Johannes Zschocke
K. Michael Gibson
Garry Brown
Eva Morava
Verena Peters *Editors*

JIMD Reports

Volume 11

SSIEM

Springer

JIMD Reports
Volume 11

Johannes Zschocke · K. Michael Gibson
Editors-in-Chief

Garry Brown · Eva Morava
Editors

Verena Peters
Managing Editor

JIMD Reports
Volume 11

Springer

ISSN 2192-8304 ISSN 2192-8312 (electronic)
ISBN 978-3-642-37327-5 ISBN 978-3-642-37328-2 (eBook)
DOI 10.1007/978-3-642-37328-2
Springer Heidelberg New York Dordrecht London

Contents

JIMD Reports
DOI 10.1007/8904_2013_214

CASE REPORT

Enzyme Replacement Therapy in a Patient with Gaucher Disease Type III: A Paradigmatic Case Showing Severe Adverse Reactions Started a Long Time After the Beginning of Treatment

Filippo Vairo • Cristina Netto • Alicia Dorneles • Suzana Mittelstadt • Matheus Wilke • Divair Doneda • Kristiane Michelin • Camila Blos Ribeiro • Amanda Quevedo • Tatiane Vieira • Tatiele Nalin • Sônia Lueska • Ida Vanessa D. Schwartz

Received: 27 October 2012 / Revised: 18 January 2013 / Accepted: 23 January 2013 / Published online: 21 February 2013

Abstract *Introduction:* There are three recombinant enzymes available for the treatment of Gaucher disease (GD): imiglucerase, velaglucerase alfa, and taliglucerase alfa.

Case report: A male GD type III patient, 14 years old, genotype p.L444P/L444, diagnosed at 2 years old. He had been treated with imiglucerase for 9 years since the diagnosis. In 2008, however, he presented a severe adverse reaction to imiglucerase, characterized by cough, laryngeal stridor, and periorbital edema. The infusions were suspended for 3 months when imiglucerase was restarted with premedication and a slower infusion rate. After 5 months, he presented a new adverse reaction with vomiting, tachypnea, cough, and periorbital edema. Intradermal testing confirmed IgE-mediated reaction but serological tests were negative. After 2 years and 10 months with no specific treatment and a significant worsening of the clinical picture, taliglucerase alfa was prescribed, with premedication and a slower infusion rate. At the first infusion, he presented moderate adverse reaction and the infusions were suspended. After 2 months, velaglucerase alfa was initiated uneventfully. He maintains day-hospital infusions without premedication and shows improvement of clinical and laboratory parameters.

Conclusion: This is the first report of the use of velaglucerase alfa in patients with GD type III. The use of recombinant enzymes is safe for the majority of GD patients, but severe reactions may occur even many years after the beginning of the treatment. Premedication and slower infusion rate reduce the incidence of adverse reactions but may not solve the problem. This case report further demonstrates the different safety profile among all the recombinant enzymes available for the treatment of GD.

Communicated by: Robin Lachmann

Competing interests: None declared

F. Vairo (✉) · C. Netto · A. Dorneles · S. Mittelstadt · M. Wilke · K. Michelin · C.B. Ribeiro · A. Quevedo · T. Vieira · T. Nalin · I.V.D. Schwartz
Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcellos, 2350, Porto Alegre, RS 90035-903, Brazil
e-mail: fvairo@hcpa.ufrgs.br

F. Vairo · I.V.D. Schwartz
Post Graduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

F. Vairo · I.V.D. Schwartz
BRAIN Experimental Research Group Laboratory, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

D. Doneda · I.V.D. Schwartz
Post Graduate Program in Medicine: Medical Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

S. Lueska
Pediatrics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

I.V.D. Schwartz
Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Introduction

Gaucher disease (GD) is the most common lysosomal storage disorder, with an estimated worldwide incidence of

1 per 57,000 live births in the general population (Meikle et al. 1999) and up to 1 per 850 live births among Ashkenazi Jews (Mistry et al. 2011). Classically, GD is subdivided into three main forms (types I, II, and III), defined by clinical characteristics, disease course, and ethnic prevalence. Nevertheless, there is a wide range of findings that overlap across the classical forms, which has led to a new assessment of GD as a continuous spectrum of disorders rather than a disease with three distinct subtypes (Beutler and Grabowski 2001; Sidransky 2004).

The incidence of subacute neuronopathic (type III) GD is approximately 1 per 100,000 live births. Its distribution is ubiquitous, although the populations of some regions in Northeast Sweden are disproportionately affected (Dahl et al. 1990). Patients with GD type III may exhibit systemic manifestations similar to those of type I patients. Neurological involvement may arise at any age, and usually presents as epilepsy, ataxia, vertical gaze palsy, or dementia (Davies et al. 2007). Some patients may have corneal opacities and valvular heart disease with progressive calcification. The life expectancy is 20–30 years (Tylki-Szymanska and Czartoryska 1999).

For many years, GD was managed with supportive care and palliative measures alone, such as splenectomy to mitigate growth delays, cytopenias, and abdominal discomfort due to splenic enlargement. Since the 1990s, enzyme replacement therapy (ERT) has been the treatment of choice. ERT has improved quality of life among GD patients by reversing many signs and symptoms (Mistry et al. 2007; Hollak et al. 2009). However, the amount of enzyme required to maintain quality of life and reverse the course of symptoms is controversial. Until 2009, imiglucerase (Genzyme Corporation, Allston, MA), obtained from Chinese hamster ovary (CHO) cell lines, was the only ERT agent available. New alternatives have since entered the market, such as velaglucerase alfa (Shire HGT, Dublin, Ireland), which is obtained from human cells and received FDA and EMA approval in 2010, and taliglucerase alfa (Protalix, Carmiel, Israel), which is obtained from carrot cells and received FDA approval in 2012. Despite the longer history of imiglucerase, studies have shown that all three recombinant enzymes are similar in terms of efficacy (Elstein 2011). Approximately 1% of patients develop adverse reactions to imiglucerase ERT, which can be related or not to the production of IgG or IgE antibodies against the synthetic enzyme, forcing judicious use of maintenance infusions. The rate of infusion reactions appears to be higher with taliglucerase alfa and lower with velaglucerase alfa (Zimran et al. 2011; Morris 2012). Substrate reduction therapy with miglustat (Zavesca®, Actelion Pharmaceuticals, Freiburg, Germany) is also available and is mostly indicated for adult GD patients in whom ERT is contraindicated (Platt et al. 1997; Cox et al. 2000; Pastores et al. 2005). The results of therapy with eliglustat (Genzyme Corporation, Allston, MA), another substrate reduction agent, appear promising, but it is still at the clinical trial stage (Lukina et al. 2010).

The management of GD type III is hindered by the fact that recombinant enzymes cannot cross the blood–brain barrier efficiently and act on the CNS. In patients with neuronopathic GD, enzyme dosage is currently adjusted according to the severity of visceral manifestations, with the maximum dosage being 60 IU/kg/infusion every 2 weeks (Vellodi et al. 2009).

This report describes the case of a patient with GD type III who has received all three recombinant ERT forms available, the adverse effects to each formulation, and the clinical outcomes obtained.

Case Report

A 14-year-old male received a diagnosis of GD type III (β-glucocerebrosidase activity, 2 nmol/h/mg [reference range: 10–45]; genotype p.L444P/L444P) at age 2 due to hepatosplenomegaly, kyphoscoliosis, horizontal supranuclear gaze palsy, and cognitive and pulmonary involvement. During workup, the patient was found to be heterozygous for a 24-bp duplication in exon 10 of the *CHIT1* gene, causing partial chitotriosidase deficiency. Shortly after diagnosis, the patient was started on imiglucerase ERT (60 IU/kg/infusion every 2 weeks) at our hospital. Two years after the start of treatment, a central venous catheter was implanted so the patient could receive infusions at his hometown, located 360 km from our service; infusions were provided this way for a total of 4 years and after this through a peripheral access, although the central venous catheter was not removed. Nine years after the start of treatment, while receiving an infusion at a local health facility in his hometown, the patient developed a severe adverse reaction characterized by cough, laryngeal stridor, and periorbital edema within 5 min of the start of infusion. The infusion was ceased at once and the patient was given IV dexamethasone and oral dexchlorpheniramine, with complete resolution of symptoms. C3 and C4 levels were within normal limits, and the IgE level was 1629 UI/mL (reference range for age, <200 UI/mL) 4 days after the adverse event. We chose to discontinue ERT and wait for the results of the serum anti-imiglucerase antibody test, which was performed by the drug manufacturer and carried out on a blood sample collected 40 days after the reaction. The patient remained ERT-free for 3 months waiting for the results of testing, which were ultimately negative for anti-imiglucerase IgG and IgE antibodies (ELISA). Therefore, imiglucerase ERT was restarted at the same dosage (60 IU/kg/infusion every 15 days), now at our service, in a hospital

