARCH REPORT

Diagnosis, Treatment, and Clinical Outcome of Patients with Mitochondrial Trifunctional Protein/Long-Chain 3-Hydroxy Acyl-CoA Dehydrogenase Deficiency

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Received: 22 January 2016 / Revised: 15 March 2016 / Accepted: 16 March 2016 / Published online: 28 April 2016

Abstract Deficiency of the mitochondrial trifunctional protein (TFP) and long-chain 3-Hydroxy Acyl-CoA dehydrogenase (LCHAD) impairs long-chain fatty acid oxidation and presents with hypoglycemia, cardiac, liver, eye, and muscle involvement. Without treatment, both conditions can be life-threatening. These diseases are identified by newborn screening (NBS), but the impact of early treatment on long-term clinical outcome is unknown. Moreover, there is lack of consensus on treatment, particularly on the use of carnitine supplementation. Here, we report clinical and biochemical data in five patients with TFP/LCHAD deficiency, three of whom were diagnosed by newborn screening. All patients had signs and symptoms related to their metabolic disorder, including hypoglycemia, elevated creatine kinase (CK), and rhabdomyolysis, and experienced episodes of metabolic decompensation triggered by illness. Treatment was started shortly after diagnosis in all patients and consisted of a diet low in long-chain fats supplemented with medium chain triglycerides (MCT), essential fatty acids, and low-dose carnitine (25 mg/kg/day). Patients had growth restriction early in life that resolved after 2 years of age. All patients but the youngest (2 years old) developed pigmentary retinopathy. Long-chain hydroxylated acylcarnitines did not change significantly with age, but increased during acute illnesses. Free carnitine levels were maintained within the normal range and did not correlate with long-chain hydroxylated acylcarnitines. These results show that patients with LCHAD deficiency can have normal growth and development with appropriate treatment. Low-dose carnitine supplements prevented carnitine deficiency and did not result in increased long-chain hydroxylated acylcarnitines or any specific toxicity.

Communicated by: Jerry Vockley, M.D., Ph.D.

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Introduction

Long-chain 3-Hydroxy Acyl-CoA dehydrogenase (LCHAD) deficiency (OMIM # 609016) is a disorder of fatty acid oxidation caused by specific mutations in the *HADHA* gene (Wanders et al. 1990; IJlst et al. 1994). This gene encodes for the alpha subunit of the trifunctional protein (TFP), which is composed of 4 alpha and 4 beta subunits and catalyzes three activities (hydratase, dehydrogenase, and thiolase) in mitochondrial long-chain fatty acids oxidation. In LCHAD deficiency, the second of the three reactions is more severely impaired, whereas in TFP deficiency (OMIM # 609015), there is decreased activity of all three enzymes. TFP and LCHAD deficiency cannot be differentiated biochemically: both are characterized by increased long-chain 3-hydroxyacylcarnitines in plasma and excessive excretion of 3-hydroxy-dicarboxylic acids

in urine, the latter only at the time of acute decompensation (den Boer et al. 2002). The diagnosis is confirmed by mutation analysis of the two genes (*HADHA* and *HADHB*) encoding for the alpha and beta subunits of the enzyme. The most common mutation causing LCHAD deficiency is c.1528 G>C (p. E510Q) in *HADHA* (IJlst et al. 1996).

Clinically, patients with TFP and LCHAD deficiency present with hypoketotic hypoglycemia after fasting or illness, with or without cardiomyopathy, liver dysfunction, and rhabdomyolysis (den Boer et al. 2002). Left untreated, morbidity and mortality are high prompting the inclusion of LCHAD and TFP deficiency in expanded newborn screening (NBS) programs (American College of Medical Genetics Newborn Screening Expert 2006). Therapy includes fasting avoidance, a diet restricted in long-chain fatty acids and supplemented with both medium chain triglycerides (MCT) and essential fatty acids. Successful treatment reduces long-chain 3-hydroxyacylcarnitines in plasma and normalizes the dicarboxylic aciduria (Spiekerkoetter et al. 2009). Fasting avoidance and supplementation with MCT and essential fatty acids are part of the clinical management of all patients with TFP/LCHAD deficiency, but the use of L-carnitine is controversial (Spiekerkoetter et al. 2009; Potter et al. 2012) since in animal models of very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency carnitine supplements might increase long-chain acyl carnitines with potential arrhythmogenic effect (Primassin et al. 2008; Tucci et al. 2014).

This manuscript reports clinical outcome and biochemical parameters in five patients with TFP/LCHAD deficiency. All patients were regularly followed in the Metabolic Clinic with an average follow-up time of nearly 10 years (9.2 ± 5.9 years).

Patients and Methods

Diagnosis

This study was reviewed and approved by the Institutional Review Board at the University of Utah. All five patients (three males and two females) were followed in the Metabolic Clinic at the University of Utah from the time of diagnosis (since birth for patients 3, 4, and 5) (Table 1). Patient 2 was last evaluated at 15 years of age. Demographic, clinical, and laboratory data were obtained by

Table 1 Clinical characteristics of patients with LCHAD/TFP deficiency

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Gender	M	F	M	F	M
Age at diagnosis	4 months	6 months	Birth (NBS)	Birth (NBS)	Birth (NBS)
Current age	22 years	19 years	10 years	5 years	24 months
Follow-up years	17 years	12 years	10 years	5 years	2 years
Genotype	c.1528 G>C [p.E510Q]	c.1528 G>C [p.E510Q]	c.1528 G>C [p.E510Q]	c.1528 G>C [p.E510Q]	c.1025 T>C [p.L342P][a]
	c.1528 G>C [p.E510Q]	c.1528 G>C [p.E510Q]	c.1528 G>C [p.E510Q]	c.1528 G>C [p.E510Q]	c.1493 A>G [p.H498R][b]
Family history	One sister died at 9 months of age; one healthy sister	No siblings	Two healthy brothers	One healthy brother, one healthy sister	One healthy brother
Pregnancy	HELLP syndrome	Unremarkable	HELLP syndrome	Preeclampsia	Placenta previa
Gestational age	Preterm	Term	37 0/7	35 0/7	35 0/7
Weight at birth (*Z*-score)	N/A	N/A	2,330 g (0.64)	1,685 g (−1.61)	2,275 g (−0.74)
Presentation at diagnosis	Lethargy	Hypoglycemia	Asymptomatic	Hypoglycemia	Hypoglycemia
Medical history	Muscle pain; depression; cardiac episode with ventricular tachycardia	Exercise-induced muscle pain; multiple hospitalizations (hypoglycemia)	Exercise-induced muscle pain	Failure to thrive; muscle pain	Failure to thrive; hypospadias
Retinitis pigmentosa	Yes	Yes	Yes	Yes	No

N/A not available
[a] Found in LCHAD deficiency compound heterozygotes (IJlst et al. 1997)
[b] Previously unreported

retrospective chart review. Patients 1 and 2 were born before the introduction of the expanded newborn screening and presented clinically: patient 1 at 4 months of age with lethargy and patient 2 at 6 months of age with severe hypoglycemia followed by cardiorespiratory arrest. The other three patients were identified by newborn screening, even though patients 4 and 5 already had hypoglycemia at birth (or shortly after). In all patients, confirmatory biochemical testing was consistent with a diagnosis of TFP or LCHAD deficiency, showing increase in plasma long-chain 3-hydroxyacylcarnitines. DNA sequencing confirmed homozygosity for the common c.1528G>C (p.E510Q) mutation in the *HADHA* gene in all patients except patient 5 who is compound heterozygote for the previously reported pathogenic mutation c.1025T>C (p.L342P) associated with LCHAD deficiency (IJlst et al. 1997) and for a novel change c.1493A>G (p.H498R) in the *HADHA* gene. This change has been identified in 1/121,392 alleles in the normal population, and it is predicted to be probably damaging by Polyphen and deleterious by SIFT (exac.broadinstitute.org).

Patient 1 had a family history of LCHAD deficiency, as his older sister died at 9 months of age with hypoglycemia, liver failure, and cardiac arrest with LCHAD deficiency diagnosed post-mortem. All other patients were the only affected individuals in their extended family.

Clinical Descriptions (Table 1)

Patient 1 (LCHAD deficiency) had intermittent muscle pain involving arms, shoulders, and legs, exacerbated by activity and cold; depression treated with psychotherapy and medications; one episode of myoglobinuria followed by renal failure and ventricular tachycardia requiring defibrillation (at 20 years of age). This episode occurred while he was in college, away from home, and he had history of poor compliance with therapy. Administration of intravenous glucose resolved both the renal failure and the ventricular tachycardia and stabilized cardiac function. He developed retinitis pigmentosa with night vision loss as a child. Therapy, followed with variable compliance, included a low-fat diet (10–20% calories from proteins; 45–65% from carbohydrates; 30% calories from fat of which 10% were from natural fat, and 20% from medium chain triglycerides), essential fatty acids (walnut oil, part of the 10% calories from natural fat), docosahexaenoic acid (DHA), CoQ10 and carnitine (25 mg/kg/day).

Patient 2 (LCHAD deficiency) had intermittent muscle pain (lower extremities) exacerbated by activity, hypoglycemia, and multiple hospitalizations for dehydration. Mild retinal degeneration was noted at 15 years of age, with modest loss of the visual field and no significant night blindness. Treatment consisted of a low-fat diet (10–20% calories from proteins; 45–65% from carbohydrates; 30% calories from fat of which 10% were from natural fat, and 20% from medium chain triglycerides such as Portagen), essential fatty acids (walnut oil, part of the 10% calories from natural fat), cornstarch at bedtime, and carnitine (25 mg/kg/day).

Patient 3 (LCHAD deficiency) was identified at birth by newborn screening. His growth and development are appropriate and he is very physically active. However, he experienced several episodes of exercise-induced muscle pain with prolonged exertion. At 3 years of age, changes consistent with retinitis pigmentosa were noted. He followed a low-fat diet (10–20% calories from proteins; 45–65% from carbohydrates; 35% calories from fat of which 10% were from natural fat, and 25% from medium chain triglycerides such as Portagen), essential fatty acids (flaxseed oil, part of the 10–15% calories from natural fat), cornstarch at bedtime, and carnitine (25 mg/kg/day). Creatine (4 g/day) was added at 5 years of age to improve exercise tolerance.

Patient 4 (LCHAD deficiency) was born prematurely at 35 weeks gestation for maternal preeclampsia. She developed severe hypoglycemia at birth that required IV glucose and was further complicated by sepsis. LCHAD deficiency was detected by newborn screening. Her medical history includes several episodes of muscle pain after illness. Mild retinal pigmentary changes were noted in the retina at 2 years of age. Therapy consisted of low-fat diet (10–20% calories from proteins; 45–65% from carbohydrates; 35% calories from fat of which 10% were from natural fat, and 25% from medium chain triglycerides such as Lipistart), essential fatty acids (flaxseed and walnut oil, part of the 10–15% calories from natural fat), cornstarch (at bedtime), and carnitine (25 mg/kg/day).

Patient 5 (LCHAD/TFP deficiency) had severe hypoglycemia at birth and difficulty regulating his temperature, but quickly recovered after IV glucose. LCHAD/TFP deficiency was detected by newborn screening. He had several preventive hospitalizations for fever and gastroenteritis. No signs of pigmentary changes have been detected yet (at 2 years of age). He has been treated with a low-fat diet (10–20% calories from proteins; 45–65% from carbohydrates; 35% calories from fat of which 10% were from natural fat, and 25% from medium chain triglycerides), DHA, cornstarch (at bed-

time), and carnitine (25 mg/kg/day). This child subsequently started therapy with triheptanoin that substituted MCT oil.

Laboratory Studies

Metabolic and routine testing was performed by the hospital laboratory or by a reference laboratory (ARUP Laboratories, Salt Lake City, UT, USA, aruplab.com) according to standard procedures. Occasional metabolic decompensation episodes, varying in number and severity among patients, required additional testing, including creatine kinase (CK) activity and transaminases (aspartate aminotransferase, AST; alanine aminotransferase, ALT) levels, and clinical evaluation. Growth parameters were assessed during routine visits. Weight, length/height, BMI percentiles, and *Z*-scores were assessed using the standard charts of the Centers for Disease Control and Prevention (CDC). Specifically, the World Health Organization (WHO) growth standards (Borghi et al. 2006) were used for data collected from birth to 2 years of age (correcting for gestational age up to 1 year of age), and the CDC growth standards (Kuczmarski et al. 2000) were used for data collected after 2 years of age. BMI percentiles were only assessed for patients >2 years of age.

Statistics

Values for descriptive statistics are presented as means ± SD. Comparison of means was performed using the paired or unpaired t-test with Welch's correction (not assuming equal variances). The correlation between parameters (patients' age and biochemical measurements) was assessed by linear regression. Results were considered statistically significant with $p < 0.05$. GraphPad Prism® software (Version 5.04 (2010), GraphPad Software Inc) was used for data analysis.

Results

Growth and Development

Table 1 summarizes the clinical characteristics of patients included in this study. Four out of five pregnancies were complicated: HELLP syndrome (patients 1 and 3), pre-eclampsia (patient 4), and placenta previa requiring emergency C-section (patient 5) resulting in preterm delivery. Patients 4 and 5 had low birth weight even after correction for prematurity (*Z*-scores −1.61 and −0.74, respectively), and experienced significant failure to thrive early in life. All patients experienced a slower growth rate in the first few years after diagnosis (Fig. 1). Cumulatively, the average *Z*-scores were significantly lower than normal ($p < 0.0001$) in the first 2 years of life for both weight (-1.74 ± 0.75, age 0–2 years, $N = 53$) and length/height (-1.44 ± 1.16, age 0–2 years, $N = 53$). After 2 years of age, all patients with TFP/LCHAD deficiency reached normal percentiles for weight, height, and BMI (Fig. 1). When compared to the normal population, there was no difference for weight (-0.068 ± 1.15, age 2–20 years, $N = 47$, $p = 0.69$) and a modest difference with height (0.56 ± 1.66, age 2–20 years, $N = 47$, $p = 0.025$), with the LCHAD/TFP deficiency patients being taller. We did not observe overweight/obesity issues after the initial slow growth in our LCHAD/TFP deficiency patients as previously reported (Haglind et al. 2013). The average *Z*-score for the BMI was -0.31 ± 0.90 SD (age 2–20 years, $N = 47$), mildly reduced when compared to the normal population ($p = 0.025$). All five patients had normal development and attended normal school.

Long-Term Follow-Up with Laboratory Testing

Laboratory testing was routinely ordered in all five patients to monitor their disease. For patients 3, 4, and 5 data are available from birth to current age (respectively, 10, 5, and 2 years of age); while for patients 1 and 2 early records from the first few years were not available. Values for long-chain hydroxyacylcarnitines (C14:0-OH, C14:1-OH, C16:0-OH, C18:0-OH, and C18:1-OH) were increased in all patients, both as individual species and cumulatively. No changes with age were observed, with nonsignificant correlation between age and levels of individual acylcarnitines or their sum (age range 0–22 years, $N = 75$, $p = 0.58$ for C14:0-OH, $p = 0.79$ for C14:1-OH, $p = 0.33$ for C16:0-OH, $p = 0.69$ for C18:0-OH, $p = 0.84$ for C18:1-OH, and $p = 0.83$ for the long-chain hydroxyacylcarnitines sum). Similar results have been reported by others (Karall et al. 2015).

All patients in our cohort experienced several illnesses, some of whom required hospitalization and administration of intravenous glucose. During illnesses, CK activity, a measure of muscle involvement, increased dramatically, normalizing with treatment. There was considerable clinical heterogeneity in levels of CK and clinical course despite the fact that four out of five patients were homozygous for the common mutation p.E510Q. Most critical values of CK were recorded for patients 4 and 5 (80%) during episodes of metabolic decompensation and patient 3 never presented with a CK > 1,000 UI/L. Transaminases (AST and ALT) levels and the AST/ALT ratio were also increased, and correlated with CK (Fig. 2), suggesting that most of the increase in transaminases was reflecting release from the muscle rather than liver involvement.

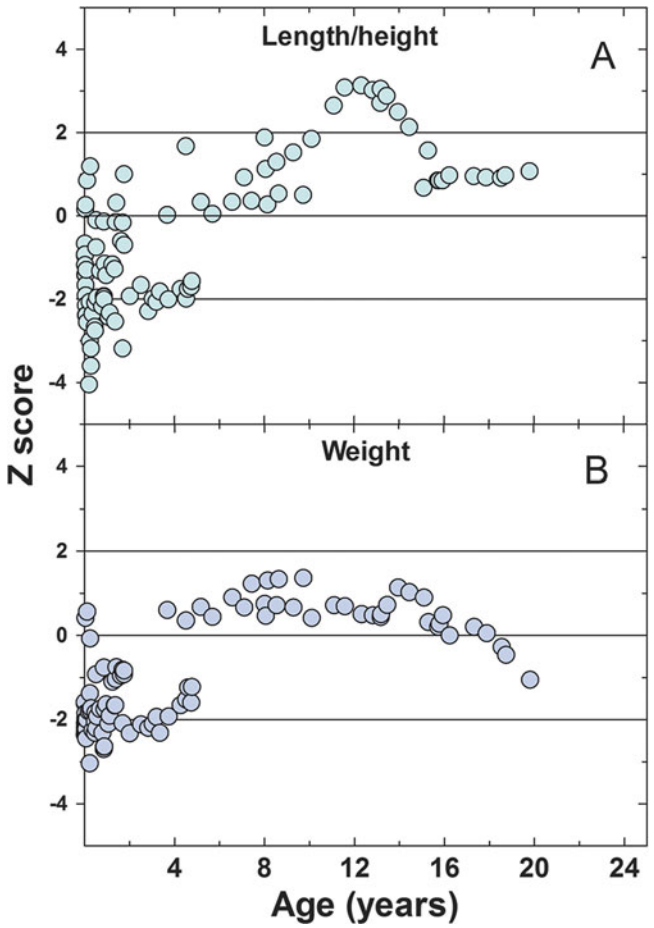

Fig. 1 *Z*-scores for length/height (**a**) and weight (**b**) normalize (*Z*-score = 0) over time (age in years) in mitochondrial trifunctional protein disorders patients. Length measurements were used for patients younger than 2 years of age, while height measurements were used for patients >2 years of age

Notably, levels of 3-OH-acylcarnitines significantly correlated with CK levels (Fig. 3a, $p < 0.0001$), but not with transaminase levels (Fig. 3b, c). The significance of the correlation was lost if values of CK during crises (>5,000 U) were omitted from the analysis ($N = 72$; $R^2 = 0.000$; $p = 0.98$).

Treatment and Clinical Outcome

Dietary intervention and medications were started shortly after diagnosis in all patients. All patients were supplemented with essential fatty acids (walnut or flaxseed oil, and/or DHA), MCT oil and/or medical food containing MCT, and carnitine (25 mg/kg/day). Free carnitine in plasma was 47.5 ± 14.5 μmol/L, ranging from 17 to 77 μmol/L ($n = 75$, normal range: 22–63 μmol/L), with all values except one (obtained at diagnosis) being within or mildly above the normal range, showing that in our cohort low-dose supplementation prevented carnitine deficiency.

Levels of medium chain acylcarnitines were mildly increased reflecting MCT oil supplements (C6 = 0.16 ± 0.10 μmol/L, normal 0–0.16 μmol/L; C8 = 0.19 ± 0.18 μmol/L, normal 0–0.21 μmol/L; and C10 = 0.28 ± 0.25 μmol/L, normal 0–0.26 μmol/L; $N = 82$). MCT oil supplements were increased during metabolic crises, probably explaining the positive correlation between the medium chain acylcarnitine cumulative concentration (C6 + C8 + C10) and CK ($p < 0.0001$; $R^2 = 0.278$; Fig. 3d) and transaminases (AST $p < 0.0001$; $R^2 = 0.333$; ALT $p < 0.05$; $R^2 = 0.052$; respectively, Fig. 3e, f). The significant relationship between medium chain acylcarnitine and CK disappeared when the outliers (CK > 5,000 IU/L) were removed ($N = 72$; $R^2 = 0.0198$; $p = 0.23$).

A mild inverse correlation was noted between free carnitine levels and medium chain acylcarnitine (Fig. 4a), possibly reflecting improved utilization or excretion of medium chain fatty acids with carnitine supplements. By

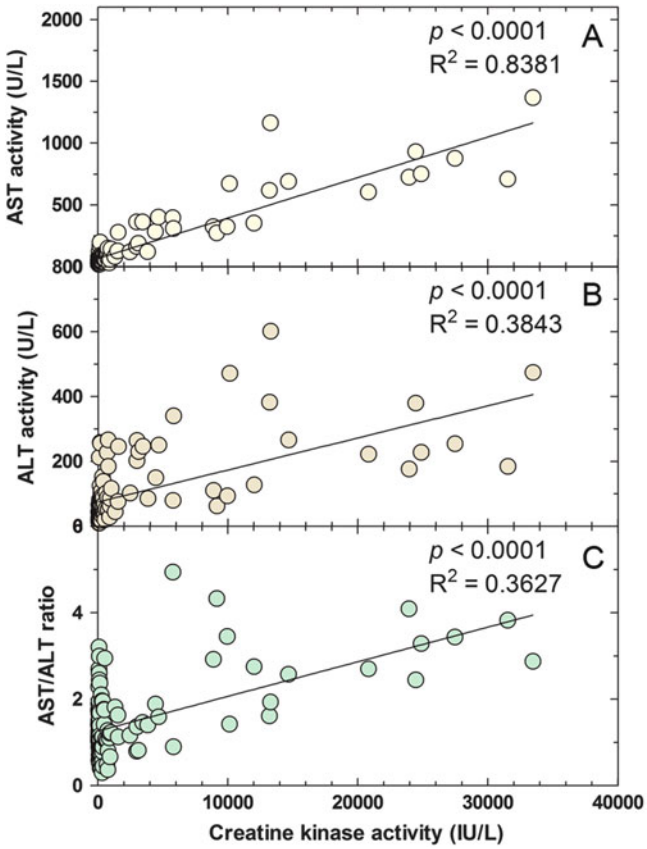

Fig. 2 Correlation between creatine kinase activity (IU/L) and (**a**) aspartate aminotransferase activity (U/L), (**b**) alanine aminotransferase activity (U/L), and (**c**) the AST/ALT ratio in mitochondrial trifunctional protein disorders patients. *Solid line* indicates the best-fit linear regression line

contrast, there was no correlation between free carnitine levels and long-chain hydroxyacylcarnitines (Fig. 4b).

Measurement of plasma essential fatty acids indicated no evidence of deficiency (not shown).

Discussion

This study evaluated the longitudinal history of five patients with mitochondrial trifunctional protein disorders (one with TFP deficiency and four with LCHAD deficiency) followed for 9.2 ± 5.7 years in the Metabolic Clinic. Three of these patients were diagnosed at birth by newborn screening. In our five patients, a diet low in long-chain fat and supplemented with MCT, essential fatty acids, and carnitine at low dose (25 mg/kg/day) was started shortly after diagnosis. Some patients also received cornstarch, DHA, and/or creatine. Our retrospective chart review found that, even after correcting for gestational age, most patients had a low birth weight and experienced failure to thrive early in life (Fig. 1). Eventually all patients were able to achieve normal growth, and did not experience overweight issues previously reported in LCHAD deficiency patients (Haglind et al. 2013). Due to the small size of our cohort, it is difficult to determine if growth normalization was due to fewer illness-triggered hospitalizations in older patients, as suggested by Karall et al. (2015). Nevertheless, these findings indicate that normal growth and development can be achieved in TFP/LCHAD deficiency patients with appropriate treatment and management of acute episodes.

It is too early to make any assessment on the effectiveness of newborn screening in preventing the complications of LCHAD/TFP deficiency. The early detection by newborn screening for TFP and LCHAD deficiencies does not prevent metabolic decompensation, which still occurs in patients treated early, before the onset of symptoms (Spiekerkoetter et al. 2009; Karall et al. 2015). In our cohort, patients identified by newborn screening still had illnesses resulting in elevation of CK, AST, and ALT (Fig. 2). There was a strict correlation between these markers suggesting that the elevation of AST and ALT was more likely secondary to muscle involvement (Fig. 2). Patients identified by newborn screening had a similar number of hospitalizations to patients

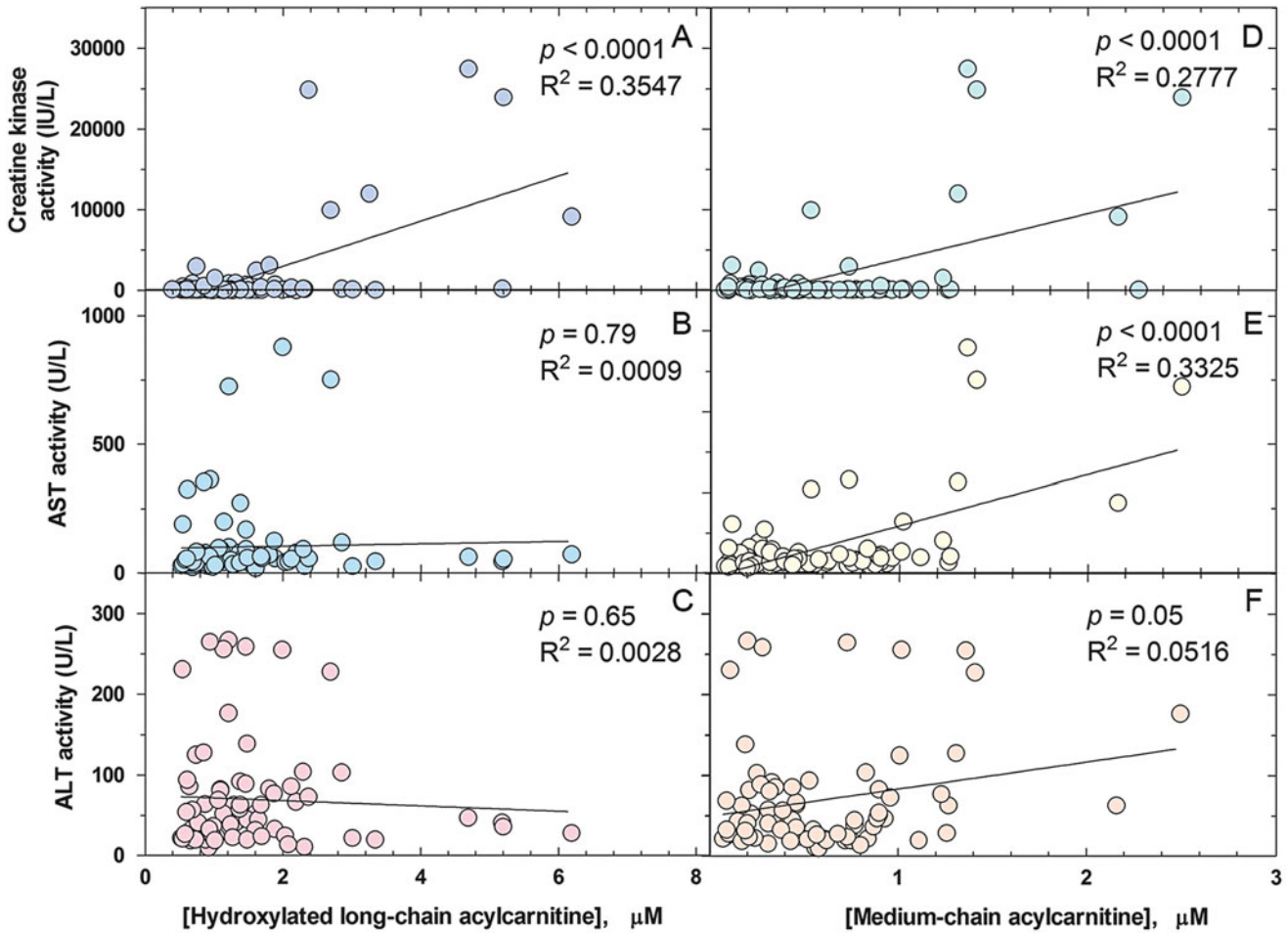

Fig. 3 Correlation between cumulative long-chain hydroxyacylcarnitines concentration (μM) and creatine kinase activity (**a**), aspartate aminotransferase activity (**b**), and alanine aminotransferase activity (**c**); and between cumulative medium chain acylcarnitines (μM) and creatine kinase activity (**d**), aspartate aminotransferase activity (**e**), and alanine aminotransferase activity (**f**). *Solid line* indicates the best-fit linear regression line

identified clinically, before the onset of newborn screening. However, these were all preventative hospitalizations and not the results of complications (rhabdomyolysis and cardiac arrest) of the disease. Hospital admissions were usually shorter. Finally, one of the patients born before newborn screening developed myopathy and neuropathy after puberty. We have to wait that our cohort reaches that age to see if these functional defects are prevented by early diagnosis and therapy. Biochemical monitoring indicated a correlation between long-chain hydroxyacylcarnitines and creatine kinase levels, with most of the correlation being driven by elevated CK levels during illnesses (Fig. 3a, $p < 0.0001$), as previously suggested (Gillingham et al. 2005; Karall et al. 2015).

There is general consensus among physicians in recommending fasting avoidance and supplementation with MCT and essential fatty acids for the treatment of TFP/LCHAD deficiencies (Spiekerkoetter et al. 2009; Potter et al. 2012). There is no consensus on the use of carnitine, in part driven by the uncertain effects on long-chain acylcarnitine species. Animal studies indicated an increase in long-chain acyl carnitines with carnitine supplementation in a mouse model of VLCAD deficiency (Primassin et al. 2008; Tucci et al. 2014). On the other hand, carnitine supplementation attenuated myocardial lipid accumulation in long-chain acyl-CoA dehydrogenase knockout mice (Bakermans et al. 2013). Carnitine supplementation is not widely used in the clinical management of patients with TFP/LCHAD deficiency (Potter et al. 2012). Acute administration of carnitine in a single patient with a biochemical diagnosis of LCHAD deficiency was ineffective in preventing death after prolonged fasting (Rocchiccioli et al. 1990). Karall

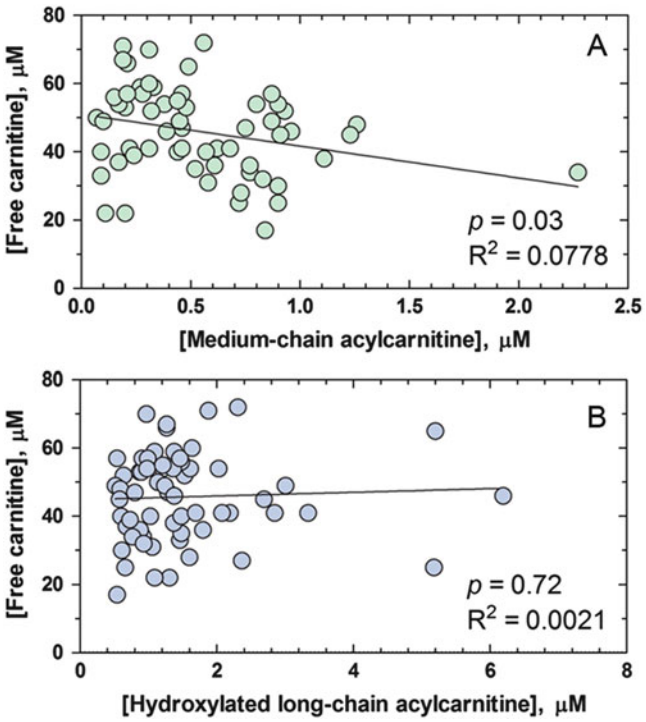

Fig. 4 Correlation between free carnitine concentration (μM) and cumulative medium chain acylcarnitines (**a**) and long-chain hydroxyacylcarnitines (**b**). *Solid line* indicates the best-fit linear regression line

et al. (2015) reported long-term follow-up data on 14 Austrian patients with LCHAD deficiency (ages ranging from 0.9 to 15.3 years), none of whom was supplemented with carnitine. In their cohort, growth and development were normal; however, long-term complications were described: cardiomyopathy (seven patients), hepatopathy (five patients), retinopathy (eight patients), and polyneuropathy (one patient).

We used low-dose oral carnitine supplementation in all five patients (age ranging from 2 to 22 years) with the goal of preventing carnitine deficiency. Free carnitine levels in treated patients were within or mildly above the normal range. There was a mild inverse correlation of plasma carnitine levels with medium chain acylcarnitines (Fig. 4a), which may suggest a better utilization or elimination of the MCT oil with carnitine supplementation. Notably, however, there was no correlation between free carnitine concentration and long-chain hydroxyacylcarnitines (Fig. 4b). None of our patients developed cardiomyopathy. Patient 1 experienced an episode of ventricular tachycardia following myoglobinuria and renal failure (at 20 years of age) triggered by an acute illness and fasting. His cardiac function returned to normal after the resolution of the acute episode. Therapeutic intervention did not prevent the onset of retinopathy in all of our patients, except possibly the youngest (still too early to determine), as previously observed by others (Gillingham et al. 2003, 2005; Fahnehjelm et al. 2008). Longer follow-up studies and a larger number of patients are necessary to determine whether carnitine has any effect on the outcome of patients with mitochondrial trifunctional protein disorders.

Acknowledgements This work was supported by the ARUP Institute for Clinical and Experimental Pathology®.

Synopsis

Patients with mitochondrial trifunctional protein or long-chain 3-Hydroxy Acyl-CoA dehydrogenase deficiency can achieve normal growth and development with proper treatment, which includes diet and low-dose carnitine supplements.

Individual Authors' Contributions

IDB, MP, and NL designed the study; NL, KSV, and LB collected the data; IDB, AL, TY, and MP contributed with data collection and interpretation; IDB, KSV, and NL wrote the manuscript. All authors were involved in revising the manuscript critically for content.

Funding

This work was supported by the ARUP Institute for Clinical and Experimental Pathology®. The authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

Conflict of Interests

Irene De Biase declares that she has no conflict of interest.

Krista S. Viau declares that she has no conflict of interest.

Aiping Liu declares that she has no conflict of interest.

Tatiana Yuzyuk declares that she has no conflict of interest.

Lorenzo D. Botto declares that he has no conflict of interest.

Marzia Pasquali declares that she has no conflict of interest.

Nicola Longo declares that he has no conflict of interest.

Compliance with Ethics Guidelines

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 waived by our Institutional Review Board in view of the retrospective nature of the study and the minimal risks to patients.

Animal Rights

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

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JIMD Reports
DOI 10.1007/8904_2016_555

RESEARCH REPORT

N-Acetylcysteine Therapy in an Infant with Transaldolase Deficiency Is Well Tolerated and Associated with Normalization of Alpha Fetoprotein Levels

Lance H. Rodan • Gerard T. Berry

Received: 11 December 2015 / Revised: 04 March 2016 / Accepted: 08 March 2016 / Published online: 30 April 2016

Abstract Transaldolase deficiency is a rare autosomal recessive disorder of the pentose phosphate pathway that presents clinically with infantile-onset hepatopathy progressing to cirrhosis, nephropathy, connective tissue abnormalities resembling cutis laxa, coagulopathy, cytopenias, and increased risk of hepatocellular carcinoma. In many cases, death occurs in infancy or early childhood. There is no established treatment for transaldolase deficiency in humans. Recent work in a knockout mouse model of transaldolase deficiency has demonstrated a benefit to supplementation with the glutathione precursor *N*-acetylcysteine (NAC). We describe an infant with genetically confirmed transaldolase deficiency with multisystem involvement, including liver enlargement and markedly elevated alpha fetoprotein. Acetaminophen was strictly avoided. Treatment with oral NAC over a 6-month period was well tolerated and was associated with a sustained normalization of alpha fetoprotein levels and stable clinical course. The clinical significance of normalized serum alpha fetoprotein in this patient is not certain, although it may reflect decreased hepatocyte injury and reduced hepatocarcinogenesis as has been suggested in the mouse disease model. NAC supplementation may provide benefit in humans with transaldolase deficiency. Longer follow-up and data on the response of additional patients with transaldolase deficiency to NAC supplementation will be required to further evaluate efficacy and optimize dosing.

Communicated by: Sylvia Stoeckler-Ipsiroglu, MD

Competing interests: None declared

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Introduction

Transaldolase deficiency is a rare autosomal recessive disorder of the pentose phosphate pathway. The latter is a major pathway for the production of NADPH, which is required for numerous biosynthetic reactions and reduction of oxidized glutathione (Wamelink et al. 2008). Transaldolase deficiency presents clinically with infantile-onset hepatopathy progressing to cirrhosis, nephropathy, connective tissue abnormalities resembling cutis laxa, coagulopathy, cytopenias, congenital heart disease, and increased risk of hepatocellular carcinoma (Eyaid et al. 2013; Leduc et al. 2014). In many cases, death occurs in infancy or early childhood. There is no established treatment for transaldolase deficiency in humans. Recent work in a knockout mouse model of transaldolase deficiency has demonstrated a benefit to supplementation with the glutathione precursor *N*-acetylcysteine (NAC) (Hanczko et al. 2009). We present the case of an infant with genetically confirmed transaldolase deficiency with multisystem disease treated with NAC for 6 months.