setting, with loratadine 10 mg PO as premedication and a slower rate of infusion (total infusion time 2 h 30 min). As the patient did not develop any adverse reactions to this scheme, infusions were restarted at his hometown after the third post-reaction infusion. Four months later, the patient developed another reaction, now presenting as vomiting, redness at the catheter site (we could not ascertain whether this was associated with a catheter-related infection), tachypnea, cough, and periorbital edema of 40 min duration. The infusion was ceased and the patient received hydrocortisone 400 mg IV, with complete resolution of symptoms. After this episode, ERT was again discontinued and the patient underwent skin testing for hypersensitivity. The test was performed in two stages, in an ICU setting, in accordance with a test protocol provided by the drug manufacturer. The first step, consisting of a similar standard prick test for common allergens, was negative. The second test included intradermal testing, whereby doses of increasingly concentrated imiglucerase were injected into the dermis. An IgE-mediated reaction was confirmed by the appearance of a >20-mm wheal-and-erythema response within 15 min of injection of imiglucerase 1:10 and 1:100. In view of the anaphylactoid nature of the reaction and the good clinical condition of the patient, we chose to discontinue imiglucerase treatment altogether. Furthermore, neither miglustat nor velaglucerase/taliglucerase alfa were available in the public health system in Brazil at the time (2008).

The patient continued to receive regular follow-up every 3 months for monitoring of clinical and laboratory parameters. At 34-month follow-up, as the patient's condition had deteriorated significantly (episodes of epistaxis, hepatosplenomegaly, hypoalbuminemia, and lower extremity edema) and taliglucerase alfa had recently become available in Brazil, and after discussing this option with the patient's family and securing their informed consent, as patients with allergic reactions to imiglucerase were excluded from clinical trials of taliglucerase alfa, we decided to attempt ERT with this novel medication. The patient was premedicated with loratadine 10 mg PO, ranitidine 150 mg PO, and hydrocortisone 400 mg IV and the infusion rate was titrated slowly (1 mL/15 min, 2 mL/15 min, 4 mL/15 min, 8 mL/15 min, 16 mL/15 min, and 32 mL thereafter). However, after infusion of 5.8 mL of taliglucerase alfa at a dosage of 60 IU/kg, the patient developed epigastric pain, vomiting, rash, and headache. Dexchlorpheniramine 2 mg PO, promethazine 25 mg IV, and metoclopramide 10 mg IV were administered and there was improvement of symptoms. The infusion was halted and the decision was made to discontinue taliglucerase alfa therapy. Two months after this reaction, velaglucerase alfa was provided for this patient as a compassionate use. After discussing this option with the patient's family and securing their informed consent, as no data were available on treatment of GD type III with this enzyme, the decision was made to attempt ERT once more. An anti-imiglucerase antibody test performed by Shire HGT in November 2011 (electrochemiluminescence immunoassay for anti-imiglucerase and anti-velaglucerase antibodies) was negative for IgG and IgE antibodies.

The patient was admitted to our hospital for stabilization of clinical parameters and a battery of tests to determine baseline laboratory values. After 2 weeks of hospitalization, velaglucerase alfa was administered at a dosage of 60 IU/kg, after premedication with hydrocortisone 400 mg IV and promethazine 25 mg IV and an infusion rate titrated to 200 mL over the course of 4 h. The infusion was completed uneventfully, and the patient was started on twice-monthly infusions on an outpatient basis. Premedication was gradually reduced over the course of five sessions, with no ill effects. After eight infusions at our hospital, the patient returned to his hometown, where he continues to receive periodic infusions. He no longer requires premedication and the infusion time has been shortened to 2 h. We chose to wait for further clinical improvement before removal of the central venous catheter.

The patient's neurological condition remains stable and his anemia, hyperproteinemia, and lower extremity edema have resolved completely. Thrombocytopenia has improved substantially and abdominal volume and chitotriosidase levels are reduced (Table 1). In addition to these improvements in objective parameters, application of the SF-36 and WHOQoL questionnaires (completed by proxy by the patient's mother) revealed improvement in quality of life (data not shown).

Discussion

Recombinant enzyme replacement therapy is safe for most GD patients, but 1.5% to 25% may develop adverse reactions, depending on the medication regimen (Starzyk et al. 2007; Zimran et al. 2011). Some reports have described premedication and manipulation of infusion rates for the management of imiglucerase-related adverse effects (Peroni et al. 2009), but these measures are not always effective. In view of a worldwide shortage of imiglucerase (Hollak et al. 2010), the Brazilian National Health Surveillance Agency (ANVISA), the regulatory counterpart of the U.S. FDA and the European EMA, granted emergency marketing authorization for taliglucerase alfa in 2010. In 2011, an updated version of the Brazilian Ministry of Health guidelines for GD disease was approved, which included all the three recombinant enzymes available on the market (imiglucerase, taliglucerase alfa, and velaglucerase alfa) and substrate reduction therapy (miglustat). Currently, there are Brazilian patients on all four forms of treatment. Although X-ray structures of all three enzymes are very similar, they show some differences in their sequence and glycan structure. Taliglucerase alfa has

Table 1 Follow-up of laboratory parameters and imaging findings

	Pre-treatment[b]	Before first imiglucerase reaction	34 months without treatment	Before first velaglucerase alfa infusion	After 6 velaglucerase alfa infusions	After 12 velaglucerase alfa infusions
Age (years)	2	11	13.3	14.3	14.6	14.9
Height (cm)[a]	73	122	132	132	132	132
Weight (kg)	9.0	23.6	29.7	29.7	30.1	31
Hemoglobin (g/dL)	8.3	13	8.6	8	10.7	12.6
Platelets (1,000/mm^3)	133	280	65	56	62	115
Chitotriosidase (nmol/mL/h)	8,627	1,808	15,117	19,878	15,814	13,074
Liver[c]	8.2 cm (longest axis)	889 cm^3	Normal	5,367 cm^3	5,369 cm^3	ND
Spleen[c] (longest axis, in cm)	12.1	9.5	17.5	27	17	ND
Albumin (g/dL)	ND	ND	3	2.9	ND	3.48
Bone changes[d]	Kyphoscoliosis	Kyphoscoliosis	Osteolytic and osteoblastic lesions, Erlenmeyer flask deformity, and kyphoscoliosis	ND	ND	ND
BMD (T score)	−5.7	ND	−4.6	ND	ND	ND
BMB score	ND	ND	ND	14	ND	ND
Spirometry	ND	FEV1/FVC: 73% – air flow preserved	FEV1/FVC: 34.5 % – severe restrictive ventilatory defect	FEV1/FVC: 31.3 % – severe restrictive ventilatory defect	ND	ND
Severity Score Index (SSI)[28]	24	28	31	33	32	29

[a] Difficult to measure due to bone changes

[b] Shortly before first imiglucerase infusion

[c] On ultrasound

[d] On X-rays

BMD Bone mineral density – DEXA (Z score was not available), *BMB score* bone marrow burden (MRI), *FEV_1* Forced expiratory volume in 1 s, *FVC* Forced vital capacity, *ND* Not done

two additional amino acids at the N-terminus, and it has additional seven amino acids at the C-terminus in relation to the "wild" human counterpart. Besides that, the amino acid composition of both imiglucerase and taliglucerase alfa differs from the human β-glucocerebrosidase at residue 495. Velaglucerase alfa has the same amino acid sequence as the human enzyme. Regarding the glycosylation process, taliglucerase alfa differs from the other two enzymes as it contains xylose and fucose derivatives, which are unique to plant-derived proteins (Brumshtein et al. 2010).

Despite no detectable serum anti-imiglucerase IgE or IgG antibodies, our patient had a positive intradermal test response and almost instant adverse response to imiglucerase (after 9 years of infusions without any intercurrence) and taliglucerase alfa (at the first infusion). This may be indicative of a hypersensitivity reaction to some element present during the manufacturing process of imiglucerase – an element possibly used in manufacturing of taliglucerase alfa as well. The patient does not seem to present an hyper-IgE syndrome since he did not present any clinical symptoms associated with hyper-IgE syndrome such as skin abscesses, recurrent pneumonia, pneumatoceles, early eczema, and late loss of primary dentition (Sowerwine et al. 2012).

Interestingly, our patient presented an anaphylactoid reaction after many years of imiglucerase ERT. This could have implications for some countries in which home therapy is widely available; for safety reasons, we suggest the patient should not be alone during home infusions.

Throughout the course of this case, we attempted to follow existing adverse reaction management protocols for patients with GD and other lysosomal storage disorders (Kim et al. 2008) and create our own, but the patient could not adapt to imiglucerase or taliglucerase alfa ERT despite these measures. Miglustat was not trialed because, despite marketing approval, there was no available stock at the time of the patient's reactions. Furthermore, the patient was extremely debilitated and underweight, and was thus not a candidate for substrate reduction therapy.

After the availability of other recombinant forms of β-glucocerebrosidase in several countries in 2010, the scenario for management of patients who tolerate imiglucerase poorly or have discontinued ERT for other reasons has improved, as the switch to substrate reduction therapy (Elstein et al. 2007) or another recombinant enzyme has proved safe and effective (Elstein et al. 2012; van Dussen et al. 2012).

This is the first report of velaglucerase alfa therapy in a patient with GD type III. We suggest, on the basis of our findings, although this enzyme has not received formal approval for use in patients with GD type III, it should be assessed for use in such patients who develop adverse reactions to imiglucerase or taliglucerase alfa. The Brazilian Ministry of Health guidelines for treatment of GD does not mention any contraindications to the use of velaglucerase alfa in patients with type III disease. In addition to describing the success of velaglucerase alfa therapy, this report demonstrates the differences in safety profile of the three enzymes available for ERT for Gaucher disease for this patient, which are most likely related to distinct manufacturing processes and can occur at any time after the beginning of therapy.

Acknowledgments We would like to thank the patient and his family; Dr. Carolina Moreno for follow-up; Dr. Ronaldo David da Costa for his assistance with hypersensitivity testing; Fabiane Oliveira, PharmD for her assistance regarding pharmacology; Professor Maria Luiza Saraiva-Pereira for her assistance with genotyping; Dra Maira Burin for her assistance with biochemical analysis; Prof. Luiz Jobim and Mariana Jobim, from the HCPA Immunology Service for their assistance regarding immunological issues; Shire Pharmaceuticals for donating velaglucerase alfa and for case discussion; Genzyme Corporation for serological analyses and for case discussion; Protalix and Pfizer, Inc. for case discussion; and FAPERGS, CNPq, CAPES, and FIPE/HCPA.

Contributors

FV designed data collection, monitored data collection, analyzed the data, drafted and revised the paper. He is the guarantor. AD, CN, SM, MW, DD, KM, CBR, AQ, TV, TN, and SL analyzed the data, and revised the paper. IVDS designed data collection, monitored data collection, analyzed the data, drafted and revised the paper.

References

Brazilian Ministry of Health Guidelines for Gaucher disease In Editor ed.^eds. *Book Brazilian Ministry of Health Guidelines for Gaucher disease.* http://portal.saude.gov.br/portal/arquivos/pdf/pcdt_doenca_de_gaucher_pdf

Brumshtein B, Salinas P, Peterson B et al (2010) Characterization of gene-activated human acid-beta-glucosidase: crystal structure, glycan composition, and internalization into macrophages. Glycobiology 20(1):24–32

Cox T, Lachmann R, Hollak C et al (2000) Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. Lancet 355(9214):1481–1485

Dahl N, Lagerstrom M, Erikson A, Pettersson U (1990) Gaucher disease type III (Norrbottnian type) is caused by a single mutation in exon 10 of the glucocerebrosidase gene. Am J Hum Genet 47(2):275–278

Davies EH, Erikson A, Collin-Histed T, Mengel E, Tylki-Szymanska A, Vellodi A (2007) Outcome of type III Gaucher disease on enzyme replacement therapy: review of 55 cases. J Inherit Metab Dis 30(6):935–942

Beutler E, Grabowski GA (2001) Gaucher disease. In: Scriver CR, Sly WS, Childs B, Beaudet AL, Valle D, Kinzler KW, Vogelstein B (eds) Metabolic and molecular bases of inherited disease, Vol II. McGraw Hill, New York, pp 3635–3668

Elstein D (2011) Recent advances in treatment approaches to Gaucher disease. Curr Pharm Biotechnol 12(6):854–860

Elstein D, Altarescu G, Maayan H et al (2012) Booster-effect with velaglucerase alfa in patients with Gaucher disease switched from

long-term imiglucerase therapy: early Access Program results from Jerusalem. Blood Cells Mol Dis 48(1):45–50

Elstein D, Dweck A, Attias D et al (2007) Oral maintenance clinical trial with miglustat for type I Gaucher disease: switch from or combination with intravenous enzyme replacement. Blood 110(7):2296–2301

Hollak CE, de Fost M, van Dussen L, Vom Dahl S, Aerts JM (2009) Enzyme therapy for the treatment of type 1 Gaucher disease: clinical outcomes and dose - response relationships. Expert Opin Pharmacother 10(16):2641–2652

Hollak CE, vom Dah S, Aerts JM et al (2010) Force majeure: therapeutic measures in response to restricted supply of imiglucerase (Cerezyme) for patients with Gaucher disease. Blood Cells Mol Dis 44(1):41–47

Kim KH, Decker C, Burton BK (2008) Successful management of difficult infusion-associated reactions in a young patient with mucopolysaccharidosis type VI receiving recombinant human arylsulfatase B (galsulfase [Naglazyme]). Pediatrics 121(3): e714–717

Lukina E, Watman N, Arreguin EA et al (2010) Improvement in hematological, visceral, and skeletal manifestations of Gaucher disease type 1 with oral eliglustat tartrate (Genz-112638) treatment: 2-year results of a phase 2 study. Blood 116(20):4095–4098

Meikle PJ, Hopwood JJ, Clague AE, Carey WF (1999) Prevalence of lysosomal storage disorders. JAMA 281(3):249–254

Mistry PK, Cappellini MD, Lukina E et al (2011) A reappraisal of Gaucher disease-diagnosis and disease management algorithms. Am J Hematol 86(1):110–115

Mistry PK, Sadan S, Yang R, Yee J, Yang M (2007) Consequences of diagnostic delays in type 1 Gaucher disease: the need for greater awareness among hematologists-oncologists and an opportunity for early diagnosis and intervention. Am J Hematol 82(8):697–701

Morris JL (2012) Velaglucerase alfa for the management of type 1 Gaucher disease. Clin Ther 34(2):259–271

Pastores GM, Barnett NL, Kolodny EH (2005) An open-label, noncomparative study of miglustat in type I Gaucher disease: efficacy and tolerability over 24 months of treatment. Clin Ther 27(8):1215–1227

Peroni DG, Pescollderungg L, Piacentini GL, Cassar W, Boner AL (2009) Effective desensitization to imiglucerase in a patient with type I Gaucher disease. J Pediatr 155(6):940–941

Platt FM, Neises GR, Reinkensmeier G et al (1997) Prevention of lysosomal storage in Tay-Sachs mice treated with N-butyldeoxynojirimycin. Science 276(5311):428–431

Sidransky E (2004) Gaucher disease: complexity in a "simple" disorder. Mol Genet Metab 83(1–2):6–15

Sowerwine KJ, Holland SM, Freeman AF (2012) Hyper-IgE syndrome update. Ann N Y Acad Sci 1250:25–32

Starzyk K, Richards S, Yee J, Smith SE, Kingma W (2007) The long-term international safety experience of imiglucerase therapy for Gaucher disease. Mol Genetics Metabol 90(2):157–163

Tylki-Szymanska A, Czartoryska B (1999) Enzyme replacement therapy in type III Gaucher disease. J Inherit Metab Dis 22(2):203–204

van Dussen L, Cox TM, Hendriks EJ et al (2012) Effects of switching from a reduced dose imiglucerase to velaglucerase in type 1 Gaucher disease: clinical and biochemical outcomes. Haematologica 97(12):1850–1854

Vellodi A, Tylki-Szymanska A, Davies EH et al (2009) Management of neuronopathic Gaucher disease: revised recommendations. J Inherit Metab Dis 32(5):660–664

Zimran A, Brill-Almon E, Chertkoff R et al (2011) Pivotal trial with plant cell-expressed recombinant glucocerebrosidase, taliglucerase alfa, a novel enzyme replacement therapy for Gaucher disease. Blood 118(22):5767–5773

JIMD Reports
DOI 10.1007/8904_2013_215

CASE REPORT

Expanding the Spectrum of *Methylmalonic Acid-Induced Pallidal Stroke*: First Reported Case of Metabolic Globus Pallidus Stroke in Transcobalamin II Deficiency

Lance Harrington Rodan • Navin Mishra • Ivanna Yau • Andrea Andrade • Komudi Siriwardena • Ingrid Tein

Received: 31 December 2012 / Revised: 28 January 2013 / Accepted: 30 January 2013 / Published online: 21 February 2013

Abstract A 10-year-old boy with transcobalamin II (TCII) deficiency on oral cyanocobalamin therapy presented with acute right hemiparesis and sensory axonal neuropathy in the context of an intercurrent viral illness. MRI demonstrated unilateral globus pallidus stroke with normal MRA. Echocardiogram was normal. Methylmalonic acid in serum was mildly elevated at 10.29 μmol/L (normal < 0.37 μmol/L), which was an 18-fold increase from his previous baseline. The patient was switched to IM cyanocobalamin and serum methylmalonic acid levels normalized over 6 months to 0.01 μmol/L. After 4 months of IM cyanocobalamin therapy, the neuropathy had resolved. Repeat MRI 4 months after the sentinel stroke demonstrated a chronic-appearing contralateral globus pallidus stroke of uncertain timing.