Case Report

The patient was of Emirati ancestry. He was the product of a non-consanguineous union. He was born at 36 3/7 weeks gestational age following an uncomplicated pregnancy. Birth weight was 2,125 g. He spent his first month in hospital for poor feeding, ultimately requiring placement of a nasogastric tube. He was also noted to have hepatomegaly, elevated transaminases, abnormal clotting profile, thrombocytopenia, and loose wrinkled skin. Based on his clinical presentation, he was diagnosed with transaldolase deficiency at 6 months of life. Molecular testing demon-

Table 1 Laboratory values at 9 and 16 months

	9 months	16 months	Normal (units)
AST	84	90	2–40 (U/L)
ALT	45	54	3–30 (U/L)
GGT	27	25	12–55 (U/L)
Alkaline phosphatase	379	293	110–400 (U/L)
Albumin	45	49	30–46 (g/L)
Bilirubin, total	6.8	6.8	5.1–20.5 (μmol/L)
Bilirubin, direct	1.7	<1.7	0–6.8 (μmol/L)
Alpha fetoprotein	457	60	1–87 (μg/L)
PTT	45	36.5	25–37 (s)
INR	1.62	1.14	
WBC	4.65	6.96	7.73–13.12 (cells/μL)
PLT	132	191	223–461 (cells/μL)
Hb	110	103	104–125 (g/L)
Creatinine	17.7	17.7	17.7–35.3 (μmol/L)
BUN	6.8	9.6	1.8–6.4 (μmol/L)
Urine beta-2 microglobulin/ creatinine	189,360	190,904	0–300 (mcg/g)
Microalbumin/ creatinine	401.2	297.7	0–20 (mg/g)

strated homozygosity for the pathogenic variant p.R192C in exon 1 of the TALDLO1 gene.

We evaluated the patient at 9 months of age. Height and weight were both less than the first percentile (z scores of −2 and −4, respectively), and head circumference was on the 35th percentile. Face was triangular in shape. There was reduced subcutaneous fat. Skin appeared loose and mildly wrinkled. He had two small capillary hemangiomas. Liver edge was palpable at the level of the umbilicus and spleen tip was palpable. Developmental assessment and neurological examination were age appropriate.

Urine polyol analysis demonstrated sedoheptulose level of 12,012 μmol/mL creatinine (normal <95), ribitol of 1,398 (normal 52–158), erythritol of 3,257 (normal 450–1,572), and arabitol of 3,335 (range 210–578) consistent with his known diagnosis. Baseline liver enzymes and function tests, serum alpha fetoprotein, CBC, creatinine, BUN, and urine tests of tubular and glomerular function are summarized in Table 1. Plasma fatty acid profile was normal. Cholesterol was reduced to 1.9 mmol/L. Total plasma glutathione was 798 μM (normal range 544–1,228). Quantitative urine organic acids demonstrated elevations of glutaric acid at 106 mmol/mol creatinine (range 0–6) and multiple citric acid cycle intermediates: citric acid 1,150 mmol/mol creatinine (normal 120–675), 2-oxoglutaric acid 560 (normal 0–152), succinic acid 133 (normal 0–80), fumaric acid 73 (normal 0–8), and malic acid 86 (normal 0–13). Plasma cystine was mildly reduced at 20 μmol/L (laboratory reference range for age 24–51). There were no additional amino acid deficiencies. Abdominal U/S demonstrated an enlarged nodular appearing liver with heterogenous course echotexture and mild splenomegaly.

The family was counselled on the risks and benefits of an empiric treatment trial with *N*-acetylcysteine (NAC). An institutional innovative therapy protocol was filed. A goal NAC dose of 100 mg/kg/day in three divided doses was selected based on reported dosing in ethylmalonic encephalopathy, another disorder associated with theoretical glutathione depletion (Banne et al. 2015). The patient was initiated on 17 mg/kg/day (100 mg) of NAC, and dosing was increased by 17 mg/kg/day approximately every 3 weeks until target dosing was reached after 4 months (13 months of age). The NAC was well tolerated with no reported adverse effects.

Over the treatment period, the only other modifications to the patient's management were replacement of the patient's longstanding infant formula, Monogen, with Pediasure 45 kcal/oz at 13 months of age, and G-tube placement at 14 months of age for failure to thrive. The family was cautioned to avoid any exposure to acetaminophen.

At most recent follow-up at 16 months of age, head circumference was on the 75th percentile, length was on the 5th percentile (z-score of −1.6), and weight was on the 2nd percentile (z-score of −1.98). Liver was palpable just below the umbilicus and spleen was not palpable.

Repeat urine polyols demonstrated sedoheptulose level of 7,178 μmol/ml creatinine (normal <95), ribitol of 776 (normal 52–158), erythritol of 2,622 (normal 450–1,572), and arabitol of 2,079 (normal 210–578). Repeat plasma total glutathione level on treatment was 865 μM, similar to baseline level. Total cholesterol level was normal at 2.6 mmol/L. Plasma cystine level was normal at 41 μmol/L (laboratory reference range for age 18–52). See Table 1 for liver enzymes and function tests, serum alpha fetoprotein, CBC, creatinine, BUN, and urine tests of tubular and glomerular function at most recent follow-up. *Note the normalization of serum alpha fetoprotein, which is plotted over time in* Fig. 1. The most recent abdominal ultrasound at 13 months of age demonstrated interval increase in hepatomegaly; spleen was normal in size. There were two echogenic lesions not visualized on the previous ultrasound that were suspicious for hemangiomas.

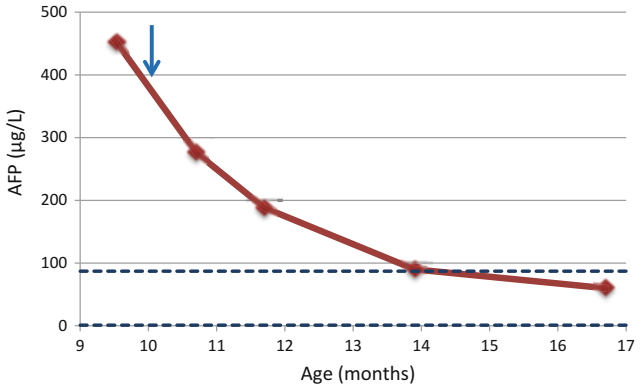

Fig. 1 Serum alpha fetoprotein levels plotted over time. The *arrow* indicates the time of initiation of NAC therapy. The normal range of AFP is indicated by the *dotted lines*

Discussion

The pentose phosphate pathway is a metabolic pathway that converts glucose-6-phosphate into fructose-6-phosphate and glyceraldehyde-3-phosphate. It consists of an irreversible oxidative phase that produces NADPH, as well as a reversible non-oxidative phase (see Fig. 2). NADPH is an important source of reducing equivalents for a number of biosynthetic reactions, including fatty acid elongation and cholesterol biosynthesis, as well as the regeneration of reduced glutathione (Wamelink et al. 2008).

Several disorders in the pentose phosphate pathway have been described to date. Perhaps the best known and most common disorder is glucose-6-phosphate dehydrogenase deficiency, which presents with drug and diet-induced hemolytic anemia. Ribulose-5-phosphate isomerase deficiency has been reported in one kindred to date in association with leukoencephalopathy (Wamelink et al. 2008).

Transaldolase deficiency has been reported in approximately 30 patients to date (Banne et al. 2015). It follows autosomal recessive inheritance, and a number of common mutations have been reported. The p.R192C mutation in the TALDO1 gene has previously been reported in the Emirati population, and a founder effect has been suggested. Despite the common genotype, there is significant phenotypic variability (Al-Shami et al. 2015). Common features include hepatosplenomegaly, liver dysfunction, anemia, thrombocytopenia, wrinkly skin, cardiac defects and cardiomyopathy, neonatal edema, renal tubulopathy, and abnormal platelet aggregation (Eyaid et al. 2013; Banne et al. 2015). The liver disease is often progressive, although it may wax and wane and there are reports of hepatomegaly with preserved liver function (Banne et al. 2015). Many patients succumb to liver failure in infancy or childhood (Eyaid et al. 2013). Patients may also develop early onset hepatocellular carcinoma (Leduc et al. 2014). Development is typically normal, although three patients with developmental delay have been described (one of whom was found to have sensorineural hearing loss) (Banne et al. 2015). Hemangiomas of skin and liver have also been reported (Eyaid et al. 2013). A number of cases have presented with hydrops fetalis (Banne et al. 2015).

The pathogenesis of transaldolase deficiency has not been entirely elucidated. It is theorized to result from a combination of toxic metabolite accumulation (e.g., sedoheptulose 7-phosphate and lipid hydroperoxides) as well as depletion of NADPH and reduced glutathione. Glutathione is a crucial antioxidant, and deficiency results in increased oxidative damage. Secondary mitochondrial dysfunction has also been described (Wamelink et al. 2008; Eyaid et al. 2013).

NAC, a precursor for glutathione synthesis, functions to replenish depleted hepatic glutathione stores, and has been used for this purpose in acetaminophen overdose and ethylmalonic encephalopathy, an inborn error of metabolism resulting in accumulation of hydrogen sulfide and glutathione depletion (Viscomi et al. 2010).

Transaldolase knockout mice spontaneously develop hepatocellular carcinoma, and when exposed to acetaminophen develop hepatic failure. Their livers are characterized by accumulation of sedoheptulose 7-phosphate and lipid hydroperoxides, depleted NADPH and glutathione, and mitochondrial dysfunction. Alpha fetoprotein expression is increased, and is associated with the development of hepatocellular carcinoma in the mice. Lifelong treatment with NAC in these mice is protective against acetaminophen-induced liver failure and blocks hepatocarcinogenesis (Hanczko et al. 2009).

We present for the first time the effects of 6 months of NAC supplementation in a patient with transaldolase deficiency. The treatment was well tolerated with no adverse effects. On therapy, we demonstrate a progressive normalization of alpha fetoprotein levels despite an interval mild increase in liver size. Although we cannot directly prove a causal relationship, we believe that this improvement is due to the NAC, and this is supported by the data from the knockout mouse model (Hanczko et al. 2009). Although it is difficult to predict the clinical implications of this normalization of alpha fetoprotein levels, we believe that this may signify decreased hepatocellular injury and a reduced risk of hepatocarcinogenesis.

We did not demonstrate a notable increase in total plasma glutathione on NAC therapy, although this measurement likely does not accurately reflex intrahepatic glutathione stores nor does it distinguish between reduced and oxidized forms. There was no change on treatment in renal tubular/glomerular dysfunction or cytopenias. Quantitative urine organic acid results in our patient were consistent with mitochondrial dysfunction, as has been

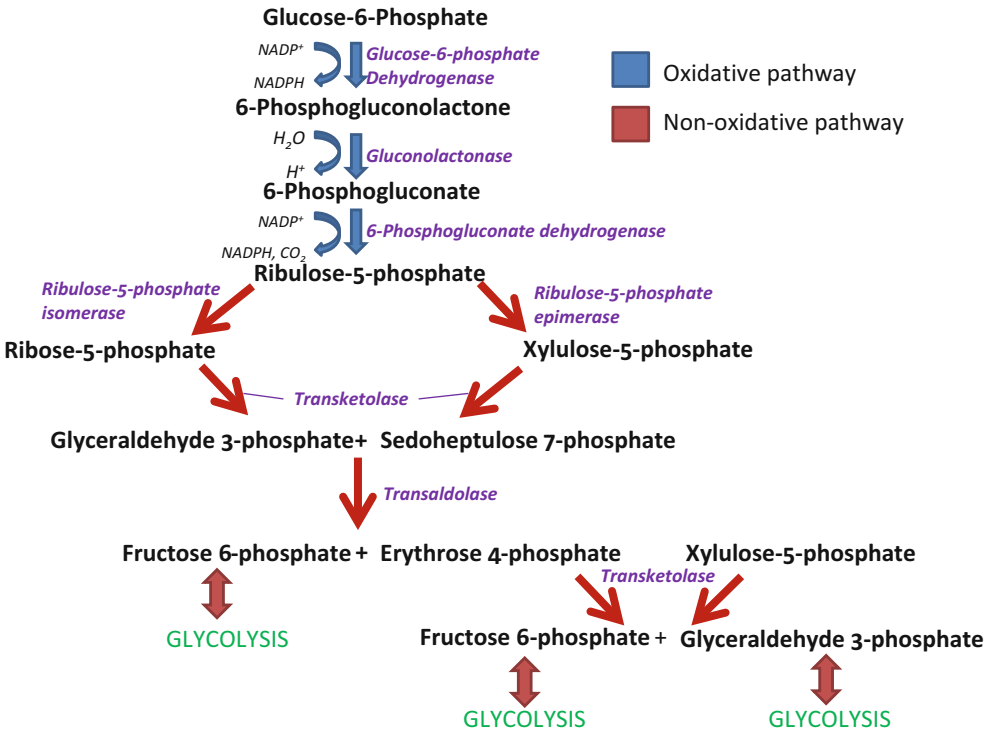

Fig. 2 Oxidative and non-oxidative components of the pentose phosphate pathway

reported in transaldolase deficiency (Wamelink et al. 2008). Baseline plasma cystine level was reduced, potentially due to increased utilization of this semi-essential amino acid in the biosynthesis of glutathione due to increased losses of glutathione in the oxidized form; repeat levels on NAC supplementation with improved nutrition were normal.

Equally important to our patient's management was the strict avoidance of acetaminophen since individuals with transaldolase deficiency may be exquisitely vulnerable to acetaminophen-induced glutathione depletion and hepatic injury based on the animal data (Hanczko et al. 2009).

Finally, it should be emphasized that treatment with NAC only addresses the reduced availability of glutathione for antioxidant defense, and does not correct the additional biochemical perturbations in this disorder including NADPH deficiency and the accumulation of toxic intermediates resulting from the enzymatic block.

In conclusion, supplementation of NAC in a patient with transaldolase deficiency appears to be well tolerated and is associated with biochemical improvement in the form of normalized alpha-fetoprotein levels. The clinical significance of this is not certain, although it may reflect decreased hepatocyte injury and reduced hepatocarcinogenesis. Longer follow-up and data on the response of additional patients with transaldolase deficiency to NAC supplementation will be required to answer these questions and optimize dosing.

Synopsis

N-acetylcysteine therapy in an infant with transaldolase deficiency was well tolerated and associated with normalization of serum alpha fetoprotein levels. NAC may provide benefit in humans with transaldolase deficiency, but additional cases and longer follow-up are required.

Author Contributions

Lance H. Rodan: Preparation of manuscript
Gerard T. Berry: Critical revision of manuscript, supervisor

Guarantor

Lance H. Rodan

Competing Interest Statement

Lance H. Rodan and Gerard T. Berry have nothing to declare.

Funding

There are no sponsors or funding to declare.

Compliance with Ethics Guidelines

Not required

Patient Consent Statement

Not applicable since the report does not contain any identifying patient information.

Conflict of Interest

There is no conflict of interest.

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JIMD Reports
DOI 10.1007/8904_2016_563

CASE REPORT

Severe Cardiomyopathy as the Isolated Presenting Feature in an Adult with Late-Onset Pompe Disease: A Case Report

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Received: 07 December 2015 / Revised: 22 March 2016 / Accepted: 23 March 2016 / Published online: 04 May 2016

The author "Mari Mori" takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Abstract Many inborn errors of metabolism can cause cardiomyopathy. Cardiomyopathy associated with glycogen storage includes *PRKAG2*-associated glycogen storage disease (GSD), Danon disease, infantile-onset Pompe disease (GSD II), GSD III, GSD IV, and phosphofructokinase deficiency (Tarui disease or GSD VII).

We present a 35-year-old female who presented with cardiomyopathy after a pregnancy complicated by primary hyperparathyroidism. She had enjoyed excellent health until her first pregnancy at age 33. One week postpartum, she developed dyspnea and an echocardiogram revealed left ventricular ejection fraction (LVEF) of 35%. A cardiac MRI was consistent with nonischemic cardiomyopathy with an infiltrative process. Endomyocardial biopsy showed striking sarcoplasmic vacuolization, excess glycogen by PAS staining, and frequent membrane-bound glycogen by electron microscopy, consistent with lysosomal GSD. Acid alpha-glucosidase (GAA) activity in skin fibroblasts was in the affected range for Pompe disease. Sequencing of the *GAA* gene revealed a paternally inherited pathogenic c.525delT (p.Glu176Argfs*45) and a *de novo* c.309C>G (p.Cys103Trp) with unknown pathogenicity. Testing of the familial mutations in her daughter indicated that the variants in the proband were *in trans*. 26-gene cardiomyopathy sequencing panel had normal results thereby excluding GSD III, Danon disease, Fabry disease, and *PRKAG2*-associated cardiomyopathy. Therefore, results strongly suggest a diagnosis of Pompe disease.

Pompe disease has a broad disease spectrum, including infantile-onset (IOPD) and late-onset (LOPD) forms. LOPD typically presents with proximal muscle weakness and respiratory insufficiency in childhood or late adulthood. Our case may represent a very unusual presentation of adult LOPD with isolated cardiomyopathy without skeletal muscle involvement or respiratory failure.

Communicated by: Marc Patterson

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Introduction

Many inborn errors of metabolism can cause cardiomyopathy. Several examples of these include mitochondrial disorders (Palecek et al. 2012), Fabry disease (Clarke 2007), glycogen storage diseases (GSDs), sphingolipid storage diseases, and mucopolysaccharidoses (Elliott et al. 2014). Cardiomyopathy associated with glycogen storage includes *PRKAG2*-associated glycogen storage (Arad et al. 2002), Danon disease (Nishino et al. 2000), infantile Pompe disease (GSD II) (Kishnani et al. 2006), GSD III (Kishnani

et al. 2010), GSD IV (Bruno et al. 2004), and phosphofructokinase deficiency (Tarui disease or GSD VII) (Musumeci et al. 2012). We report an adult with late-onset Pompe disease (LOPD) presenting with cardiomyopathy during pregnancy complicated by primary hyperparathyroidism without evidence of skeletal or respiratory muscle involvement.

Case Report

The proband is a 35-year-old female who had enjoyed excellent health and competitive athletic activities until her first pregnancy at age 33. Her history was notable for a cardiac murmur at age 23 without clinical symptoms. Clinical evaluation showed normal cardiac findings. A murmur was again noted during a job application at age 33 with reassuring results on an electrocardiogram, echocardiogram, and treadmill stress test. She had no family history of cardiomyopathy or sudden death.

During the fifth month of her pregnancy, she developed sudden-onset dyspnea lasting for a week. One week after a normal delivery, dyspnea recurred and an echocardiogram revealed dilated cardiomyopathy with a left ventricular ejection fraction (LVEF) of 35% (normal 54–76%). A follow-up echocardiogram 7 months later showed moderate LV enlargement [LV volume of 81 ml (systolic) and 127 ml (diastolic)], mild hypertrophy [LV mass index (2D) of 141 g/m^2; LV septum thickness of 12 mm (normal 7–10 mm); LV posterior wall thickness of 9 mm (normal 7–11 mm); and end-diastolic LV diameter of 55 mm (normal 39–53 mm)], and reduced systolic function [LVEF of 34% and LV longitudinal peak systolic strain of ~8% (normal $< -18\%$)]. Grade 3/4 LV diastolic dysfunction consistent with severely elevated LV filling pressure as well as bi-atrial enlargement and mild right ventricular hypertrophy was also noted. The heterogeneous echo-bright appearance of myocardium was suggestive of an infiltrative cardiomyopathic process. A subsequent cardiac magnetic resonance imaging (MRI) confirmed LV enlargement (maximal end-diastolic wall thickness of 12 mm at mid septum) with pronounced wall motion abnormalities and moderately reduced systolic function with LVEF of 32% (normal 54–85%); LV volume/Body Surface Area (BSA) of 91 ml/m^2 (systolic, normal 12–36 ml/m^2) and 134 ml/m^2 (diastolic, normal 49–90 ml/m^2); and LV mass/BSA of 66 g/m^2 (diastolic, normal 59–103 g/m^2). Extensive biventricular delayed myocardial enhancement predominantly in the epicardial and transmural muscles was noted.