Conclusions: We are describing the first case of metabolic stroke and peripheral neuropathy in TCII deficiency. The neuropathy was responsive to parenteral hydroxycobalamin. Unilateral globus pallidus stroke in the appropriate clinical context should not exclude a metabolic etiology as it may herald contralateral involvement and may provide an opportunity for early recognition and treatment. IM hydroxycobalamin should be strongly considered in all patients with TCII, particularly when they reach later childhood. This case highlights the selective vulnerability of the globus pallidus to increased levels of methylmalonic acid of various causes, which is important for both diagnosis and ultimately understanding the mechanisms of neurological injury in this group of conditions. Metabolic stroke may occur with lower levels of methylmalonic acid than previously reported in the context of an intercurrent bioenergetic stressor.

Communicated by: Ertan Mayatepek

Competing interests: None declared

L.H. Rodan · N. Mishra · I. Yau · A. Andrade · I. Tein
Division of Neurology, University of Toronto, Toronto, Canada M5G 1X8

K. Siriwardena
Division of Clinical and Metabolic Genetics, Dept. of Pediatrics, Hospital for Sick Children, University of Toronto, Toronto, Canada M5G 1X8

I. Tein
Dept. of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada M5G 1X8

L.H. Rodan (✉)
Division of Neurology, Hospital for Sick Children, 555 University Ave., Toronto, ON, Canada M5G 1X8
e-mail: lance.rodan@sickkids.ca1

Introduction

The globus pallidus is an area of the brain that shows a selective vulnerability in a number of conditions associated with methylmalonic aciduria (MMA), including methylmalonyl-CoA mutase deficiency, cobalamin complementation group disorders, and severe pernicious anemia (Heidenreich et al. 1988; Trinh et al. 2001; Sharrief et al. 2012). The pathophysiology behind this selective vulnerability is currently unknown, although it has been postulated to result from respiratory chain dysfunction that preferentially affects the highly metabolically active globus pallidi (Heidenreich et al. 1988). Here we present the first reported case of globus pallidus stroke associated with methylmalonic aciduria in a patient with transcobalamin II (TCII) deficiency (OMIM #275350), thereby expanding the spectrum of *MMA-induced pallidal stroke*.

Case Report

The index patient is a 10-year-old, left-handed boy who was diagnosed with TCII deficiency at the age of 10 weeks when he presented with failure to thrive, lethargy, myoclonus, pancytopenia, and megaloblasts in his bone marrow. He had elevated methylmalonic acid and plasma homocysteine with normal serum B12 and folic acid. Enzymatic testing confirmed a defect of TCII. He was initially treated with IM hydroxycobalamin, with rapid normalization of homocysteine and methylmalonic acid levels. At 1 year of age, he was transitioned to oral cyanocobalamin (2,500 μg daily) with sustained normalization of laboratory parameters. The genetic diagnosis was later established as a homozygous sequence variant in the TCN2 gene (c.497_498de/tc). He remained systemically well following the early infantile period, with no history of metabolic decompensations. He did preferentially toe walk and on serial neurological assessments had decreased reflexes in his lower extremities; two prior nerve conduction studies were normal. Serum methylmalonic acid level performed at the age of 9 years was 0.58 μmol/L (normal < 0.37 μmol/L).

At the age of 10 years, the patient presented with a 2-day history of gradually progressive right hemiparesis (arm and leg) 2 weeks following a viral exanthem. The family had not noticed any additional weakness, encephalopathy, or seizures. Other than a moderate right hemiparesis, the examination was significant for bilateral lower extremity areflexia, with preserved reflexes in the upper extremities.

MR brain with spectroscopy (Fig. 1) demonstrated a well-defined area of restricted diffusion involving the left globus pallidus. MR angiography was normal. MRS with voxel placed in the left basal ganglia demonstrated a small lactate doublet at 1.33 ppm. Echocardiogram with bubble study was normal.

On first presentation, serum methylmalonic acid was elevated at 8.57 μmol/L, which rose to 10.29 μmol/L over the following 4 days (normal < 0.37 μmol/L). The latter represented an 18-fold increase from his previous level at 9 years of age. Urine methylmalonic acid was also elevated at 184 mmol/mol creatinine. Quantitative acylcarnitines demonstrated only a borderline elevation of C3 at 1.11 μmol/L (normal <1.08) with normal total and free carnitine. Serum amino acids, homocysteine, hemoglobin, mean corpuscular volume (MCV), WBC count, venous blood gas, and serum immunoglobulins were normal.

Nerve conduction studies in the lower extremities were abnormal with decreased sensory nerve action potential (SNAP) amplitude of the sural nerve (with normal conduction velocity and distal latency) and absent superficial peroneal nerve SNAPs. Motor studies in the lower extremities and sensory/motor studies in the upper extremities were normal. These findings were in keeping with a length-dependent sensory axonal polyneuropathy.

The etiology of stroke in our patient at this point was not entirely clear, and both primary metabolic and vascular etiologies were entertained. The patient was switched from his oral cyanocobalamin formulation to 1 mg IM hydroxycobalamin daily. He was also started on 3 mg/kg ASA.

On repeat bloodwork at approximately 2, 3, and 6 months' follow-up, the serum methylmalonic acid gradually normalized from 2.70 μmol/L to 1.99 μmol/L, and finally to 0.01 μmol/L, respectively. On clinical assessment at 4 months, the patient's right hemiparesis was mildly improved. Reflexes were 1+ in the lower extremities. There was no evidence of dystonia or chorea. Repeat MR brain (Fig. 2) at 4 months demonstrated interval development of a new, chronic-appearing right globus pallidus stroke as well as gliotic changes of the original left globus pallidus stroke. Spectroscopy was normal. Repeat nerve conduction studies of sural and superficial peroneal nerves were normal.

Discussion

Transcobalamin II (TCII) is one of the three human transporters of cobalamin (vitamin B12). It binds and transports cobalamin absorbed in the terminal ileum to tissue cells throughout the body, where it ultimately participates in cell surface adhesion and internalization of the cobalamin (Quadros 2009). TCII is also synthesized by astrocytes and is the primary enzyme involved in the neuronal uptake of cobalamin in the central nervous system (Begley et al. 1994). Cobalamin ultimately has an important role in the metabolism of both homocysteine and methylmalonic acid as a cofactor for methionine synthase and methylmalonyl-CoA mutase, respectively (Takahashi-Iñiguez et al. 2012; Banerjee and Ragsdale 2003). Methylmalonyl-CoA mutase isomerizes mitochondrial methylmalonyl-CoA to succinyl-CoA, a crucial substrate in energy provision via the citric acid cycle and complex II of the respiratory chain (Dutra et al. 1993; Okun et al. 2002). Inherited deficiency of TCII has been described in over 40 patients (Watkins and Rosenblatt 2011). TCII deficiency is an autosomal recessive condition resulting from mutations in the TCN2 gene on chromosome 22q12 (Watkins and Rosenblatt 2011; Regec et al. 1995). It typically presents in the first 2 months of life with pancytopenia, failure to thrive, and gastrointestinal symptoms (Kaikov et al. 1991; Gimpert et al. 1975). Laboratory markers typically include increased homocysteine, reduced methionine, increased methylmalonic acid, metabolic acidosis, and megaloblastic anemia (Kaikov et al. 1991). Stroke and peripheral neuropathy have not previously been reported in association with TCII deficiency.