Pro-BNP level was elevated at 3,894 pg/ml. The patient was started on a loop diuretic, potassium sparing diuretic, digoxin, an angiotensin II receptor blocker, and a beta-blocker though the patient admitted to intermittent noncompliance. A placement of an implantable cardioverter-defibrillator was recommended but was declined by the patient. An endomyocardial biopsy was performed based on the cardiac MRI findings. Histologic analysis of formalin-fixed tissue revealed prominent myocyte vacuolization. The vacuoles contained glycogen by Periodic acid–Schiff (PAS) staining, with a minor component of diastase-resistant material on PAS diastase (PASD). Frequent membrane-bound glycogen was seen on electron microscopy; no abnormal glycogen structures were seen. The underlying cause of the myocardial glycogen accumulation was investigated. Acid alpha-glucosidase (GAA) activity was measured on dried blood spot in two different clinical labs. The first lab reported a level in the affected range, the second lab in the carrier range. GAA activity in skin fibroblasts (16.7% normal: 260±80 nmol/h/mg protein) and in heart biopsy tissue (0.07; normal: 0.89±0.42 μmol/min/g tissue) was clearly in the affected range and partially reduced in the muscle biopsy tissues (5.86; 38% of normal: 22.91±8.4 nmol/h/mg protein). However, both muscle and heart tissue glycogen content and structure tested in the low or normal ranges (muscle glycogen content 0.11 umol glucose/mg tissue; normal 0.39–1.5; muscle glycogen structure measured as glucose-1-phosphate/glucose ratio 72.22%; normal $> 5\%$; heart glycogen content 0.14 umol glucose/mg tissue; normal > 0.047; and heart glycogen structure 23.91%; normal $> 5\%$). Branching enzyme activity in the heart and muscle tissue was also normal.

Sequencing of the *GAA* gene and follow-up parental testing revealed a paternally inherited heterozygous c.525delT (p.Glu176Argfs*45) and a heterozygous *de novo* c.309C>G (p.Cys103Trp) variant. Mutations of cysteine 103 (p.Cys103Arg and p.Cys103Gly) have been reported in Pompe disease, while the pathogenicity of c.309C>G (p.Cys103Trp) is currently unknown. This mutation was not found in the proband's mother. Polyphen, SIFT, and MutationTaster algorithms, that predict the effect of amino acid changes, predicted this change to be deleterious to protein function. The proband's daughter was tested for the familial mutations in order to determine the phase of the variants in the proband, and was found to harbor the c.525delT but not c.309C>G. These results indicate that the *GAA* variants in the proband are in trans. A 26-gene cardiomyopathy sequencing panel was sent to exclude other causes of cardiomyopathy and yielded no pathogenic mutation in any of the tested genes that included *AGL* (GSD III), *LAMP2* (Danon disease), *GLA* (Fabry disease), and *PRKAG2*. Urine glucose tetrasaccharides (Hex4) biomarker level was normal at 1.0 mmol/mol cr (<4). Creatine kinase (CK) activity levels were measured in the normal range.

The patient was also noted to have elevation of calcium (11.6 mg/dl, reference 8.9–10.1 mg/dl) and PTH (541 pg/ml, reference 15–65 pg/ml) and underwent partial parathyroidectomy due to a diagnosis of primary hyperparathyroidism; histologic analysis showed parathyroid hyperplasia. The onset of hyperparathyroidism could not be determined. Skeletal muscle biopsy performed at the same time as the parathyroidectomy showed only denervation atrophy; the specimen was not available for our review.

Several months after the surgery, she developed sudden decline in her cardiac function to an LVEF of 20–25% (normal 54–76%) with global hypokinesis and no evidence of LV dilation by echocardiography. CK-MB was 36 U/l (normal 7.0–25.0 U/l). An urgent placement of a centrifugal continuous-flow left ventricular assist device (LVAD) was required. Right ventricular myocardial tissue taken during the LVAD placement, 17 months after the first cardiac biopsy, showed progression in lysosomal glycogen storage with myocyte hypertrophy with striking sarcoplasmic vacuolization, excess glycogen by PAS staining, and diffusely increased lysosomal activity by acid phosphatase staining on frozen tissue (Fig. 1). PASD showed only minimal diastase-resistant material. Phosphofructokinase and myophosphorylase enzymatic activities were intact. There was widespread replacement fibrosis. Electron microscopy showed frequent membrane-bound glycogen, degenerative changes, and mildly increased mitochondrial numbers (Fig. 1); Rectus skeletal muscle obtained at the same time showed mild degenerative features and fiber atrophy. Extensive cautery artifact limited the interpretation, although PAS and PASD staining showed no evident glycogen deposition in the few well-preserved areas.

The patient had multiple pulmonary function tests due to the diagnosis of LOPD with normal results with normal vital capacity in the upright and supine positions. Her neurological examination before and after the LVAD placement were both normal without muscle weakness. She had excellent muscle strength except slightly weak back extensors and normal grip and pinch strength. Her motor speech function, inspiratory/expiratory muscle strength, and lingual strength were essentially normal. Her 6-min walk test showed 76.9% predicted for her age and gender. Optimal cooperation from the patient was not possible for this test as she was fatigued from international traveling. She recovered to the point where she can practice Pilates and low weight resistance exercises after the LVAD placement. Enzyme replacement therapy (ERT) with alglucosidase alfa was discussed after the diagnosis of LOPD was made. It was felt that the therapy would not reverse the extensive fibrotic and irreversible damage evident in the cardiac muscle, nor alleviate cardiomyopathy as penetration of the enzyme through the fibrotic tissue was questionable. She was maintained on warfarin and aspirin but suffered an extensive thrombotic stroke that has precluded further ongoing evaluation for possible heart transplant.

Discussion

Based on myocardial histology, low enzyme activity in fibroblast tissue and cardiac muscle, and the identification of two *GAA* changes in trans, LOPD is the favored diagnosis in this case.

Pompe disease has a broad disease spectrum, including infantile-onset (IOPD) and late-onset (LOPD) forms. LOPD typically presents with proximal muscle weakness and respiratory insufficiency in childhood or late adulthood (Kishnani et al. 2006). Cardiac involvement in LOPD include aortic aneurysm (El-Gharbawy et al. 2011), arrhythmias, ventricular dysfunction, but cardiomyopathy is rare and, if found, is not the primary presentation (Soliman et al. 2008; Morris et al. 2015). Lee et al. reported six children with Pompe disease who developed cardiomyopathy at ages ranging from 1 month to 3 years (Lee et al. 2014). All of the patients also had muscular involvement with hypotonia or developmental delay at the time of presentation. Ben-Ami et al. reported a 45-year-old man with dilated cardiomyopathy and marked myocardial hypertrophy, in whom endomyocardial biopsy and staining indicated abnormal glycogen storage. Enzyme and genetic tests and other clinical history were not described in this case (Ben-Ami et al. 2001). In the current case, a diagnosis of LOPD is most likely based on histology, ultrastructure, enzymology, and *GAA* genotype. Two myocardial biopsies showed membrane-bound glycogen associated with lysosomal structures, suggesting a lysosomal GSD. The presence of glycogen granules with a normal structure argues against a polyglucosan disorder. *PRKAG2*-associated cardiomyopathy, Danon disease, Fabry disease, GSD IV, and GSD III were ruled out by sequencing and/or enzymology. Normal staining of phosphofructokinase and myophosphorylase activity by immunohistochemistry ruled out GSD VII and GSD V. Some mitochondrial disorders can cause isolated cardiomyopathy but would not cause glycogen storage in histology. *GAA* sequencing revealed a pathogenic mutation c.525delT and a missense variant c.309C>G *in trans.* Notably, *de novo* variants in GAA are rare and the *de novo* variant in our case may represent a post-zygotic or germline event. GAA activity in skin fibroblasts, the current gold standard for laboratory diagnosis (Kishnani et al. 2006), was clearly in the affected range. Urine-Hex4 and CK can be normal in Pompe disease. In our extensive experience, skeletal muscle biopsy can often be normal in affected in patients with LOPD if the biopsy is not taken from an affected tissue. It is possible that at the current age our proband has yet to manifest skeletal myopathy

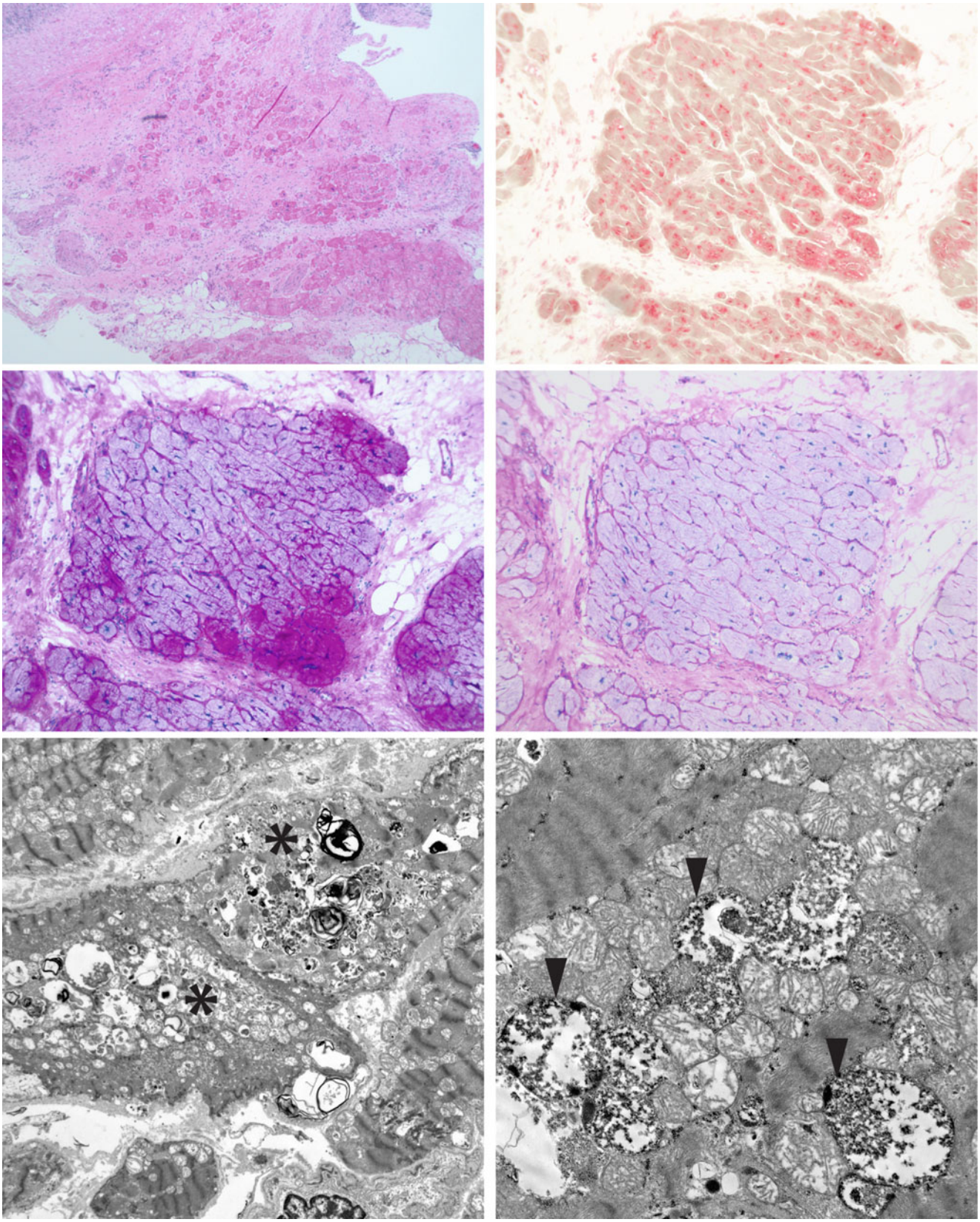

Fig. 1 Histologic and ultrastructural features of myocardial tissue taken during LVAD placement, showing features of a lysosomal glycogen deposition disorder. *Top row, left*: paraffin-embedded H&E section showing extensive fibrosis and myocyte loss, myocyte hypertrophy, and vacuolization, with reactive, pericardial, mononuclear inflammation. *Top row, right*: frozen section stained for acid phosphatase activity

or respiratory failure and other LOPD symptoms, whereas pregnancy and hyperparathyroidism triggered exacerbation of cardiomyopathy (Andersson et al. 2004).

Conclusion

We described a rare presentation of adult LOPD that presented with isolated cardiomyopathy after a pregnancy, complicated by hyperparathyroidism. Histology, enzymology, and molecular analysis of *GAA* support the diagnosis. LOPD can present with cardiomyopathy even in an absence of skeletal muscle involvement. Cardiac muscle pathology with specific staining supported by genetic and enzyme testing can lead to the diagnosis.

Synopsis

LOPD can present with an isolated cardiomyopathy and endomyocardial biopsy can be diagnostic.

Compliance with Ethics Guidelines

Conflict of Interest

Mari Mori, Lauren A. Bailey, Catherine W. Rehder, Januario Estrada, Deeksha S. Bali, Anne F. Buckley, Jennifer S. Li, Joseph G. Rogers, and Priya S. Kishnani declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by the any of the authors.

Contributions of Individual Authors

Mari Mori and Priya S. Kishnani: Patient evaluation, literature search, drafting and revisions of the manuscript.

Lauren A. Bailey: Collection of clinical data.

Catherine W. Rehder: Molecular analysis of the proband and daughter, and critical revision of the manuscript.

Januario Estrada: Preparation and interpretation of skeletal and cardiac muscle tissues.

Deeksha S. Bali: Enzyme activity analysis of fibroblast, and skeletal/cardiac muscle tissues, and critical revisions of the manuscript.

Jennifer S. Li and Joseph G. Rogers: Patient evaluation and critical revisions of the manuscript.

Anne F. Buckley: Interpretation of skeletal and cardiac muscle tissues and critical revisions of the manuscript.

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Fig. 1 (continued) showing diffusely increased lysosomal activity. *Middle row, left*: PAS staining of frozen section showing increased glycogen in myocytes, in an uneven pattern. *Middle row, right*: PASD (frozen) showing only minimal diastase-resistant material. *Bottom row, left*: electron microscopic image at 2200× showing vacuoles (*asterisks*) containing glycogen and lysosomal structures. *Bottom row, right*: electron microscopic image at 9300× showing that the glycogen is frequently membrane-bound (*arrowheads*)

JIMD Reports
DOI 10.1007/8904_2016_550

RESEARCH REPORT

Chronic Diarrhea in L-Amino Acid Decarboxylase (AADC) Deficiency: A Prominent Clinical Finding Among a Series of Ten French Patients

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Received: 24 September 2015 / Revised: 09 February 2016 / Accepted: 29 February 2016 / Published online: 05 May 2016

Abstract Aromatic L-amino acid decarboxylase (AADC) deficiency is an autosomal recessive inborn error of metabolism, affecting catecholamines and serotonin biosynthesis. Cardinal signs consist in psychomotor delay, hypotonia, oculogyric crises, dystonia, and extraneurological symptoms.

Patients and methods: We present a retrospective descriptive multicentric study concerning ten French children with a biochemical and molecular confirmed diagnosis of AADC deficiency.

Results: Clinical presentation of most of our patients was consistent with the previous descriptions from the literature (hypotonia (nine children), autonomic signs (nine children), sleep disorders (eight children), oculogyric crises (eight children), motor disorders like hypertonia and involuntary movements (seven children)). We described however some phenotypic particularities. Two patients exhibited normal intellectual abilities (patients already described in the literature). We also underlined the importance of digestive symptoms like diarrhea, which occurred in five among the ten patients. We report in particular two children with chronic diarrhea, complicated by severe failure to thrive.

Communicated by: Nenad Blau, PhD

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Vanillactic acid (VLA) elevation in urines of one of these two patients led to suspect the diagnosis of AADC deficiency, as in two other patients from our population.

Conclusion: Some symptoms like chronic diarrhea were atypical and have been poorly described in the literature up to now. Diagnosis of the AADC deficiency is sometimes difficult because of the phenotypic heterogeneity of the disease and VLA elevation in urines should suggest the diagnosis.

Introduction

Aromatic L-amino acid decarboxylase (AADC) deficiency is an inborn error of neurotransmitter biosynthesis with an autosomal recessive inheritance. The enzyme AADC is the second enzyme in the catecholamines (dopamine, noradrenaline, and adrenaline) and serotonin biosynthetic pathway (Pons et al. 2004). Currently less than 100 cases are reported in the world (Hyland and Clayton 1990, Hyland et al. 1992; Korenke et al. 1997; Maller et al. 1997; Swoboda et al. 2003; Chang et al. 2004; Pons et al. 2004; Lee et al. 2009; Ito et al. 2008; Brun et al. 2010). This pathology is clinically characterized by oculogyric crises, dystonia and other involuntary movements, hypotonia, global developmental delay and autonomic dysfunctions (Swoboda et al. 2003). The diagnosis relies on the analysis of neurotransmitters in the cerebral spinal fluid (CSF) showing a reduced concentration of homovanillic acid (HVA, main metabolite of dopamine), 5-hydroxyindoleacetic acid (5-HIAA, main metabolite of serotonin), and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG); an increased concentration of 3-methoxytyrosine (3MT), 3-O-methyldopa (3OMD, main metabolite of L-dopa), L-dopa and 5-hydroxytryptophan (5HTP). Vanillactic acid (VLA) elevation investigated by organic acids profile in urine was also reported in the pathology (Hyland et al. 1992). The confirmative diagnosis is based on enzyme activity in the plasma (Hyland et al. 1992; Verbeek et al. 2007) and DDC gene (coding for the enzyme AADC) analysis (Hyland et al. 1992; Verbeek et al. 2007; Haavik et al. 2008). The therapeutic response is variable (Allen et al. 2009), often disappointing and in only single cases clinical improvement has been observed (Swoboda et al. 2003; Pons et al. 2004; Lee et al. 2009; Allen et al. 2009; Manegold et al. 2009; Brun et al. 2010). Here we report the phenotype of ten patients recruited in France, and we describe particularly two patients with severe chronic diarrhea.