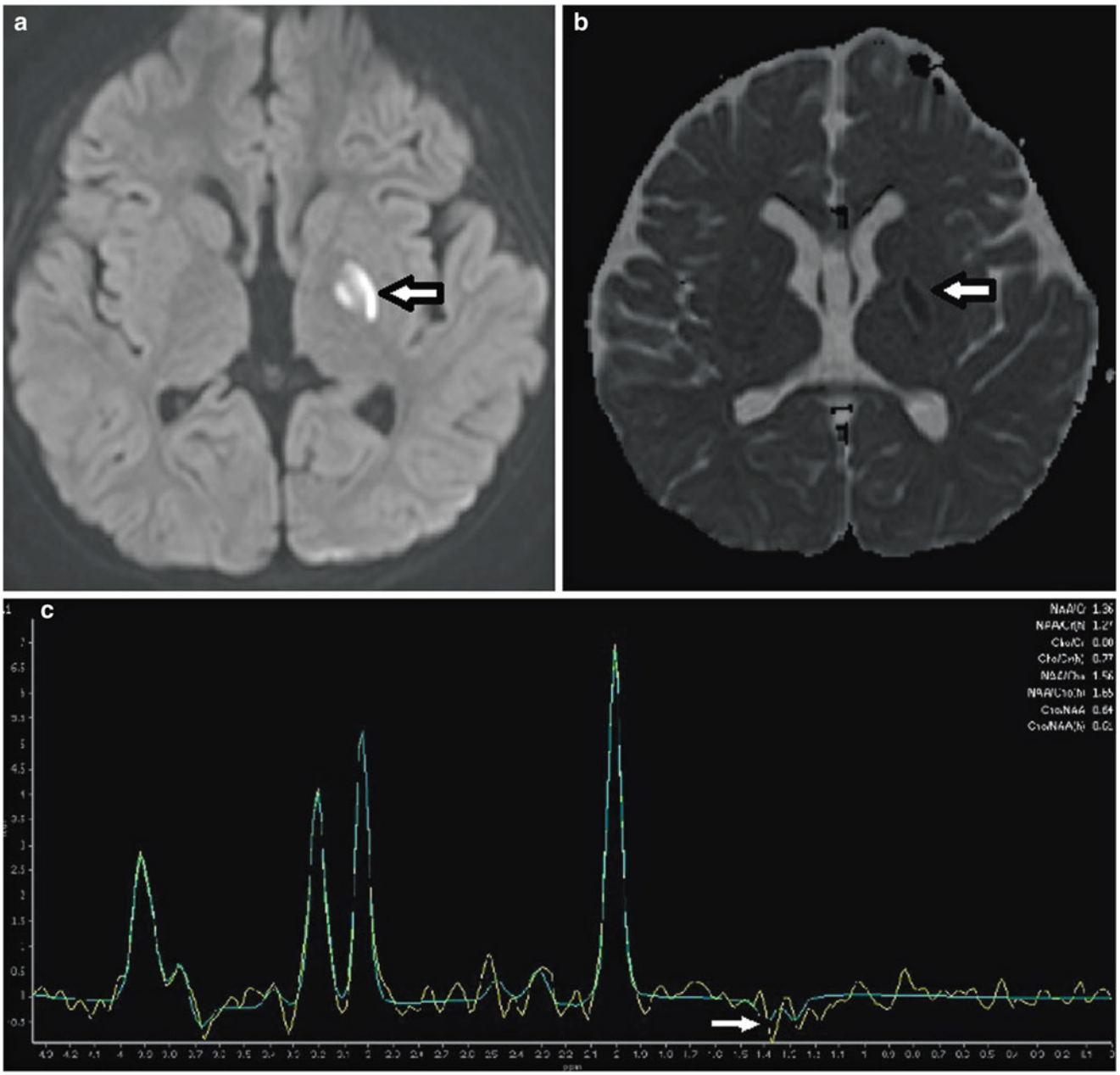

Fig. 1 MR brain with spectroscopy at time of sentinel stroke. Diffusion-weighted imaging (**a**) with apparent diffusion coefficient (**b**) demonstrates restricted diffusion in the left globus pallidus (*arrows*). Proton spectroscopy (**c**) with voxel placed in the left globus pallidus demonstrates a small lactate doublet at 1.33 ppm (*arrows*)

We presume that the mechanism of stroke in our patient was MMA-induced pallidal stroke for the following reasons. Firstly, our patient presented with staggered, bilateral globus pallidus strokes, without any additional parenchymal involvement in the lateral lenticulostriate or anterior choroidal vascular territories bilaterally. The latter vessels supply the globus pallidus, as well as the putamen, caudate, and internal capsule. *It would be highly implausible for a vaso-occlusive stroke to selectively affect bilateral pallidi in isolation (the identical portion of the vascular territory bilaterally), sparing the remainder of the vascular territory.* This is especially the case with normal vascular imaging. *The selective bilateral pallidal involvement is, therefore, more consistent with a "metabolic" stroke in a vulnerable region of the basal ganglia,* as previously described in MMA of various etiologies. While globus pallidus stroke has not been specifically described in patients with TCII deficiency, it is a logical extension given

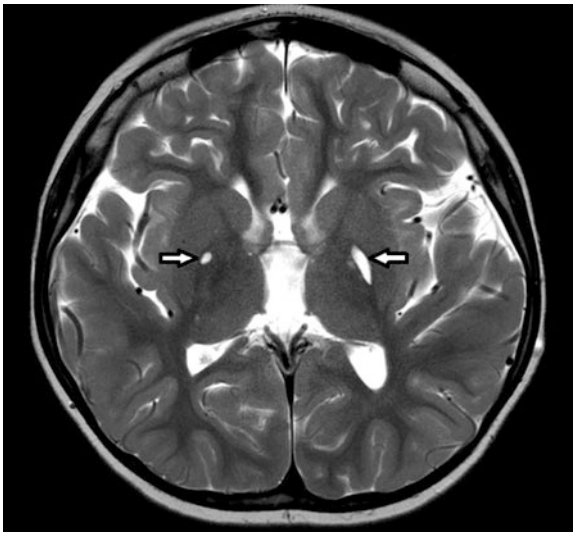

Fig. 2 MR brain at 4-month follow-up. Axial T2-weighted sequence demonstrates bilateral hyperintensities in the globus pallidi (*arrows*)

the role of TCII in the metabolism of methylmalonic acid. It is admittedly unusual that our patient presented initially with unilateral globus pallidus involvement, as asymmetries have not been previously described with MMA. Repeat neuroimaging was done 4 months after the incident stroke, making it difficult to determine the precise timing of the chronic-appearing contralateral globus pallidus stroke. The asymmetry in the initial presentation may suggest subtle differences in the pallidal neuronal micromilieu in this patient. In addition, although our patient's peak level of MMA was lower than that previously reported in association with MMA-associated metabolic stroke (lowest reported level is 89 μmol/L as per Sharrief et al. 2012), our patient's stroke did occur in the context of an almost 20-fold elevation of MMA from his previous baseline. The threshold for metabolic stroke in our patient may have been lowered in part by the bioenergetic stress of his intercurrent viral illness.

In addition to stroke, our patient presented with a new, electrophysiologically confirmed peripheral neuropathy. A predominantly demyelinating peripheral neuropathy has been previously described in both early-onset and late-onset forms of cobalamin C deficiency and methylene tetrahydrofolate reductase deficiency (Frattini et al. 2010; Nishimura et al. 1985). In addition, sensory neuropathy has been described in patients with other forms of homocystinuria, although this has been attributed to toxicity from supplementation with pyridoxine (Ludolph et al. 1991). Dietary B12 deficiency is also associated with a reversible sensorimotor demyelinating polyneuropathy (Sakly et al. 2005). The presumed pathophysiology relates to deficient methylation of myelin basic protein, an important component of myelin that influences myelin compaction (Frattini et al. 2010). To our knowledge, this is the first report of sensory axonal peripheral neuropathy in a patient with TCII deficiency. Importantly, abnormalities on nerve conduction studies reversed with IM hydroxycobalamin.

Previous authors have suggested aggressive treatment of TCII deficiency with IM hydroxycobalamin and have cited reports of treatment failure in late childhood and adolescence in patients transitioned from IM treatment to oral cyanocobalamin. The patients with treatment failure presented with cognitive decline, visual dysfunction from maculopathy, and proprioceptive difficulties from presumed dorsal column involvement of the spinal cord. Symptoms improved when IM therapy was resumed (Schiff et al. 2010).

In conclusion, we are describing the first case of metabolic stroke and peripheral neuropathy in TCII deficiency. The neuropathy was responsive to parenteral hydroxycobalamin. This case highlights a number of important issues. Firstly, unilateral globus pallidus stroke in the appropriate clinical context should not exclude a metabolic etiology as it may herald contralateral involvement; in fact, it may provide an opportunity for early recognition and treatment. Secondly, this case suggests that IM hydroxycobalamin should be strongly considered in all patients with TCII, particularly when they reach later childhood. Finally, this case highlights the selective vulnerability of the globus pallidus to increased levels of methylmalonic acid of various causes, which is important for both diagnosis and ultimately understanding the mechanisms of neurological injury in this group of conditions. It appears that metabolic stroke may occur with lower levels of MMA than previously reported in the context of an intercurrent bioenergetic stressor.

Synopsis

Transcobalamin II deficiency may present with metabolic pallidal stroke and peripheral neuropathy, reversible with IM cyanocobalamin.

Contributions

Lance H Rodan – reviewed patient, concept, manuscript preparation, literature review, discussion, manuscript writing, guarantor

Navin Mishra – reviewed patient, manuscript editing, literature review, discussion

Ivanna Yau – reviewed patient, manuscript editing, discussion

Andrea Andrade – reviewed patient, manuscript editing, discussion

Komudi Siriwardena – contributed patient and patient data and reviewed patient, manuscript editing, discussion

Ingrid Tein – reviewed patient, concept, manuscript editing, discussion, supervisor

Conflict of Interest

No authors report a conflict of interest related to the publication of this manuscript.

References

Banerjee R, Ragsdale SW (2003) The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. Annu Rev Biochem 72:209–247

Begley JA, Colligan PD, Chu RC (1994) Synthesis and secretion of transcobalamin II by cultured astrocytes derived from human brain tissue. J Neurol Sci 122:57–60

Dutra JC, Dutra-Filho CS, Cardozo SE et al (1993) Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. J Inherit Metab Dis 16:147–153

Frattini D, Fusco C, Ucchino V, Tavazzi B, Della Giustina E (2010) Early onset methylmalonic aciduria and homocysteinuria cblC type with demyelinating neuropathy. Pediatr Neurol 43:135–138

Gimpert E, Jakob M, Hitzig WH (1975) Vitamin B12 transport in blood in congenital deficiency of transcobalamin II. Blood 45:71–82

Heidenreich R, Natowicz M, Hainline BE, Kelley RI, Hillman RE et al (1988) Acute extrapyramidal syndrome in methylmalonic academia: "metabolic stroke" involving the globus pallidus. J Pediatr 113(6):1022–1027

Kaikov Y, Wadsworth LD, Hall CA, Rogers PC (1991) Transcobalamin II deficiency: case report and review of the literature. Eur J Pediatr 150:841–843

Ludolph AC, Ullrich K, Bick U, Fahrendorf G, Przyrembel H (1991) Functional and morphological deficits in late-treated patients with homocysteinuria: a clinical, electrophysiologic, and MRI studies. Acta Neurol Scand 83:161–165

Nishimura M, Yoshino K, Tomita Y, Takashima S, Tanaka J et al (1985) Central and peripheral nervous system pathology of homocysteinuria due to 5,10 –methylene tetrahydrofolate reductase deficiency. Pediatr Neurol 1(6):375–378

Okun JG, Horster F, Farkas LM, Feyh P, Hinz A, Sauer S et al (2002) Neurodegeneration in methylmalonic aciduria involves inhibition of complex II and tricarboxylic acid cycle, and synergistically acting excitotoxicity. J Biol Chem 277:14674–14680

Quadros EV (2009) Advances in the understanding of cobalamin assimilation and metabolism. Brit J Hematol 148:195–204

Regec A, Quadros EV, Platica O, Rothenberg SP (1995) The cloning and characterization of the human transcobalamin II gene. Blood 85:2711–2719

Sakly G, Hellara O, Trabelsi A, Dogui M (2005) Reversible peripheral neuropathy induced by B12 deficiency. Neurophysiol Clin 35 (5–6):149–153

Schiff M, Ogier de Baulny H, Bard G, Barlogis V, Hamil C, Moat SJ et al (2010) Should transcobalamin deficiency be treated aggressively. J Inherit Metab Dis 33:223–229

Sharrief AZ, Raffel J, Zee DS (2012) Vitamin B12 deficiency with bilateral globus pallidus abnormalities. Arch Neurol 69(6):769–772

Takahashi-Iñiguez T, Garcia-Hernandez E, Arreguin-Espinosa R, Flores ME (2012) Role of vitamin B12 on methylmalonyl-CoA mutase activity. J Zhejiang Univ-Sci B (Biomed & Biotechnol) 13(6):423–437

Trinh BC, Melhem ER, Barker PB (2001) Multi-slice proton MR spectroscopy and diffusion weighted imaging in methylmalonic academia: report of two cases and review of the literature. AJNR 22:831–833

Watkins D, Rosenblatt DS (2011) Inborn errors of cobalamin absorption and metabolism. Am J Med Genet 157:33–44

JIMD Reports
DOI 10.1007/8904_2013_216

CASE REPORT

A Large Intragenic Deletion in the *ACADM* Gene Can Cause MCAD Deficiency but is not Detected on Routine Sequencing

Claire Searle • Brage Storstein Andresen • Ed Wraith • Jamie Higgs • Deborah Gray • Alison Mills • K. Elizabeth Allen • Emma Hobson

Received: 10 January 2013 / Revised: 10 January 2013 / Accepted: 06 February 2013 / Published online: 2 April 2013

Abstract We report of a family who has three members affected by medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, one of whom sadly died in the neonatal period prior to diagnosis. Routine sequencing, available on a service basis in the UK, identified only a heterozygous mutation in *ACADM* gene (c.985A>G, p.Lys329Glu) in this family. Linkage analysis suggested a possible intragenic deletion which was confirmed by the use of array-based comparative genomic hybridization (aCGH). This second mutation was a large intragenic deletion encompassing at least exons 1–6 of the *ACADM* gene. Now that this deletion has been identified, several family members have come forward for carrier testing which was not possible previously. Larger deletions (20bp or more) have only previously been reported twice, but these may be a more frequent cause of MCAD deficiency than hitherto believed, due to fact that these are not anticipated and, therefore, the routine diagnostic techniques used will not identify them. This finding represents a useful learning point in the management of families with MCAD deficiency, and highlights that we should be routinely looking for larger deletions, when only one of the mutations can be identified on standard sequencing.

Communicated by: John Christodoulou

Competing interests: None declared

C. Searle (✉)
Yorkshire Regional Genetic Service, Chapel Allerton Hospital, Chapeltown Rd, Leeds LS7 4SA, UK
e-mail: cjs29@doctors.org.uk

B.S. Andresen
Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense, Denmark

E. Wraith
Royal Manchester Children's Hospital, Oxford Road, Manchester M13 9WL, UK

J. Higgs
GeneDx, 207 Perry Parkway, Gaithersburg MD20877, USA

D. Gray · A. Mills
Cytogenetics, Ashley Wing, St. James's University Hospital, Beckett St., Leeds LS9 7TF, UK

K.E. Allen
Sheffield Diagnostic Genetics Service, Sheffield Children's NHS Foundation Trust, Western Bank, Floor C, Blue Wing, Sheffield S10 2TH, UK

E. Hobson
Yorkshire Regional Genetic Service, Chapel Allerton Hospital, Chapeltown Rd, Leeds LS7 4SA, UK

Abbreviations

MCAD	Medium Chain Acyl-CoA Dehydrogenase
MLPA	Multiplex Ligation-dependent Probe Amplification

Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD; OMIM 201450; EC 1.3.99.3) deficiency (MIM 201450) is an autosomal recessive disorder resulting from a defect of fatty acid oxidation. Clinical presentation of the condition is varied. Some with the condition will never present with symptoms, others, after metabolic stress, will present with vomiting, lethargy, and coma. Unfortunately, a few are diagnosed after death in childhood. A newborn screening programme is in place in the UK. If treatment is established early, then long-term morbidity and mortality is low. Mutation screening by means of direct sequencing of exons 1 to 12 of the *ACADM* gene will not detect large deletions when present in the heterozygous state. We present a family

where a large deletion in the *ACADM* gene had wider implications.

Case Report

This family had three members affected by MCAD deficiency as depicted in Fig. 1. The twin brothers (C2 and C3) were diagnosed in 1994, at the age of 18 months, when one of them presented to hospital acutely unwell with a low glucose level. The second twin has never had any acute episodes requiring hospital admission. Sadly the other affected member of this family died, in 2009, at the age of 2 days from undiagnosed MCAD deficiency (C4).

Routine sequencing, available on a service basis in the UK, identified only the common heterozygous mutation in the *ACADM* gene (c.985A>G p.Lys329Glu) in this family. This testing was reported to exclude the vast majority (>99%) of all reported mutations (Andresen et al. 2001; Maier et al. 2005; Morris et al. 1995; Andresen et al. 1993; Maegawa et al. 2008).

To try and find the other mutation linkage analysis was performed (Fig. 2). Patient C4 was found to be heterozygous for the polymorphic silent variation c.1161 A>G in exon 11[4] of the *ACADM* gene. This polymorphic silent variation, in our experience, is normally observed in linkage with other polymorphic variations, including intron 1 (IVS1-32G), intron 3 (IVS3+10C), intron 5 (IVS5+32G), and intron 6 (IVS6-22A). Patient C4 was apparently homozygous for polymorphisms normally only seen with the c.985A>G haplotype, which included intron 1 (IVS1-32C), intron 3 (IVS3+10T), intron 5 (IVS5+32C), and intron 6 (IVS6-22C). This suggested the presence of a deletion spanning at least intron 1 to intron 6 in C4 c.1161G allele. The analysis found the mother (B6) to be heterozygous for the c.985A>G mutation. The analysis in the father (B5) found him to be heterozygous for the polymorphic silent variation c.1161 A>G in exon 11, but homozygous for the polymorphisms, which are usually not linked to c.1161A>G. This indicates that he also may have a deletion affecting exons 1–6 of the *ACADM* gene (Fig. 2). Targeted array-based comparative genomic hybridization (aCGH) with exon-level resolution (ExonArrayDx) was then employed to establish if such a deletion was present in the father. It confirmed the presence of an intragenic deletion encompassing at least exons 1–6 of the *ACADM* gene would have knocked out standard primer sites used in routine PCR-based sequencing in at least exons 2–5. The array contained multiple oligonucleotide probes in all exons and/or their flanking regions in the *ACADM* gene. Hybridization data was analyzed with Genomic Workbench v5 software (Agilent Technologies) to evaluate the copy number at the exon level. The Exon Array was designed to detect most single exon deletions and duplications. This result was confirmed by repeat analysis. Multiplex ligation-dependent probe amplification (MLPA) analysis with probes that test for deletions that span at least exons 2–4 was then employed to confirm the presence of the deletion in the father (B5), B4, C2, and C3. Testing by means of MLPA is now available on request through the Leeds Cytogenetics Laboratory in the UK.