Materials and Methods

Patients

Ten patients with a diagnosis of AADC deficiency (confirmed by enzymatic and/or genetic analysis) established in France were included in this study. Our patients, six boys and four girls, came from nine families with various ethnic background. Two of these patients have already been described in the literature (case 1 and case 5) (Barth et al. 2012; Arnoux et al. 2013) (cf. Table 1).

Detailed data on patients were collected in the medical files. Information on DNA variations was compared with BIOMDB data (http://www.biopku.org) and the literature. Informed consent according to the Declaration of Helsinki was obtained from the parents to collect the data of the patients.

Biochemical and Molecular Investigations

Neurotransmitters in CSF were investigated by high-performance liquid chromatography (HPLC); organic acids profile in urine was investigated by gaseous chromatography with mass spectrometry; enzyme activity was measured in plasma, after adding PLP and L-dopa, reaction products were then quantified by HPLC.

Results

Clinical Findings

Symptoms were present before the age of 1 year in all children except case 5. Oculogyric crises became evident at the median age of 13 months (ranging from 3 to 42 months), and were not documented in two children at the time of investigation. Hypotonia was observed in nine children except case 5; four patients lost head control and four patients never acquired head control. Seven patients presented with a severe encephalopathy although patients 1, 5, and 6 were less severely affected. A good eye contact was however reported in nine children. Two patients had a normal intellectual efficiency: patient 1 composite Intellectual Quotient was 85 (Wechsler Intelligence Scale for Children III) and patient 5 had normal education in elementary school with speech therapy. Limb hypertonia was noticed in seven children, seven children presented with dystonia, four with dyskinesia/chorea, one with non-epileptic myoclonia, one with ballic movements. Autonomic signs were observed in nine children except case

Table 1 Clinical and paraclinical features in our population

Patient/Sex	1/F (Barth et al.)	2/F	3/M	4/M	5/F (Arnoux et al.)	6/M[a]	7/M[a]	8/M	9/F	10/M
Consanguinity	No	Yes	Yes	Yes	No	Yes	Yes	No	Yes	No
Perinatal history	No	No	No	No	No	No	No	No	No	No
Age at first consultation	3 m	3 m	3 m	1 m	20 m	4 m 1/2	2.5 y	1 y	3 m	2 m
Age at diagnosis (CSF profile)	7 y	3.5 y	8 m	8.5 y	6 y	5 m	NT	5.5 y	3.5 m	3 m
Clinical signs										
Neurologic symptoms										
Severe encephalopathy	–	+	+	+	–	+	+	+	+	+
Ocular contact	+	–	+	+	+	+	+	+	+	+
Acquired microcephaly	–	+	–	–	–	–	+	–	–	–
Axial hypotonia	+	+	+	+	–	+	+	+	+	+
Limb hypertonia	+	+	+	+	–	+	+	+	–	–
Hypokinesia	–	–	–	–	–	–	–	–	–	–
Involuntary movements	+	+	+	+	–	+	+	+	+	+
Functional prehension	+	–	–	–	+	+	–	–	–	–
Tongue thrusting	–	–	–	–	–	–	+	–	–	–
Oculogyric crises	+	–	+	+	–	+	+	+	+	+
Seizures	–	–	–	–	–	+	+	–	–	–
Autonomic signs										
Hyper salivation	+	+	+	+	–	+	+	–	+	+
Excessive sweating	+	+	+	+	–	+	+	–	+	–
Ptosis	+	–	–	–	–	–	–	–	+	–
Nasal obstruction	+	+	+	+	+	+	–	–	+	–
Nonneurological signs										
Sleeping disorders	+	–	+	+	+	+	–	+	+	+
Diarrhea/age at onset	+/3 y	–	–	+/5.5 y	+/3 y	–	–	+/6 y	+/6 m	–
Gastrostomy	–	+	–	+	–	–	–	+	–	+
Hypoglycemia	–	–	–	–	+	–	–	–	+	–
GH deficiency	–	–	–	+	–	–	–	–	–	–
Malaise with cyanosis	–	+	–	–	–	–	–	–	–	–
Stridor	–	–	+	–	–	–	–	–	+	–
Biochemical investigations										
VLA increase in urine	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Enzyme activity pmol/m in/ml (16–130)	4	0	0	0	5	<2	NT	NT	5	0.05

(continued)

Table 1 (continued)

Patient/Sex	1/F (Barth et al.)	2/F	3/M	4/M	5/F (Arnoux et al.)	6/M[a]	7/M[a]	8/M	9/F	10/M
Neurotransmitters CSF values nmol/L normal range)										
HVA	**110** (202–596)	**12** (304–658)	**30** (295–932)	<**10** (202–596)	**248** (304–658)	**27** (310–1,328)	NT	**19** (144–801)	**61** (310–1,100)	**89** (543–1,142)
HIAA	**12** (87–366)	**5** (106–316)	<**5** (114–336)	<**10** (87–366)	**40** (106–316)	**3** (150–1,142)	NT	**20** (88–316)	**19** (150–800)	**74** (383–1,028)
OMD	**520** (5–60)	**740** (3–64)	**NT**	**729** (5–60)	**592** (3–64)	**2,500** (20–162)	NT	**1,048** (3–64)	**NT**	**3,273** (<25)
5–HTTP	**56** (2–16)	55 (4–23)	**74** (<10)	**139** (2–16)	**50** (4–23)	**300** (2–26)	NT	**41** (4–23)	**107** (<10)	**264** (<200)
Mutation analysis										
Mutations DDC gene	c.1040G>A p. R347Q	c. 781+6T>c	c. 1040G>A p. R347Q	c. 106G>A p. G36R	c. 97G>C p. V33L	c. 1040G>A p. R347Q	c. 1040G>A p. R347Q	c. 1040G>A p. R347Q	c. 823G>A p. A275T	c. 1379T>G p. V460G
	c. 478C>T p. R160W	c. 781+6T>	c. l040G>A p. R347Q	c. 1340G>A p. R447H	c. 1385G>C p. R462P	c. 1040G>A p. R347Q	c. 1040G>A p. R347Q	c. 1040G>A p. R347Q	c. 823G>A p. A275T	c. 73G>Ap. E25K

F female, *M* male, *m* months, *y* years, *CSF* cerebrospinal fluid, + present, − absent, *VLA* vanillactic acid, HVA homovanillic acid, *HIAA* 5-hydroxyindoleacetic acid, *OMD* 3-O-methyldopa, *5-HTP* 5-hydroxytryptophan, *DDC* dopa decarboxylase, *NT* not tested

[a] Patients 6 and 7 are brothers, AADC deficiency concerning patient 7 was established after his death according to the diagnosis of his younger affected brother

8 (cf. Table 1) and sleep difficulties in eight children. Seizures occurred in two children, hypoglycemia in two patients, growth hormone (GH) deficiency in one child. Psychiatric symptoms were not reported in our group of patients.

A total of five patients suffered from diarrhea, which was severe in three and in two children diarrhea resulted in severe failure to thrive and malnutrition (a detailed description of these two patients is provided thereafter). Diarrhea was permanent and chronic in three patients (patients 4 and 8 described thereafter and patient 5 described by Arnoux et al.) with an age of onset ranging from 3 to 6 years; diarrhea was transient and/or alternating with constipation in two patients (patient 1 described by Barth et al. and patient 9) (cf. Table 1).

Biochemical Investigations

VLA dosage in urine was performed in all of the patients, and was elevated in 9/10 patients (except patient 7). For three of the patients this elevation suggested the diagnosis of AADC deficiency. CSF analysis was performed in nine children and showed a typical profile in all patients. Enzyme activity was measured in eight patients, and was always very low (maximum 5% of total activity) or not detectable (cf. Table 1).

Molecular analysis

Ten different mutations were detected, five of them had not been described earlier: c.478C>T, c.781+6T>c, c.106G>A, c.1379T>G, c.73G>A (cf. Table 1). New variations were considered as pathogenic according to prediction databases (BIOMDB). The NCBI nucleotide reference sequence used was NM_000790.3 and the protein sequence reference NP_000781.1.

Treatment

The patients benefited from various treatments, with limited benefit in four. Only two patients with a moderate phenotype exhibited significant clinical improvement concerning motor function and oculogyric crises (patient 1) and concerning motor function, diarrhea, and nasal obstruction (patient 5) (cf. Table 2).

Patient 4

This patient was the first child of consanguineous Moroccan parents. He was born full term and had feeding difficulties from birth. Hypotonia was noticed at the age of 1 month. He progressively exhibited limbs hypertonia, dystonia, autonomic signs (excessive sweating, nasal obstruction), sleep disorders. Oculogyric crises appeared at the age of 3.5 years, occurred two times a week, and were associated with dystonic crises. Between the attacks, this patient had a good ocular contact. Due to swallowing difficulties and growth failure, gastrostomy procedure was proposed but the parents finally agreed for gastrostomy feeding when the child was 5 years of age. At this age his neurological examination showed severe axial hypotonia and limb hypertonia, good ocular contact, no language and no prehension. From the age of 5.5 years, this patient suffered from severe chronic diarrhea, which became the most disabling symptom for the family. Diarrheic stools occurred 10–15 times a day; aqueous diarrhea was described, particularly after enteral feeding. This patient also had a GH deficiency, but despite a GH supplementation we noticed only a small positive effect on trophic state. At the age of 8, he weighted 14 kg (below three standard deviations) and his height was 115 cm (below two standard deviations). Biochemical investigations were performed in order to find out the etiology of this chronic diarrhea. Stools infectious investigations (bacteriological, viral, parsitological and mycotic analysis) were normal. There was no ionic, hepatic or pancreatic disorder, no inflammation. Malabsorption (D-Xylose test, steatorrhea, vitamins), celiac disease, CDG syndrome, intolerance to saccharides were excluded. Hormonal tests (TSH, VIP Gastrin, calcitonin) and serotonin dosage in blood were normal. Red Carmin test was concordant with the diagnosis of motor diarrhea. Metabolic investigations were also performed and urine analysis disclosed increased VLA dosage, which suggested AADC deficiency. Typical AADC deficiency profile of CSF neurotransmitters was identified, AADC enzyme activity was not detectable. Levodopa 160 mg/day (10 mg/kg/day), oxitriptan 100 mg/day (6 mg/kg/day), and serotonin were ineffective on diarrhea or other symptoms. Loperamide 2 mg/day (0.15 mg/kg/day) moderately improved diarrhea frequency.

Patient 8

This patient is the third child of nonconsanguineous parents. Pregnancy and delivery were uneventful. Hypotonia was noticed from the age of 3 months. Oculogyric attacks occurred at the age of 1 year; the attacks lasted a few minutes, recurred several times a day. Seizures were suspected; antiepileptic treatment was initiated without any benefit. A gastrostomy was performed at the age of 2 because of swallowing difficulties, which improved with time and gastrostomy was then only occasionally used. The patient was lost of follow-up until the age of 5.5 years, when he was referred for feeding difficulties with hematemesis. Due to these symptoms, a new gastrostomy and a Nissen surgery were performed. At this time, clinical

Table 2 Treatments and clinical evolution with treatments

Patients	Treatments	Evolution with treatment
1	Pyridoxine 250 mg/day Bromocriptine 7.5 mg/day Levodopa 12 mg/day Folinic acid	Positive effects: bromocriptine (transitory effects), levodopa: better balance, fatigue improvement, better intelligibility, better fine motor function, oculogyric crises improvement Side effects: not reported
2	Bromocriptine 3 mg/day (0.3 mg/kg/day) Pyridoxal phosphate 600 mg/day (65 mg/kg/day) Folinic acid L-carnitine 900 mg/day (100 mg/kg/day)	Positive effects: no clinical improvement Side effects: pyridoxal phosphate: vomiting
3	Pyridoxine 50 mg/day (5 mg/kg/day) Folinic acid 5 mg/day (0.5 mg/kg/day) Bromocriptine 7.5 mg/day (0.75 mg/kg/day) Levodopa-carbidopa 150 mg/day (15 mg/kg/day) Serotonin Oxitriptan (Levotonine®) 5 mg/day (7.5 mg/kg/day) Trihexyphenidyl (Artane®) 3 mg/day (0.3 mg/kg/day) Ropinirole (Requip®) Rotigotine patch (Neupro®)	Positive effects: no clinical improvement Side effects: not reported
4	Levodopa 160 mg/day (10 mg/kg/day) Oxitriptan l00 mg/day (6 mg/kg/day) Serotonin	Positive effects: no clinical improvement Side effects: not reported
5	Pyridoxine 600 mg/day (30 mg/kg/day)	Positive effects: diarrhea improvement, nasal obstruction improvement, handwriting improvement Side effects: not reported
6	Pyridoxine 500 mg/day (60 mg/kg/day) Folinic acid 30 mg/day (3.5 mg/kg/day) Bromocriptine 1.25 mg/day (0.15 mg/kg/day) Amitriptyline 4 mg/day (0.5 mg/kg/day) Ropinirole 0.5 mg/day (0.06 mg/kg/day) Rotigotine Patch (Neupro®) Clonazepam Phenobarbital	Positive effects: ropinirole: mild improvement of head control, but persistence of an important axial hypotonia Side effects: not reported
7[a]	Antiepileptic drugs	
8	Pyridoxine 250 mg/day (19 mg/kg/day) Folinic acid l0 mg/day (0.8 mg/kg/day) Ropinirole 0.75 mg/day (0.06 mg/kg/day) L-carnitine l,000 mg/day (75 mg/kg/day) Clobazam 15 mg/day (1. 2 mg/kg/day)	Positive effects: ropinirole: vigilance improvement Side effects: ropinirole: vomiting
9	Pyridoxine 360 mg/day (50 mg/kg/day) Pramipexole 0.56 mg/day (0.08 mg/kg/day) Folinic acid 10 mg/day (1.4 mg/kg/day) Selegiline 4 mg/day (0.6 mg/kg/day) Apomorphine pump 1.8 mg/h (0.25 mg/kg/h) Amitriptyline 8 mg/day (1.l mg/kg/day) Melatonine 6 mg/day (0.9 mg/kg/day)	Positive effects: no clinical improvement Side effects: apomorphine pump: skin rash, vomiting
10	Pyridoxine Selegiline Levodopa	Positive effects: selegiline: sleeping disorders improvement, interaction improvement Side effects: levodopa: vomiting, tremor

[a] As the AADC deficiency diagnosis was established after the death of the patient, patient 7 did not receive treatment specific for AADC defect, data concerning the antiepileptic drugs that were administered are not available

examination showed a severe encephalopathy, with truncal hypotonia with no head control, lower limbs pyramidal tract signs, movement disorders including non-epileptic myoclonia, ballic movements, dyskinesia, dystonia, possible ocular contact but no language; sleep disorders were also reported. The patient exhibited aqueous diarrhea (about 6 per day spontaneously, and after every feeding attempt), associated with severe failure to thrive (weight 12 kg at the age of 6 years, below three standard deviations). Repeated stools infectious investigations were negative. Stools pH was 7, which is concordant with normal oligosaccharides tolerance. There was no ionic or thyroid hormone abnormalities, blood analysis did not disclose inflammation. Hepatic and pancreatic investigations were normal. Malab-

sorption, celiac disease, and cow's milk proteins allergy were excluded. Red Carmin Test was concordant with the diagnosis of motor diarrhea. Loperamide 2 mg/day (0.15 mg/kg/day) did not provide any improvement of diarrhea, lacteol 680 mg/day (50 mg/kg/day) was also ineffective. Further etiological investigations were performed, with exhaustive metabolic analysis including CSF neurotransmitters profile, which was consistent with AADC deficiency. Enteral feeding was rapidly challenged by the severity of the diarrhea, which persisted despite dramatic decrease of the ration and various specific regimens; pharmacological treatments used for AADC deficiency (ropinirole 0.75 mg/day (0.06 mg/kg/day) (dopa agonist), pyridoxine 250 mg/day (19 mg/kg/day), and folinic acid 10 mg/day (0.8 mg/kg/day)) did not provide any improvement. Total parenteral feeding through a central catheter was undertaken, nevertheless the child still exhibited diarrheic stools several times a day. The patient suffered from several nosocomial infections and died at 7 years of age during a severe sepsis, due to central catheter infection.

Discussion

In this paper we describe the phenotype of ten patients with AADC deficiency, and we emphasize the occurrence of motor diarrhea with variable age of onset in half of them. Among these five patients, two children (case 4 and 8) exhibited devastating digestive symptoms, resulting in malnutrition, and requiring surgical procedures whose benefit was very limited (Nissen surgery, gastrostomy, parenteral feeding). Diarrhea occurred in patients with variable severity of neurological involvement, as it was also a main symptom of patient 5 with a milder phenotype characterized by normal motor function and normal learning abilities. Although extraneurological manifestations are common in AADC deficiency, digestive disorders (except swallowing difficulties) are poorly described in the literature. In the largest series of 78 patients reported up to now, Brun described feeding difficulties in 42% of the patients, but diarrhea is not recorded among this series of patients (Brun et al. 2010). Lee et al. reported that four out of their eight patients suffered from diarrhea (Lee et al. 2009), Manegold et al. reported constipation in four patients and diarrhea in two out of nine patients (Manegold et al. 2009); recently, Graziano et al. reported a patient with a complex phenotype including chronic diarrhea (Graziano et al. 2015). Nevertheless, no details are provided concerning the semiology of diarrhea of these patients. The relationship between drug administration and diarrhea in our patients must be discussed. Levodopa, oxitriptan, or L-carnitine might induce diarrhea; in the patients who received these drugs, diarrhea occurred before the introduction of these drugs. The other drugs used in our patients (pyridoxine, ropinirole, selegiline, clobazam) are not known to be responsible for diarrhea, or might rather induce constipation (bromocriptine, pramipexole, apomorphine pump, amitriptyline). Therefore we do not consider that the drugs administered to our patients could be responsible for diarrhea. In our two described patients, aqueous stools are described, and an exhaustive evaluation by gastro pediatricians was concordant with motor diarrhea. Loperamide treatment was ineffective in case 4, and improved moderately the digestive symptoms of case 8. Loperamide, with its antisecretory action and also its action on intestinal motility, is indeed the first line therapy for motor diarrhea (Ooms et al. 1984) but its benefit may vary among patients. Physiopathologic pathway of diarrhea in AADC deficiency might only be hypothesized. The role of serotonin and its metabolites in motor diarrhea has been largely described: serotonin increases intestinal motility, especially by stimulating 5-HT_4 and 5-HT_3 receptors. In AADC deficiency, even though serotonin rate is low, the increased level of the serotonin precursor 5-HTP might be implicated in the occurrence of digestive troubles (Hyland et al. 1992). Autonomic dysfunction is a common cause of diarrhea, and such autonomic dysfunction, which is part of AADC pathophysiology, might also be implicated in chronic diarrhea observed in AADC patients (Swoboda et al. 2003).

Among our group of ten patients, two children (case 1 and case 5) (Barth et al. 2012; Arnoux et al. 2013) are noteworthy because they had normal intellectual efficiency, and they attended a normal school at last follow-up (10 years old for patient 1 and 6 years old for patient 5). Although mental retardation is not reported in all the patients reported in the literature (Brun et al. 2010; Tay et al. 2007), in most cases AADC deficiency causes a severe encephalopathy. However, patients display severe hypotonia and abnormal involuntary movements which dramatically interfere with motricity and communication abilities; therefore, their cognitive level is difficult to evaluate. Other milder phenotypes have been recently described (Helman et al. 2014; Leuzzi et al. 2015), suggesting the heterogeneity and phenotypic widening of the disease.

The diagnosis of AADC deficiency in patient 4 of this series was suggested by increased VLA in the urinary organic acid profile, which was included in an exhaustive metabolic screening for chronic diarrhea. Urine VLA was increased in all but one patient of this series, and actually this elevation is not a constant finding in cases reported in the literature (Brun et al. 2010). This molecule can pass

unnoticed (Braütigam et al. 2000), and it can be necessary to ask specifically for it. Although this peak is not specific for AADC deficiency (Clayton et al. 2003; Mills et al. 2005; Hyland 2007), and is only exhibited by a subgroup of AADC patients, it must be considered as a red flag for AADC deficiency diagnosis, which will be confirmed by CSF neurotransmitters profile analysis. Therefore, organic acid profile in urine (including VLA dosage) is actually an interesting untangling test whatever the phenotype. Recently Atwal et al. reported a novel and less invasive approach to diagnose AADC deficiency using plasma metabolomic profiling (Global MAPS platform) (Atwal et al. 2015). This new technique in case of increased VLA in the urinary organic profile could increase the specificity of urinary organic profile, but is available in a limited number of laboratories up to now.

Molecular analysis identified ten different pathogenic variations including five novel mutations. Four children with a severe clinical phenotype were homozygous for the same mutation. The severity of clinical manifestations might be correlated to the nature of the genetic defect (Tay et al. 2007) and mild phenotypes recognition is important since the treatment might be more efficient (Tay et al. 2007; Allen et al. 2009). All of the patients, except patient 7, were treated according to the recommendations (Allen et al. 2009; Brun et al. 2010). Diarrhea in patient 5 was ameliorated after pyridoxine treatment, which also resulted in a decrease of dysautonomic symptoms like nasal obstruction; pyridoxine is converted to PLP (pyridoxal-5′-phosphate), an AADC cofactor which might result in increased residual enzyme activity. Overall, the various treatments were well tolerated, but therapeutic strategy resulted in poor improvement, and was very disappointing, as previously reported in the literature (Brun et al. 2010). Gene replacement therapy is currently developed and may represent an attractive therapeutic approach for this devastating disorder (Hwu et al. 2012).

Conclusion

Our observations expand the phenotype associated with AADC deficiency as we highlight that diarrhea might be a major symptom of the disease, associated or not with severe encephalopathy; more subtle symptoms suggesting autonomic dysfunction (nasal obstruction, excessive sweating, hypersalivation, ptosis), or sleep disorders might represent minor associated signs that are important to identify, as they might help the clinician to suggest the diagnosis of AADC deficiency and prompt urinary organic acid profile analysis. Diagnosis of the pathology is however difficult because of its clinical heterogeneity and VLA elevation in urine can be useful for the diagnosis.

Acknowledgments We thank the patients and their families. We are indebted to Dr Lagavulin for generating helpful discussions.

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Take-Home Message

Diarrhea might be a major symptom of AADC deficiency and subtle associated symptoms (autonomic dysfunction, sleep disorders) are important to identify, as they might help the clinician to suggest the diagnosis of AADC deficiency and prompt urinary organic acid profile analysis.

Compliance with Ethics Guidelines

Disclosure of Conflicts of Interest

MA Spitz, MA NGuyen, S Roche, B Heron, M Milh, P de Lonlay, L Lion-François, H Testard, S Napuri, M Barth, S Fournier-Favre, L Christa, C Vianey-Saban, C Corne, A Roubertie declare that they have no conflict of interest.

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JIMD Reports
DOI 10.1007/8904_2016_565

CASE REPORT

Hyperammonemia due to Adult-Onset *N*-Acetylglutamate Synthase Deficiency

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Received: 04 January 2016 / Revised: 25 March 2016 / Accepted: 29 March 2016 / Published online: 05 May 2016

Abstract A 59-year-old woman, with a medical history of intellectual disability after perinatal asphyxia, was admitted because of coma due to hyperammonemia after she was treated for a fracture of the pelvis. The ammonia level was 280 μM. Acquired disorders as explanation for the hyperammonemia were excluded. Metabolic investigations showed an elevated glutamine and alanine and low citrulline, suspect for a urea cycle defect (UCD). Orotic acid could not be demonstrated in urine. DNA investigations were negative for mutations or deletions in the *OTC* and *CPS1* gene, but revealed a homozygous c.603G>C mutation in exon 2 of the *N*-acetylglutamate synthase (*NAGS*) gene (NM_153006.2:c.603G>C), which mandates p.Lys201Asn. This is a novel mutation in the *NAGS* gene.

After the diagnosis of NAGS deficiency was made carbamylglutamate was started in a low dose. In combination with mild protein restriction the ammonia level decreased to 26 μM.

This is one of the first patients in literature in whom the diagnosis of a UCD is made at such an advanced age. It is important for the adult physician to consider a metabolic disorder at every age.

Communicated by: Nancy Braverman, M.D.,M.Sc.

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Introduction

In humans, detoxification of ammonia occurs in the liver via the urea cycle, a biochemical pathway consisting of six enzymes and two mitochondrial membrane transporters: *N*-acetyl glutamate synthase (NAGS, EC 2.3.1.1), carbamyl phosphate synthetase I (CPSI, EC 6.3.5.5), ornithine transcarbamylase (OTC, EC 2.1.3.3), argininosuccinate synthetase (AS, EC 6.3.4.5), argininosuccinate lyase (ASL, EC 4.3.2.1), arginase (EC 3.5.3.1), the aspartate transporter (citrin), and the ornithine transporter (ORNT1) (Walker 2009; Cartagena et al. 2013). All disorders except for X-linked OTC deficiency (OMIM 311250) are inherited in an autosomal recessive manner (Cartagena et al. 2013). The metabolic consequence of a deficiency in the urea cycle is an elevated blood ammonia which may lead to mental retardation, encephalopathy, coma, and possibly death. Urea cycle disorders typically present in the neonatal period or during the first months of life (Nakamura et al. 2014). However, there is a growing knowledge concerning urea cycle disorders that have been recognized in adulthood (Serrano et al. 2010; Roberts et al. 2013). A partial or mild enzyme deficiency may permit an individual to function relatively normally, sometimes for decades, until a stressful medical situation such as infection, starvation, surgery, pregnancy, or trauma occurs, that triggers a hyperammonemic crisis (Summar et al. 2005). Such an underlying urea cycle disorder might be difficult to recognize, because the patients can be ill because of other reasons. However, prompt recognition is critical to prevent a fatal outcome (Blair et al. 2015).

We report a case of hyperammonemic encephalopathy due to late-onset NAGS deficiency (OMIM 237310) as a consequence of a novel mutation. We also give an overview

of the patients reported in literature up till now with adult-onset NAGS deficiency.

Case Description

A 59-year-old woman, with a medical history of intellectual disability ascribed to perinatal asphyxia, diabetes mellitus type 2, a cerebrovascular accident, and myelodysplastic syndrome, was referred to our hospital because of coma due to hyperammonemia after she was treated conservatively for a fracture of the pelvis. She had been found confused by the home health care after she had been sent home with pain killers (paracetamol, NSAIDs, and morphine). On presentation at the referring hospital her vital signs were stable, but she had a decreased EMV score of E3M4V2. On examination she had isocoric pupils and pareses of both arms and legs. Laboratory examinations showed an elevated ammonia level of 280 μM ($N < 50$), normal liver enzymes, albumin, and coagulation factors (PT, APTT, and Factor V). Blood gas demonstrated respiratory alkalosis PH 7.52, PCO_2 3.7 kPa, PO_2 15.3 kPa, HCO_3 22.3 mmol/L, BE −0.6 mmol/L. Toxicology screening in urine was negative. Because of further deterioration of her EMV score that night, mechanical ventilation was initiated and she was transferred to our ICU. She was treated with high dose glucose intravenously, laxatives, and sodium benzoate. She became fully conscious at an ammonia level of 100 μM and remained stable on the internal medicine ward using a mild protein restriction, laxatives, and sodium benzoate treatment.

Acquired disorders as explanation for the hyperammonemia were excluded: liver failure, portosystemic shunt, infection with urease positive bacteria, urinoma, and bacterial overgrowth. After exclusion of these acquired disorders, the suspicion of a urea cycle defect (UCD) became stronger. Upon a more thorough anamnestic evaluation, patient reported a self-inflicted protein-restricted diet and she did not eat meat. Thirteen years before this presentation she visited a neurologist with complaints of headache, tiredness, dizziness, dyspnea during exercise, and vision disturbances. No clear diagnosis was made at that time and it was considered to be secondary to the mental retardation. Respectively 5 and 8 years before the current admission, the patient was seen at the hospital because of a delirium during infection. Family history was negative: her son did not have any complaints, although he had learning difficulties and drug addiction problems. She has one healthy sister and two brothers who are also healthy. One sister died at birth because of prematurity. Our patient did not have any problems during pregnancy or delivery of her son.

Three days after admission and treatment at the ICU metabolic investigations didn't show any abnormalities suggestive of a UCD; normal levels of glutamine (777 μM, range 463–797), alanine (449 μM, range 150–450), citrulline (33 μM, range 20–46), arginine (52 μM, range 31–117), and ornithine (72 μM, range 53–153) were measured. All acquired causes as explanation for the hyperammonemia were excluded however and the suspicion of a urea cycle disorder was still very strong. After discharge to the general ward metabolic investigations were repeated after an adequate protein load and this demonstrated an elevated glutamine (1,121 μM, range 463–797) and alanine (845 μM, range 150–450) level and a decreased citrulline (11 μM, range 20–46) and arginine level (22 μM, range 31–117), with a normal ornithine level (61 μM, range 53–153). Orotic acid could not be demonstrated in urine as assayed by specific tandem mass spectrometry analysis. DNA investigations were negative for the *OTC* and *CPS1* genes, but revealed a "homozygous" (not proven, parents unavailable for testing) c.603G>C mutation in exon 2 (NM_153006.2:c.603G>C), which mandates a substitution of lysine by asparagine in the protein: p.Lys201Asn. This is a novel mutation in the *NAGS* gene.

Before the DNA diagnosis of NAGS deficiency was established, the patient was treated with protein restriction (0.5 g per kg bodyweight per day) and sodium benzoate. The combination of protein restriction and a diabetes diet can be a real challenge for the dietician and the patient (Grunert et al. 2013). We decided to give her no dietary carbohydrate restriction. She was readmitted a couple of times because of a hyperammonemic crisis. It turned out that she was not capable of keeping her diet and taking the medication on a regular basis living on her own. After she had been transferred to a nursery home her behavior and cognitive functions markedly improved. Her ammonia levels remained stable around 80 μM. After the diagnosis of NAGS deficiency was made, oral *N*-carbamylglutamate (NCG) was started in a low dose 600 mg bid and sodium benzoate was stopped. With this treatment and a protein load of 0.8 g per kg bodyweight per day the ammonia level decreased to 26 μM and she was doing well.

Discussion

Inherited NAGS deficiency is the rarest of UCD, its true incidence is unknown (Ah Mew and Caldovic 2011). This is the first patient in literature in whom the diagnosis of NAGS deficiency is established at such an advanced age. Retrospectively it is very likely that her earlier medical problems such as the intellectual disability, headache, and

delirium during infection were due to metabolic decompensation of the UCD. In a recent review 35 previously reported cases with confirmed NAGS deficiency were presented, including only five patients that were diagnosed in adulthood (age at diagnosis: 20–57 years) (Cartagena et al. 2013) (Table 1).

NAGS Deficiency

NAGS produces *N*-acetylglutamate (NAG) from glutamate and acetyl coenzyme A (Acetyl CoA). NAG is an essential allosteric activator of mitochondrial carbamyl phosphatase 1 (CPS1), the first enzyme of the urea cycle (Caldovic et al. 2004). Hyperammonemia can result once CPS1 is deprived of its cofactor/activator NAG. Deficiencies of NAGS activity can be inherited as in this case. Sometimes they are acquired by secondary inhibition of NAGS activity in conditions which cause short chain fatty acid accumulation such as some organic acidemias and the use of valproic acid (Cartagena et al. 2013). In addition, conditions of compromised acetyl-CoA formation, such as fatty acid oxidation disorders, lead to a reduced formation of NAG with subsequent lack of CPS1 stimulation.

Clinical Presentation of NAGS Deficiency

The classical presentation for NAGS deficiency is in the first few days of life (Nakamura et al. 2014). The infant usually presents with vomiting after feeding (as a consequence of the protein load) and lethargy, seizures, and coma can follow quickly. Patients with late-onset NAGS deficiency may present with cyclical nausea and vomiting and chronic headaches (Ah Mew and Caldovic 2011). Almost all survivors of a hyperammonemic coma suffer from developmental delay (Cartagena et al. 2013). Most patients self-select a low protein diet. Symptom onset coincides with a precipitating factor such as infection, surgery, psychological stress, excess protein intake, or a trauma as in this case. Laboratory findings in NAGS deficiency include an elevated plasma ammonia, high levels of glutamine and alanine. Plasma citrulline is frequently low or undetectable and urinary orotic acid is not elevated. The

Table 1 Summary of findings in reported adult cases of confirmed *N*-acetylglutamate synthase (NAGS) deficiency

Patient	Diagnosis/onset of symptoms; sex	Presentation	Genotype	Peak ammonia level	Outcome	References
1	Diagnosis 20 years (1.5 years onset of symptoms); male	Confusion, combative behavior	Partial NAGS deficiency. Liver biopsy: NAGS activity <50% control	>100 μmol/l	Critical illness polyneuropathy, cerebral dysfunction, and paraplegia	Hinnie et al. (1997)
2	Diagnosis 33 years (27 years onset of symptoms); female	Seizures, coma during pregnancy	L312P/T431I	4,781 μmol/l	Not indicated	Grody et al. (1994) Caldovic et al. (2007)
3	Diagnosis 33 years (5 years onset of symptoms); male	Post-operative combativeness, confusion, seizures	V173E/T431I	621 μmol/l	Death	Caldovic et al. (2005)
4	Diagnosis 57 years (40 years onset of symptoms); female	Intermittent staring spells, nausea, recurrent vomiting, lethargy, ataxia, migraine headaches, eventually coma	V350I/L442V	500 μmol/l	Normal intellect at 57 years	Tuchman et al. (2008)
5	Diagnosis 38 years (20 years onset of symptoms); male	Episodic confusion, nausea and vomiting	E433G/IVS6+5 G>A	434 μmol/l	Short term memory loss	Cartagena et al. (2013)
6	Diagnosis 59 years (46 years onset of symptoms); female	Confusion, coma	Exon 2 (c 603 G>C) in Lys201Asn	280 μM	Behavior and cognitive functions markedly improved	

concentrations of other urea cycle intermediates are low-to-normal (Ah Mew and Caldovic 2011).

The timing of metabolic investigation is important, however, because a strict protein restriction (with a high dose of glucose intravenously as in our patient) can result in a false negative result.

Novel Mutation in NAGS Gene

NAGS deficiency is an autosomal recessive disorder and the last urea cycle disorder for which molecular testing became available. In 2002 the *NAGS* gene was cloned, which was found to be located on the long arm of chromosome 17 within band 17q21.31 (Caldovic et al. 2002). There are 23 mutations in the *NAGS* gene published up to date (Ah Mew and Caldovic 2011; Cartagena et al. 2013). Although at present two mutations occurred in more than one family (Thr431Leu and Trp324Ter), there does not appear to be any mutational hot spot in the *NAGS* gene (Ah Mew and Caldovic 2011).

In our patient, an apparently homozygous mutation c.603G>C in exon 2, which mandates a substitution of lysine by asparagine in the protein: p.(Lys201Asn), was found. The mutation is not found in the online HGMD database and also not in the NHLBI exome variant server. The c.603G>C, p.(Lys201Asn) variant/mutation was not detected in 13,000 control alleles (http://evs.gs.washington.edu/EVS/). Parents were not available for analysis to prove homozygosity. Theoretically, a deletion on one allele may have escaped detection. Nevertheless, such a genetic constellation would also lead to NAGS deficiency. The mutation p.Lys201Asn lies in the kinase domain and affects an amino acid residue that is not conserved in evolution. A mutation affecting the adjacent p.Cys200Arg mutation was found in another patient with late-onset NAGS deficiency and has been characterized with some residual enzyme activity (Caldovic et al. 2005; Schmidt et al. 2005). We therefore postulate that p.Lys201Asn is also compatible with residual enzyme activity and a late-onset presentation, although there are currently no enzyme data on this. The clinical presentation and the positive reaction on NCG support this. In summary, this mutation acts as a typical late-onset NAGS deficiency causing mutation.

Management of NAGS Deficiency

A hyperammonemic crisis is an emergency situation and stabilization is most important; treatment includes intravenous glucose, ammonia scavengers, and sometimes hemodialysis for ammonia removal. Therapeutic principles for management of NAGS deficiency, as with all UCDs, include minimizing endogenous ammonia production by limiting protein intake and avoiding periods of catabolic stress; administration of urea cycle substrates that are lacking as a consequence of the enzymatic defect (arginine and citrulline), and administration of compounds that facilitate the removal of ammonia through alternative pathways (sodium benzoate and sodium phenylacetate) (Walker 2009; Cartagena et al. 2013). However NAGS deficiency is the only inherited urea cycle disorder that can be specifically and effectively treated by a drug (Morris et al. 1998). In patients with NAGS deficiency, a 3-day trial of NCG at a dose of 2.2 g/m^2/day was demonstrated to restore ureagenesis and normalize blood ammonia, as demonstrated by isotopic studies (Caldovic et al. 2004). NCG activates CPS1, therefore leading to a reduction in ammonia levels and obviates the necessity for a protein restriction.

Conclusion

Hyperammonemia due to urea cycle disorders can also occur at advanced age. It is important for the adult physician to consider a metabolic disorder at every age. Because of growing knowledge and better diagnostic tools this diagnosis will be made more often, especially in our patient population that is still getting older.

Take Home Message

It is important for the adult physician to consider a metabolic disorder at every age; we describe one of the first patients in literature in whom the diagnosis of a UCD is made at such an advanced age.

Conflict of Interest

A vd Logt, L Kuijtmans, M Huigen, and M Janssen declare that they have no conflict of interest

Compliance with Ethics Guidelines

Informed Consent

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

Details of the Contributions of Individual Authors

A vd Logt and MC Janssen wrote the manuscript. All authors interpreted and discussed the results. The manuscript was read and corrected by all authors.