Now that this deletion has been identified, several family members have come forward for carrier testing which was not possible previously.

Discussion

Larger deletions (20bp or more) have only previously been reported twice in *ACADM* (Morris et al. 1995; Maegawa et al. 2008), but these may be a more frequent cause of MCAD deficiency than hitherto believed, due to fact that these are not anticipated, and therefore, the routine diagnostic techniques used will not identify them. Arnold et al. reported on the mutational spectrum in newborns identified by routine screening for MCAD deficiency in New York (Arnold et al. 2010). They reported one case with a similar deletion to the one found in our family being a large deletion of exons 1–6 and heterozygosity for the c.985A>G mutation in the other allele. This patient demonstrated profound fasting intolerance in the first years of life, requiring gastrostomy feeding for several years. They also reported 7 cases out of 23 cases where the second mutation in the *ACADM* gene was unknown. We, therefore, speculate that some of these unknown second mutations could be due to deletions not usually detected by use of a service genetic laboratory. Another study of the mutational spectrum in newborns with MCAD deficiency (Kennedy et al. 2010) also reported a high proportion (5/25) of individuals with MCAD deficiency, who have an unknown second mutation, whereas a recent study from Denmark (Andresen et al. 2012) did not find such high proportions of individuals with MCAD deficiency who have an unknown second mutation. This may be due to differences in the mutational spectrum in the investigated populations.

Conclusion

This finding represents a useful learning point in the management of families with MCAD deficiency. It highlights that we should be routinely looking for larger deletions, when only one of the mutations can be identified on standard sequencing, in patients where we have high clinical suspicion or biochemical evidence of MCAD deficiency.

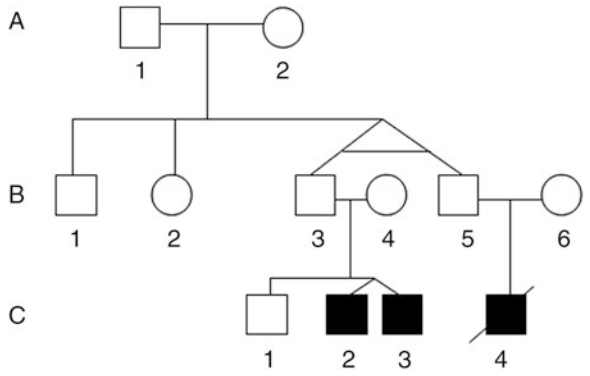

Fig. 1 Pedigree. Family members affected by MCAD deficiency are in *black*

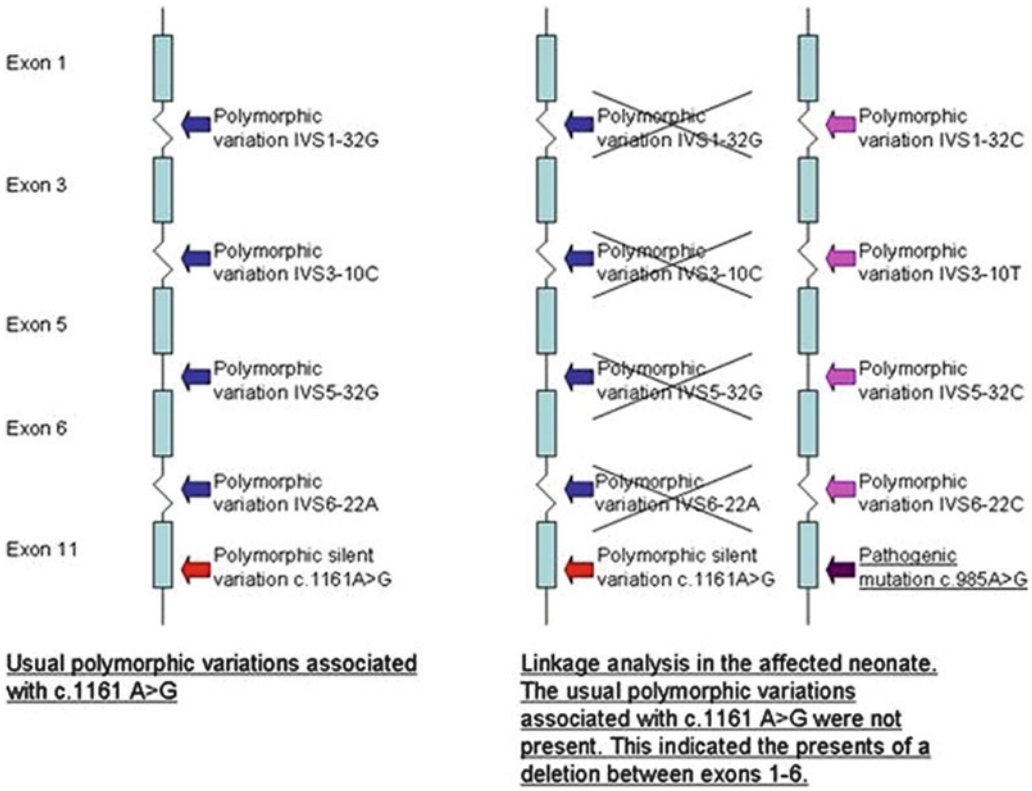

Fig. 2 Linkage analysis of the *ACADM* gene

Acknowledgements We would like to thank the family involved in this article.

Take-Home Message

Large deletions in *ACADM* can cause of MCAD deficiency, and routine diagnostic techniques in common use will not identify them.

Details of the Contributions of Individual Authors

1. Claire Searle – Co-wrote the article, collated the information about the family and test results, and met with several family members.
2. Prof Brage Storstein Andresen – Gave very valuable advice pertaining to the family, performed the linkage analysis, and co-wrote the article.
3. Prof Ed Wraith – Headed up clinical care for a number of affected family members and proofread the article.
4. Jamie Higgs GeneDx, performed the array CGH analysis of the *ACADM* gene and proofread the article.
5. Deborah Gray – Performed the MLPA analysis and proofread the article.
6. Alison Mills – Designed the MLPA primers and proofread the article.

7. K. Elizabeth Allen – Reported the initial sequencing work of the *ACADM* gene and proofread the article.
8. Emma Hobson – Identified and counselled all family members involved, collated the clinical information, instigated testing to confirm the presence of the deletion and co-wrote the article.

Guarantor for the Article

Claire Searle

Conflict of Interests Statement

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

Ethics Approval

No ethics approval was required for this case report.

Patient Consent Statement

No patient-identifiable data has been included in this article.

References

Andresen BS, Dobrowolski SF, O'Reilly L et al (2001) Medium-chain acyl-CoA dehydrogenase (MCAD) mutations identified by MS/MS-based prospective screening of newborns differ from those observed in patients with clinical symptoms: identification and characterization of a new prevalent mutation that results in mild MCAD deficiency. Am J Hum Genet 68:1408–1418

Andresen BS, Bross P, Jensen TG et al (1993) Am J Hum Genet 53:730–739

Andresen BS, Lund AM, Hougaard DM et al (2012) MCAD deficiency in Denmark. Mol Genet Metab 106:175–188

Arnold GL, Saavedra-Matiz CA, Galvin-Parton PA et al (2010) Lack of genotype-phenotype correlations and outcome in MCAD deficiency diagnosed by newborn screening in New York State. Mol Genet Metab 99:263–268

Kennedy S, Potter BK, Wilson K et al (2010) The first three years of screening for medium chain acyl-CoA dehydrogenase deficiency (MCADD) by newborn screening in Ontario. BMC Pediatr 10:82

Maegawa GHB, Poplawski N, Andresen BS et al (2008) Interstitial deletion of 1p22.2-p31.1 and medium-chain acyl-CoA dehydrogenase deficiency in a patient with developmental delay. Am J Med Genet A 146A(12):1581–1586