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JIMD Reports
DOI 10.1007/8904_2016_543

CASE REPORT

Glycine *N*-Methyltransferase Deficiency: A Member of Dysmethylating Liver Disorders?

Ivo Barić · Sahin Erdol · Halil Saglam · Mila Lovrić · Robert Belužić · Oliver Vugrek · Henk J. Blom · Ksenija Fumić

Received: 17 November 2015 / Revised: 30 January 2016 / Accepted: 02 February 2016 / Published online: 21 May 2016

Abstract Glycine *N*-methyltransferase deficiency is an inherited disorder of methionine metabolism, reported so far in only four patients and characterised by permanent hypermethioninemia. This disorder has been considered as probably benign because moderate hepatomegaly in two patients was the only obvious symptom and mild to moderate elevation of aminotransferases the only laboratory abnormality. Our experience with the current novel patient points out that this disease, due to very high hypermethioninemia, is not harmless and that there may be diagnostic pitfalls in interpretation of biochemical hallmarks of the disease. Since the first description of glycine *N*-methyltransferase deficiency, other disorders of this metabolic pathway affecting the liver have been reported pointing to dysmethylation as the common pathogenetic mechanism. Therefore, we suggest the whole group to be named dysmethylating liver diseases.

Communicated by: Viktor Kožich

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2016_543) contains supplementary material, which is available to authorized users.

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Introduction

Glycine *N*-methyltransferase (GNMT) deficiency (OMIM 606664; E.C. 2.1.1.20) is an autosomal recessive inherited disorder of methionine metabolism (Fig. 1), which has been reported in extenso so far in only three patients from two families (Mudd et al. 2001; Augoustides-Savvopoulou et al. 2003). In 2015, the fourth patient was described in an abstract (Rakic et al. 2015). This disorder has been considered to be probably benign because moderate hepatomegaly in two patients was the only obvious symptom and mild to moderate elevation of aminotransferases (up to 5× normal) was the only routine laboratory abnormality. Liver biopsy, done in two patients, showed only mild centrilobular fibrosis and presence of some eosinophils in one patient and virtually normal result with only a few hepatic cells with mild hydropic degeneration in the other one. During follow-up the three in extenso reported patients have been asymptomatic (R. Cerone, P. Augoustides-Savvopoulou – personal communications). Biochemical hallmarks of the disease are remarkable hypermethioninemia, highly elevated plasma *S*-adenosylmethionine (AdoMet) with normal *S*-adenosylhomocysteine (AdoHcy) and total homocysteine (tHcy). Here we report

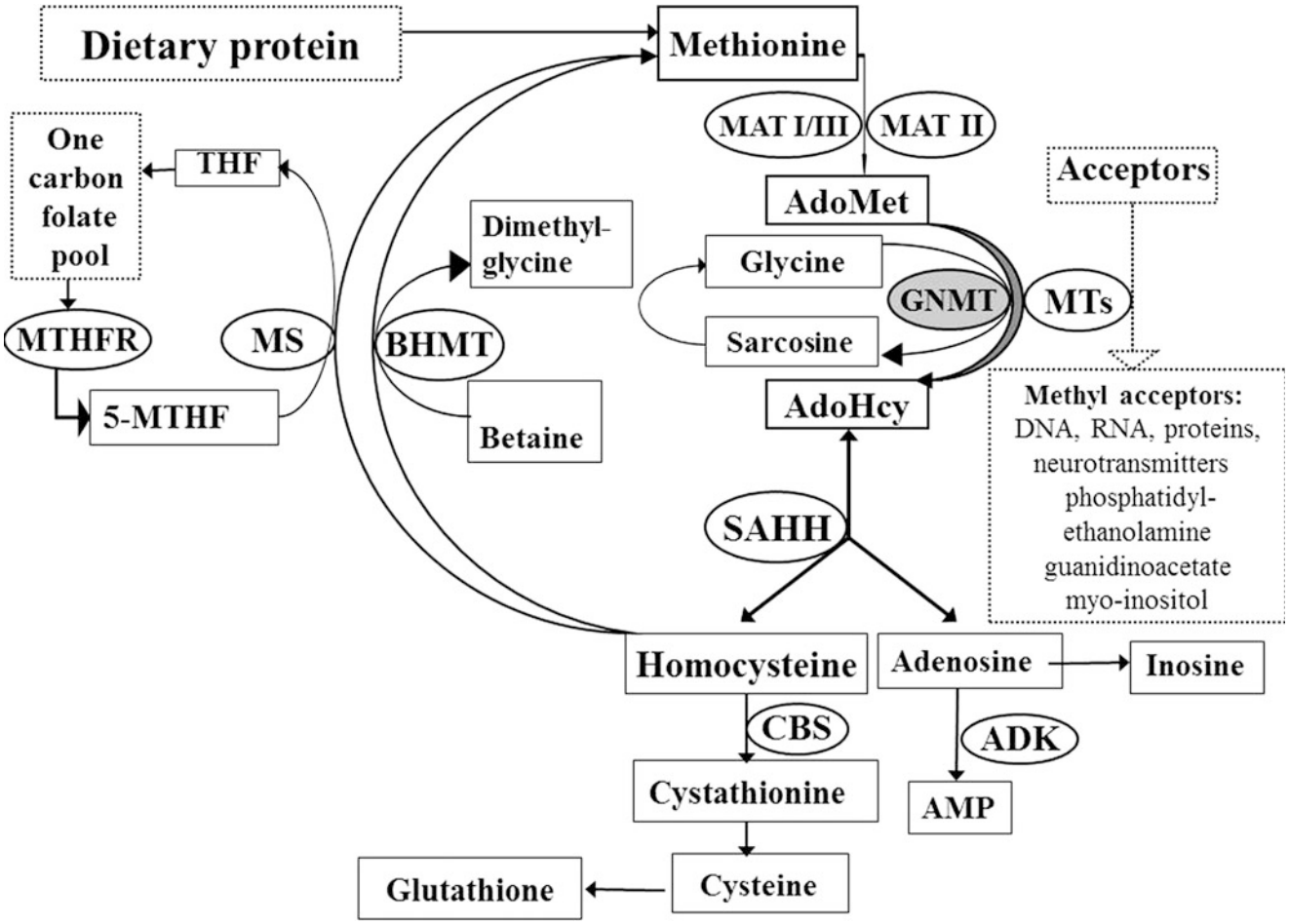

Fig. 1 Methionine metabolism. *AdoMet* *S*-adenosylmethionine, *AdoHcy* *S*-adenosylhomocysteine, *THF* tetrahydrofolate, *5-MTHF* 5-methyltetrahydrofolate, *AMP* adenosine monophosphate. The following enzymes are in circles: *MAT* methionine adenosyltransferase (E.C.2.5.1.6), *GNMT* glycine *N*-methyltransferase (E.C.2.1.1.20), *MTs* a variety of AdoMet-dependent methyltransferases, *SAHH* AdoHcy hydrolase (E.C.3.3.1.1), *CBS* cystathionine β-synthase (E.C.4.2.1.22), *MS* methionine synthase (5-MTHF-homocysteine methyltransferase) (E.C.2.1.1.13), *BHMT* betaine-homocysteine methyltransferase (E.C.2.1.1.5), *MTHFR* methylenetetrahydrofolate reductase (E.C.1.5.1.20), *ADK* adenosine kinase (E.C.2.7.1.20)

the fifth patient with this disease whose data indicate that the disease may not be a benign one.

Since the first description of GNMT deficiency, other disorders of this metabolic pathway affecting the liver have been reported pointing to the common pathogenetic mechanism of this group of the diseases, which we therefore suggest may be named dysmethylating liver diseases.

Case Report

The subject of this report is a Turkish boy at the age of 5 and half years. He is the second child of consanguineous healthy parents. His brother had etiologically unexplained muscle weakness and epilepsy from the age of 4 years and died at the age of 7 years. The mother's brother and sister had muscle weakness and could not walk since the age of 11 years and had epilepsy since the age of 15 years. Neither their samples nor the tissue from the patient's brother is available for additional analyses. Our patient was born after unremarkable pregnancy and delivery. He was considered healthy until the age of 2 years and 8 months, when he experienced a single episode of febrile convulsions. At that time his aminotransferases were checked for the first time and found to be permanently elevated, up to 6× normal (AST) and 3× normal (ALT). Physical findings have always been normal. Diagnostic work-up showed highly elevated plasma methionine, which turned out to be a permanent abnormality (815, 1,018, 1,246, and 1,247 μmol/L, reference range 7–47 μmol/L). tHcy was mildly elevated at 21.4 and 28.3 μmol/L (reference range 5–12 μmol/L). Other amino acids were either normal or showed mild unspecific changes. Creatine kinase, albumin, gamma-

glutamyl transpeptidase, bilirubin, alkaline phosphatase, alpha-fetoprotein, coagulation tests, folic acid, vitamin B_{12}, and liver ultrasound were normal. Liver biopsy was denied. Because of unexplained isolated hypermethioninemia, AdoMet and AdoHcy were measured. In whole blood, AdoMet was 1,790 nmol/L (reference range 1,000–1,800 nmol/L) and AdoHcy 30 nmol/L (reference range 20–170). In plasma, which became available after the diagnosis has been confirmed by the gene analysis, AdoMet was highly elevated – 3,348 nmol/L (reference range 71–118 nmol/L), whilst AdoHcy was 63 nmol/L (reference range 9.3–14.1 nmol/L). Increased whole blood ratio of AdoMet to AdoHcy, in combination with clinical presentation, raised suspicion on GNMT deficiency which was confirmed by *GNMT* gene analysis showing the previously unreported homozygous c.296 G>A (R99H) mutation. Parents were heterozygotes for this mutation. Expression studies confirmed pathogenicity of the mutation (see details in supplementary material). During the follow-up period, the patient has been well, without any treatment.

Materials and Methods

Amino Acid Measurement

Plasma amino acids were measured with a kit for quantitative LC-MS/MS analysis of amino acids in biological fluids (Zivak Technologies, Holland) using HPLC-MS-MS technology according to manufacturer's protocol.

Total Homocysteine Measurement

Plasma tHcy was measured by chemiluminescence immunoassay (CLIA) on IMMULITE 2000 (Siemens Healthcare Diagnostics) according to manufacturer's instructions.

S-Adenosylmethionine and *S*-Adenosylhomocysteine Measurement

In whole blood, AdoMet and AdoHcy were measured in perchloride acid-treated whole blood according to Fux and coworkers (Fux et al. 2005) and modified as described in supplementary material. In plasma, AdoMet and AdoHcy were measured as previously described (Gellekink et al. 2005).

Gene Expression Studies and Enzymatic Activity

Heterologous expression of recombinant GNMT was performed in prokaryotic (*E. coli*) and eukaryotic (HEK293) hosts. Major differences in protein solubility in between recombinant wild-type and mutant R99H GNMT were observed. Namely, wild-type protein showed high solubility, whereas only a minor fraction of mutant protein was remained soluble during extraction indicating significant structural changes leading to high amounts of misfolded protein.

Enzymatic activity was determined using a modified coupled assay including two major steps based on the protocol from R&D systems (https://www.rndsystems.com). Firstly, recombinant GNMT is incubated with glycine to generate sarcosine and AdoHcy. Addition of recombinant adenosine deaminase and *S*-adenosylhomocysteine hydrolase leads to hydrolysis of AdoHcy into homocysteine and inosine. Subsequently, free homocysteine is reacted with thiol reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) (Belužić et al. 2006), and the amount of thiols produced is measured spectrophotometrically at a wavelength of 405 nm. The retrieved soluble fraction of mutated GNMT lacked measurable enzymatic activity, whereas wild-type protein exhibits specific activity of >200 pmol/min/μg.

To overcome known limitations of prokaryotic expression, human HEK293 cells were transfected with either wild-type or R99H GNMT, cloned as a N-terminal GFP fusion in order to be able to distinguish between endogenous and foreign mRNA levels. Subsequently, mRNA and protein levels were determined using real-time PCR and Western blotting, respectively. Whilst we could not detect any differences in transcript levels of wild-type and mutant GNMT, again the GFP-R99H protein was barely detectable. Taken together, these findings strongly support the adversive effect of R98H mutation on GNMT translation or stability and thereby its function. Detailed experimental procedures are available in supplementary material.

Discussion

Data from our patient point to an important aspect of GNMT deficiency which has not been discussed as yet in the literature. Although his clinical presentation and routine liver tests support the thesis on a benign disorder, his plasma methionine was on three occasions above 1,000 μmol/L, which was estimated as approximate borderline for increased risk of various neurological complications related to hypermethioninemia regardless of the cause (Barić and Fowler 2014; Mudd 2011). Plasma methionine levels above 1,000 μmol/L were observed also in the third reported patient (Augoustides-Savvopoulou et al. 2003). Very recent data from patients with methionine adenosyltransferase I/III deficiency (MAT I/III; OMIM 250850; E.C. 2.5.1.6) suggest that patients with mean values above 800 μmol/L almost always have central nervous system abnormalities, whereas those with means less than 800 μmol/L usually do not (Chien et al. 2015). The most characteristic brain imaging

changes related to high hypermethioninemia are demyelination and oedema of white matter, more pronounced in the dorsal brain stem, resulting in separation of myelin layers – the so-called vacuolating myopathy (Braverman et al. 2005; Chamberlin et al. 1996; Devlin et al. 2004). Accordingly, treatment recommendations could be regular monitoring and low-methionine diet if symptoms appear or if methionine is very high.

Experience with this patient revealed interesting possible diagnostic pitfalls of GNMT deficiency. Plasma AdoHcy was unexpectedly more than four times above the reference range. If clinical data and plasma AdoMet are neglected, this could suggest other diseases, for instance, adenosine kinase deficiency (OMIM 614300, E.C. 2.7.1.20). A plausible explanation for increased AdoHcy in this patient could be extremely high plasma AdoMet which secondarily caused an increase of AdoHcy. Actually, AdoMet is an unstable compound and can be converted in part due to inappropriate sampling, shipment or storage to AdoHcy, particularly if the sample is not properly acidified. Whether plasma AdoHcy in our patient was normal or decreased or even slightly increased, we cannot be sure. The instability of AdoMet could be the reason also for the AdoMet levels in the upper normal range when measured in the whole blood, whilst in the same sample, AdoHcy was in the lower normal range. This indicates that, when measured in the whole blood, AdoMet/AdoHcy ratio could be more accurate diagnostic key than isolated AdoMet value and, even more importantly, that plasma is the sample of choice for assaying AdoMet and AdoHcy. The plasma tHcy elevation in our patient (not reported in other patients) may seem confusing because hypermethioninemia and hyperhomocysteinemia characterise cystathionine beta-synthase deficiency (classic homocystinuria; OMIM 263200, E.C. 4.2.1.22), although in the latter tHcy is usually higher. The elevation of homocysteine may be explained by inhibition of betaine-homocysteine methyltransferase or methylenetetrahydrofolate reductase by methionine or AdoMet. Finally, the GNMT activity assay does not seem to be feasible in peripheral white blood cells, lymphocytes or cultured skin fibroblasts, thus making the way to diagnosis more difficult, i.e. requiring either liver biopsy or enzyme assay in a specialised laboratory or gene analysis. Even then, the pathogenicity of mutations may not always be clear.

In our patient, there are several factors indicating that the R99H mutation is pathogenic. First of all, the mutated residue is in the conserved region of the gene. Secondly, the mutant protein in *E. coli* is almost insoluble and completely inactive. Thirdly, mutant GNMT protein levels in eukaryotic HEK293 cells were at the threshold of detection, whereas wild-type protein was overexpressed as expected. We excluded mechanisms of transcript control/degradation, e.g. 'no-go decay', which would lower the mRNA levels. Taken together, the most plausible explanation is that this single amino acid change results in drastically lowered stability of GNMT protein, thereby leading to rapid degradation and/or proteolysis by cellular mechanisms.

Concerning pathogenesis of the GNMT deficiency, it seems that at the moment more can be learned from conditions associated with similar biochemical pattern, i.e. elevated AdoMet and disturbed AdoMet/AdoHcy ratio than from just monitoring patients with GNMT deficiency. These conditions, i.e. AdoHcy hydrolase deficiency and adenosine kinase deficiency, are two disorders sharing the same metabolic pathway with GNMT deficiency. In all three, elevated AdoMet and disturbed AdoMet/AdoHcy ratio possibly lead to complex changes in numerous methylation processes in the body, some of them affecting the liver. Remarkably elevated plasma AdoMet, with much less elevated AdoHcy, was found in all patients with adenosine kinase deficiency, reported first time in 2011 (Bjursell et al. 2011). In that report all six patients had signs of liver disease. In a recent review of 11 patients, nine of them had liver disease, and liver biopsies from four out of five patients showed fibrosis (Staufner et al. 2015). Liver disease is a frequent finding also in AdoHcy hydrolase deficiency (OMIM 613752; E.C. 3.3.1.1), described for the first time by our group (Barić et al. 2004), where AdoMet elevation is a constant finding in untreated patients. In this disorder, the methylation ratio is decreased due to highly elevated AdoHcy. Interestingly, high AdoMet and high methylation index were recently found also in hepatic mtDNA depletion syndromes with poor outcome (Mudd et al. 2012).

All these examples indicate that changes in AdoMet concentration and methylation index are harmful for the liver and that dysmethylation has to be considered as one of pathogenetic mechanisms of liver diseases or, in other words, that this group of disorders affecting the liver may be pathogenetically considered as 'dysmethylating liver diseases'. GNMT deficiency seems to be an underdiagnosed disorder, but a valuable natural model for studying effects of methylation changes on the liver, which in the long run may cause, not only in GNMT deficiency, serious liver pathology, as shown in animal and in vitro models. In GNMT knock-out mouse, *Gnmt−/−* mice developed liver steatosis, fibrosis, cirrhosis, chronic hepatitis and hepatocellular carcinoma (Luka et al. 2006; Liu et al. 2007; Martínez-Chantar et al. 2008; for review see also Luka et al. 2009). This is in accordance with previous reports that GNMT is down-regulated in the liver of patients with hepatitis C virus-induced and alcohol-induced cirrhosis who are at risk for hepatocellular carcinoma (Avila et al.

2000) and is not expressed in hepatocellular carcinoma (Chen et al. 1998). Therefore, it seems recommendable to regularly check the liver status in GNMT-deficient patients, particularly for development of hepatocellular carcinoma. An important, very recently reported similarity to GNMT-deficiency in mice is the occurrence of hepatocellular carcinoma in an adult with AdoHcy hydrolase deficiency (Stender et al. 2015). This points to the possible common, liver-related pathogenetic mechanism of these two disorders.

Acknowledgements The study was supported by the project "European Network and Registry for Homocystinurias and Methylation Defects (E-HOD)" which is co-funded by the European Union in the framework of the Health Program (No.2012_12_02). OV and RB were supported by FP7-REGPOT-2012-2013-1, Grant Agreement Number 316289-InnoMol.

Take-Home Message

Glycine-*N*-methyltransferase deficiency does not seem a harmless disease and interpretation of diagnostic biochemical abnormalities requires caution.

Description of Authors' Contributions to the Study

Ivo Barić- study design and supervision, writing of the manuscript; he contributed pertinent aspects of the planning, conducting and reporting of the work described in the article.

Sahin Erdol and Halil Saglam- diagnosis and follow-up of the patient, providing medical data of the patient for the manuscript and critical revision of the manuscript.

Mila Lovrić- biochemical analyses, contributing to the manuscript writing and critical revision of the manuscript.

Robert Belužić- gene analysis and expression studies, contributing to the manuscript writing and critical revision of the manuscript.

Oliver Vugrek- gene analysis and expression studies, contributing to the manuscript writing and critical revision of the manuscript.

Henk J. Blom- biochemical analyses, contributing to the manuscript writing and critical revision of the manuscript.

Ksenija Fumić- biochemical analyses, contributing to the manuscript writing and critical revision of the manuscript.

Guarantor for the Article

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Conflict of Interest

Ivo Barić, Sahin Erdol, Halil Saglam, Mila Lovrić, Robert Belužić, Oliver Vugrek, Henk J. Blom, and Ksenija Fumić declare that they have no conflict of interest.

Details of Funding

The study was supported by the project "European Network and Registry for Homocystinurias and Methylation Defects (E-HOD)" which is co-funded by the European Union in the framework of the Health Program (No.2012_12_02). OV and RB were supported by FP7-REGPOT-2012-2013-1, Grant Agreement Number 316289-InnoMol. The author (s) confirm(s) independence from the sponsors; the content of the article has not been influenced by the sponsors.

Compliance with Ethics Guidelines

Ethics approval was not required for all research studies.

Informed Consent

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

Animal Rights

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

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JIMD Reports
DOI 10.1007/8904_2016_546

CASE REPORT

Disease Heterogeneity in Na^+/Citrate Cotransporter Deficiency

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Received: 19 November 2015 / Revised: 4 February 2016 / Accepted: 09 February 2016 / Published online: 10 March 2016

Abstract We report a novel mutation found in two siblings, a male and a female aged 8 and 5 years, respectively. Both patients presented with developmental delay and intractable seizures consistent with previous reports of SLC13A5 transporter deficiency. Both had two mutations in the *SLC13A5* gene, c.655G>A (G219R) and the novel mutation c.245A>G (Y82C). However, the phenotypes were not identical as the female had focal cortical dysplasia that led to brain surgery. This is another example of the heterogeneity in disease expression even when the genotype is identical in the affected individuals.

Introduction

The human Na^+/citrate cotransporter (Na^+CT; symbol, *SLC13A5*) was first cloned and characterized by Inoue et al. (2002). It is a plasma membrane transporter with preferential selectivity toward citrate as a substrate. Na^+CT mediates the cellular entry of citrate, and, as an active transporter, it is probably responsible for maintaining a cytosolic concentration gradient for citrate in certain cells such as neurons and hepatocytes. Citrate plays a major role in several important biochemical pathways. Most importantly, it is an intermediate in the citric acid cycle. Playing a role in the maintenance of the cytosolic pool of citrate, the Na^+CT may assist in the establishment of an adequate pool of citrate in the mitochondrial matrix. Citrate is also the major precursor for cytoplasmic acetyl CoA (generated by ATP citrate lyase) and thus serves as an obligatory starting material for the synthesis of fatty acids, cholesterol, and, in neurons, acetylcholine. As such, Na^+CT is likely to be a very important protein in neuronal homeostasis and cholinergic signaling.

In support of this hypothesis, mutations in the *SLC13A5* gene were recently associated with encephalopathic epilepsy (Thevenon et al. 2014; Hardies et al. 2015). We report two siblings with this neuronal citrate transporter defect with seizures but with different phenotypes, i.e., the presence or absence of intractable seizures and focal brain lesions, which underscore the heterogeneity in SLC13A5 disease expression.

Methods

Whole exome sequencing (WES) was performed in proband-parent quad analysis at GeneDX (Gaithersburg, MD, USA). Informed consent was obtained for each individual. Genomic DNA was extracted from whole blood from the two affected children and their unaffected parents, and the resultant DNA sequences were mapped to the reference human genome sequence (UCSC Genome

Communicated by: Garry Brown

Competing interests: None declared

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Browser hg19). Targeted coding exons and splice junctions of known protein-coding RefSeq genes were assessed for average depth of coverage and a minimum depth of 10×. Variants were filtered as appropriate on the basis of inheritance patterns, lists of genes of interest, and phenotype and population frequencies. Resources including the Human Gene Mutation Database, 1000 Genomes, the NHLBI Exome Variant Server, ExAc, OMIM, PubMed, and ClinVar were used for evaluating genes and detecting sequence changes of interest, which were identified and confirmed in all members of the family by conventional dideoxy DNA sequence analysis. Those rare functional variants that were homozygous and compound heterozygous were prioritized using the PhenoDB Variant Analysis Tool. Variants with a minor allele frequency >0.01 were excluded. At the time of the results reporting, two novel compound heterozygous variants of uncertain significance, c.245A>G (Y82C) and c.655G>A (G219R), were found in the *SLC13A5* gene in both children. Since the time of the report, the c.655G>A (G219R) mutation was also reported by Thevenon et al. (2014) and Hardies et al. (2015).

Clinical Report

We present two siblings with NaCT deficiency. Both siblings were found to be compound heterozygotes for mutations in the *SLC13A5* gene: c.245A>G (Y82C) and c.655G>A (G219R). They have similar histories and presentations, although Patient 2 has greater developmental delay.

Patient 1

Patient 1, an 8-year-old male, was born via repeat cesarean section after a full-term pregnancy. Birth weight was 7 lb, 3 oz. Apgar scores were 9 and 9. The pregnancy was notable for frequent rhythmic fetal movements in the third trimester initially attributed to "hiccups." Seizures began within the first 24 h of life. He developed prolonged clonic seizures at 3 weeks of age, which required a phenobarbital coma to resolve. He was seizure free for approximately 10 months following that episode but then experienced recurrence of daily seizures. His semiology consisted of frequent myoclonic jerks as well as tonic seizures with arm and leg extension. Multiple electroencephalograms (EEGs) over the years have been abnormal and consistent with generalized epilepsy. The most recent EEG shows multifocal sharp waves, potentiated by sleep and absence of well-formed sleep feature. He has been treated with numerous antiseizure medications. Seizures improved at 4 years of age and now are predominantly myoclonic, occurring one to two times per month. Brain MRIs initially showed delayed myelination and subsequently have showed no abnormal findings. Magnetic resonance spectroscopy (MRS) was normal with no lactate peak.

Patient 1 had several unrevealing CSF studies. Additional studies included normal chromosomal microarray analysis, and genetic panel for myoclonic epilepsies (including MERRF, Lafora body disease, EPM2A, EPM2B, and SCN1A). Skin biopsy revealed no inclusion bodies.

Patient 1 developed head control late and was able to sit independently between 3 and 4 years of age. At age 5, he was able to pull to stand. Now, at age 8, he is able to scoot independently and walk with a special walker. He attends school in a substantially separate classroom, where he is one of the most capable students. He is making consistent progress. He does not yet speak, but he is able to participate in classroom activities.

On physical examination at age 8, Patient 1 exhibited small stature, with height and weight less than the first percentile (-3SD) and head circumference in the first percentile. His general physical examination was otherwise normal. He was alert and interactive. Neurological examination revealed truncal hypotonia with mildly increased tone in his legs. He had hyperreflexia in his lower extremities.

Patient 2

Patient 2 is the younger sister of Patient 1, a 5-year-old female. She was born via repeat cesarean section at 38-week gestation. Her mother described no rhythmic fetal movements during the pregnancy. Apgar scores were 9 and 9. Birth weight was 7 lb. Seizures began in the first 24 h of life, consisting of lip smacking, eye rolling, and twitching of the hands. Since that time, she has continued to have daily intractable seizures resistant to multiple medications and the ketogenic diet. A vagus nerve stimulator was placed but produced no benefit.

Patient 2 had negative testing for peroxisomal disorders, unrevealing CSF studies and skin biopsy negative for inclusions.

Patient 2 has had several brain MRIs over the years, which have revealed blurring of the gray and white matter junction in the right superior frontal gyrus and at the right frontoparietal junction, including the precentral gyrus, with FLAIR/hyperintensity extending into periventricular white matter. Similar findings were seen to a lesser degree on the left side. This appearance was consistent with areas of focal cortical dysplasia. Patient 2 underwent resection of the right frontoparietal lesion at 4.5 years with resolution of left focal seizures for several weeks following the procedure. However, frequent seizures have since recurred. Her multiple EEGs have been abnormal, consistent with generalized epilepsy. Her most recent EEG demonstrates near continuous sharp waves in the right frontal area and continuous generalized slowing. Her most recent brain MRI shows postsurgical changes after right frontal lobe surgery

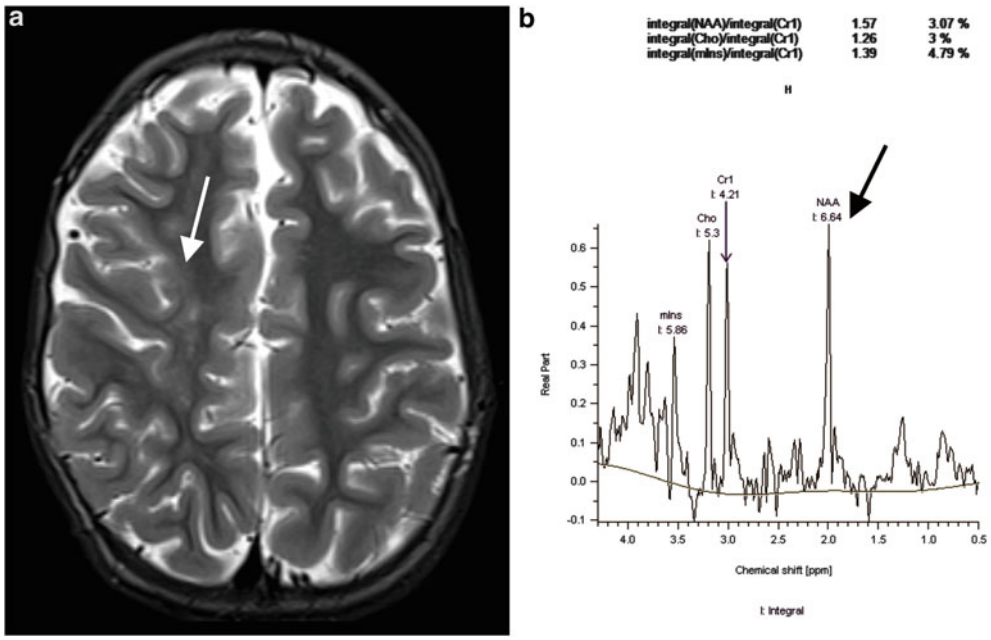

Fig. 1 (**a**) Axial T2-weighted image from Patient 2 shows blurring of the *gray and white matter* in the right frontal lobe and frontoparietal junction extending to the periventricular white matter. (**b**) Sample short TE spectrum from a processed voxel from the left thalamus of Patient 2 shows reduced NAA peak and reduced NAA/Cr ratio (1.57) compared to normal controls (1.8–2.1)

and residual abnormality at the gray and white matter junction in the right frontoparietal region extending to the periventricular white matter (Fig. 1a). Multivoxel 3D MRS performed with both short (30 ms) and long (135 ms) echo times revealed reduced ratio of *N*-acetylaspartate to creatine (NAA/Cr) within the gray and white matter compared to a normal age-matched control and no other abnormal metabolites (Fig. 1b). Specifically, there was no evidence of elevated lactate peak at several processed voxels.

Patient 2's hearing and vision are normal. She has a history of grade II vesicoureteral reflux and is otherwise healthy.

Developmentally, Patient 2 is delayed more than her brother was at her age. She is able to roll, but she has no head control. She cannot sit unsupported. She does not have purposeful use of her hands and is unable to reach or grab objects. In spite of severe motor delay, however, Patient 2 is a very sociable child. She is able to babble. Weight and height are less than the first percentile (-5SD and -2SD) and head circumference is in the 9th percentile. On the neurologic exam, her muscle tone is increased in both upper and lower extremities with severe truncal hypotonia. She has generalized weakness and is normo-reflexic.

Discussion

In 2014, Thevenon et al. reported seven patients from three families, all of whom presented with early epileptic encephalopathy and severe global developmental delay (2014). All of these patients developed seizures in the first few days of life; five developed seizures in the first few hours. Most had profound developmental delay. On neurological examination, they had axial hypotonia and appendicular hypertonia. None of the patients had facial dysmorphism, and all had normal head size. Metabolic and imaging studies were unrevealing. Seizures were persistent in all patients with several being in subclinical status epilepticus. All were found to have mutations in the *SLC13A5* gene. Two patients from the consanguineous family were homozygous, four patients from other families were compound heterozygotes, and one patient from the fourth family was heterozygous for c.655G>A mutation.

In 2015, Hardies et al. reported eight additional patients belonging to four different families with seven different autosomal recessive mutations in the *SLC13A5* gene (2015). All of these patients had focal clonic seizures in the first days of life. Status epilepticus was common in the course of their disease. Neurological and developmental outcome ranged from mild to severe intellectual disability. Patients had combinations of ataxia, choreoathetosis, and spasticity. All patients had teeth hypoplasia/hypodontia. This was also described by Thevenon but was not a feature in our patients. Three patients were treated with ketogenic diet for their seizures and had favorable response. The authors studied the effect of seven identified mutations in vitro, demonstrating that cells expressing mutant sodium-dependant citrate transporter had a complete loss of citrate uptake.

One of the two *SLC13A5* mutations found in our patients, c.655G>A (G219R), was present in the patients reported by Thevenon et al. (2014) and Hardies et al. (2015). This mutation causes nonconservative amino acid substitution of small, neutral, nonpolar glycine to be replaced with a large, positively charged arginine residue at a position that is highly conserved across species. In silico analysis predicts this variant to be damaging to the protein structure/function. This mutation was not observed in 6,500 individuals of European and African-American ancestry in the NHLBI Exome Sequencing Project.

The second variant found in our patients, c.245A>G (Y82C), has not been reported before. It causes a semiconservative amino acid substitution as a neutral polar tyrosine residue is replaced with neutral polar cysteine residue. The cysteine residue may impact disulfide bonding in the protein. Tyrosine is conserved at this position across species. In silico analysis predicts this variant to be pathogenic. This mutation was also not observed in 6,500 individuals in the NHLBI Exome Sequencing Project. Theoretically, this mutation may interfere with the tertiary structure of the protein as it may lead to an unwanted disulfide bridge. Alternatively, the loss of the tyrosine residue may perturb phosphorylation of the protein at that position.

It was suggested in the past that patients with SLC13A5 deficiency may benefit from a ketogenic diet for the treatment of their seizures. Three patients reported by Hardies et al. (2015) responded to the diet favorably. Patient 2, however, was placed on the ketogenic diet with no benefit. She was also found to have small cortical dysplasia/gliosis that was resected, but surgery produced no benefit, as she continues to have intractable seizures.

Brain malformations, predominantly agenesis of corpus callosum (ACC), were reported previously with disorders of energy metabolism and Krebs cycle. These conditions include pyruvate dehydrogenase (E1α) deficiency (Shevell et al. 1994), the Amish SLC25A19 (thiamine pyrophosphate transporter) defect (Siu et al. 2010), fumarase deficiency (Mroch et al. 2012), SLC25A1 mitochondrial citrate transporter (Edvardson et al. 2013), and several others. Normal function of the citrate transporter is important in prenatal brain development, and we believe that it is possible that the cortical dysplasia found in Patient 2 may be due to SLC13A5 deficiency. Given the fact that the transporter is expressed in the liver, it is curious that none of the patients reported to date have had liver disease.

In summary, we report a novel mutation in the *SLC13A5* gene. Our patients have presentation similar to seven previously reported cases with clonic seizure onset in the first hours of life, epileptic encephalopathy with multifocal EEGs, lack of dysmorphic features, and the absence of significant medical problems. Unlike patients reported by Hardies et al. (2015), our patients do not exhibit hypodontia but rather evidence of altered enamel. While Patient 1 has significant delay, he is more advanced in his development than most previously reported cases.

All reported cases of patients with SLC13A5 transporter deficiency presented with seizures in the first day of life. We believe that testing for this condition should be strongly considered in such cases.

Lastly, we hypothesize that triheptanoin or 1,3-Di (heptanoyloxy)propan-2-yl heptanoate may benefit patients with neuronal citrate transporter deficiency. The reasoning behind this offering is as follows. The SLC13A5-mediated transport of citrate across the plasma membrane from the extracellular space into the cytoplasm may play a role in maintaining the pool size of citrate in both the cytoplasm and mitochondrial matrix. We hypothesize that triheptanoin will increase the metabolism of odd-chain fatty acids in neuronal mitochondria and thereby increase the levels of succinyl-CoA, subsequently leading to an increase in citrate concentrations. This possibility was raised by Thevenon et al. as well (2014). The increased level of citrate in the mitochondrial matrix may lead to an increased efflux of citrate from the matrix to the cytoplasm, thus increasing the cytoplasmic pool of citrate and causing the malfunctioning citrate transporter to have less of an impact on the cytoplasmic pool size. We hypothesize that triheptanoin therapy will improve neuronal function and lead to an improvement in CNS function for patients with citrate transporter deficiency, perhaps related to improved cholinergic neurotransmission and energy metabolism.

Synopsis

We report a novel mutation in the *SLC13A5* gene, c.245A>G (Y82C), in two siblings with different phenotypic expression of the Mendelian disease.

Compliance with Ethics Guidelines

Conflict of Interest

Irina Anselm, Morgan MacCuaig, Sanjay Prabhu, and Gerard T. Berry declare that they have no conflict of interest.

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

Author Contributions

Irina Anselm drafted the manuscript. Morgan MacCuaig contributed to data collection. Gerard Berry reviewed and edited the manuscript. Sanjay Prabhu contributed neuroimaging data. All authors have seen and approved this version of the manuscript.

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JIMD Reports
DOI 10.1007/8904_2016_577

CASE REPORT

Erratum to: Disease Heterogeneity in Na^+/Citrate Cotransporter Deficiency

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Received: 19 November 2015 / Revised: 4 February 2016 / Accepted: 09 February 2016 / Published online: 21 June 2016

Erratum to: JIMD Reports
DOI: 10.1007/8904_2016_546

Dr. Prabhu's middle initial was mistakenly published as "B." and should be "P." (should be Sanjay P. Prabhu).

The online version of the original chapter can be found under DOI 10.1007/8904_2016_546

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