Maier EM, Liebl B, Roschinger W et al (2005) Population spectrum of ACADM genotypes correlated to biochemical phenotypes in newborn screening for medium-chain acyl-CoA dehydrogenase deficiency. Hum Mutat 25:443–452

Morris AAM, Taylor RW, Lightowlers RN et al (1995) Medium-chain acyl-CoA dehydrogenase deficiency caused by a deletion of exons 11 and 12. Hum Mol Genet 4:747–749

JIMD Reports
DOI 10.1007/8904_2013_217

CASE REPORT

Infantile Hypophosphatasia Secondary to a Novel Compound Heterozygous Mutation Presenting with Pyridoxine-Responsive Seizures

Dina Belachew • Traci Kazmerski • Ingrid Libman • Amy C. Goldstein • Susan T. Stevens • Stephanie DeWard • Jerry Vockley • Mark A. Sperling • Arcangela L. Balest

Received: 12 October 2012 / Revised: 31 January 2013 / Accepted: 07 February 2013 / Published online: 12 March 2013

Abstract Hypophosphatasia (HPP) is a rare metabolic disease with the hallmark finding of deficient serum tissue nonspecific alkaline phosphatase (TNSALP) activity. TNSALP is primarily known for its role in mineralization; hence, HPP is characterized by defective mineralization of bone and/or teeth. TNSALP is also necessary for proper vitamin B6 metabolism and its participation as a cofactor for neurotransmitters in the central nervous system. Defective TNSALP activity in the brain can result in intractable seizures responsive to pyridoxine. The pathophysiology of pyridoxine-responsive seizures (PRS) in severe HPP remains to be clearly defined. We review the case of a 2-month-old Caucasian boy presenting with seizures refractory to conventional antiepileptic medications. Empiric treatment with favorable response to pyridoxine in conjunction with severe metabolic bone disease, extremely low serum alkaline phosphatase, elevated phosphoethanolamine, hypercalcemia, hypercalciuria, and nephrocalcinosis led to a clinical diagnosis of infantile HPP. Sequence analysis revealed compound heterozygosity of the TNSALP gene with a novel mutation in exon 9 and a previously reported mutation in exon 12. This case reminds the physician that severe infantile HPP can present with PRS as its major initial manifestation and should alert clinicians to consider HPP in their differential of PRS. In addition, despite this severe genotype, the clinical diagnosis of our patient was delayed because of minimal phenotypic features initially. This highlights that the phenotype-genotype correlation could be variable even in severe disease. This case also demonstrates that HPP should be classified as PRS and not a form of pyridoxine-dependent epilepsy (PDE) as our patient was able to stop the pyridoxine supplementation without seizure recurrence once enzyme replacement was initiated. With the advent of enzyme replacement therapy, this once fatal disease may have improved morbidity and mortality.

Communicated by: K. Michael Gibson

Competing interests: None declared

D. Belachew
Department of Pediatric Endocrinology, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

T. Kazmerski
Department of Pediatrics, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

I. Libman
Department of Pediatric Endocrinology, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

A.C. Goldstein
Department of Pediatric Neurology, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

S.T. Stevens
Kids Plus Pediatrics, Pittsburgh, PA

S. DeWard
Department of Medical Genetics, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

J. Vockley
University of Pittsburgh, School of Medicine, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

M.A. Sperling
Department of Pediatric Endocrinology, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh of UPMC Pittsburgh, USA

A.L. Balest (✉)
Department of Pediatrics, Children's Hospital of Pittsburgh of UPMC, 4401 Penn Avenue, Pittsburgh, PA 15224
e-mail: Arcangela.Balest@chp.edu

Abbreviations

ALP	Alkaline phosphatase
CSF	Cerebrospinal fluid
CT	Computerized tomography
EEG	Electroencephalogram
HPP	Hypophosphatasia
HSV	Herpes simplex virus
MRI	Magnetic resonance imaging
PCR	Polymerase chain reaction
PLP	Pyridoxal-5′-phosphate
PRS	Pyridoxine-responsive seizures
PTH	Parathyroid hormone
TNSALP	Tissue nonspecific alkaline phosphatase

Introduction

Hypophosphatasia (HPP) is a rare metabolic disease defined by a deficiency of serum tissue nonspecific alkaline phosphatase (TNSALP). It was first described in 1948 (Rathbun 1948) and has a variable clinical presentation. Seven forms have been reported based primarily on the age at which skeletal lesions are discovered (Whyte 2012).

- Perinatal – usually with clinically apparent skeletal deformities and pathognomonic radiographic changes with rapidly progressive clinical course and early death
- Benign prenatal – gradual improvement of bone disease after birth
- Infantile – symptoms similar to, but typically less severe, than perinatal form and recognized before 6 months of age
- Childhood – diagnosed after 6 months of age, predominantly skeletal manifestations and premature deciduous tooth loss
- Adult – osteopenia, recurrent fractures, and pseudo-fractures with early loss of adult dentition common
- Odontohypophosphatasia – isolated dental manifestations
- Pseudohypophosphatasia – clinical findings similar to infantile HPP but with unremarkable ALP levels

The incidence of HPP has been estimated at 1 per 100,000 births in Canada where HPP was first described (Fraser 1957). In France, infantile and lethal forms have been reported to be less common (1/250,000 births), while milder adult disease is more frequent (approximately 1/6,000 individuals) (Mornet et al. 2011). No race or sex predilection exists, but a Canadian Mennonite isolate has been described with a 1:2,500 incidence (Greenberg et al. 1993). Molecular testing has proven the defect in HPP occurs in the TNSALP gene on chromosome 1p36.1-p34 (Greenberg et al. 1990), with more severe forms having an autosomal recessive inheritance pattern (Mornet 2008). Milder expressions of disease may be autosomal dominant (Moore et al. 1999).

Alkaline phosphatase (ALP) is a dephosphorylating enzyme found throughout the body. Tissue-specific ALPs are encoded by individual genes and are found in the intestines, placenta, and germ cells (McComb et al. 1979). TNSALP is encoded by a single gene and ubiquitously expressed (Harris 1990). It is primarily known for its role in bone growth by providing inorganic phosphate for hydroxyapatite crystal production (Robison 1923) and by hydrolyzing inorganic pyrophosphate (Whyte 2010; Moss et al. 1967) which is an inhibitor of bone mineralization (Fleisch et al. 1966). TNSALP is also involved in regulation of neurotransmission in the cerebral cortex (Négyessy et al. 2011; Spentchian et al. 2003; Whyte 2010). More than 200 mutations in the TNSALP gene have been described, 80% of these being missense mutations (Mornet 2008). A recurrent point mutation has been described in the Japanese population (Watanabe et al. 2011). Large deletions are rare (The Tissue Nonspecific Alkaline Phosphatase Gene Mutations Database 2011).

In severe HPP, defective TNSALP activity in the cerebral cortex has been associated with "pyroxidine-responsive seizures" (PRS) (Baumgartner-Sigl et al. 2007). TNSALP is necessary for the conversion of pyridoxal 5′ phosphate (PLP) to pyridoxal in order to cross the blood–brain barrier. Pyridoxal is then converted back to PLP, where it is a cofactor for over 140 enzymatic reactions, including regulators of GABA synthesis (Gospe 2010). GABA is an inhibitory neurotransmitter, and when reduced, the unopposed excitatory neurotransmitters lead to seizure activity (Plecko and Stöckler 2009; Stockler et al. 2011; Plecko et al. 2007; Smilari et al. 2005; Nunes et al. 2002; Balasubramaniam et al. 2010; Narisawa et al. 2001). The differential diagnosis of PRS includes those that are pyridoxine dependent, such as antiquitin deficiency due to mutations in ALDH7A1 and folinic acid-responsive seizures which are also caused by antiquitin deficiency. The category of pyridoxine-dependent epilepsy (PDE) is reserved for those conditions where a biochemical or molecular defect has been confirmed and removal of pyridoxine leads to return of seizure activity (Baxter 1999, Basura et al. 2009). The moniker PRS is applied when clinical seizures do not recur after the withdrawal of pyridoxine, indicating that the seizure disorder is not dependent upon the vitamin (Basura et al. 2009). Mutational analysis is available for ALDH7A1, and biochemical testing includes the accumulation of α-aminoadipic semialdehyde (AASA), piperideine-6-carboxylate (P6C), and pipecolic acid, which serve as diagnostic markers in urine, plasma, and CSF. Other conditions which cause pyridoxine-responsive seizures include hypophosphatasia, familial hyperphosphatasia (PIGV deficiency), and nutritional vitamin B deficiency

(Plecko and Stöckler 2009). Pyridoxal phosphate-dependent seizures result from a deficiency of pyridox(am)ine 5′-phosphate oxidase (PNPO) which is necessary for the conversion of pyridoxine and pyridoxamine to PLP (Mills et al. 2005; Hoffmann et al. 2007). PNPO is not required for the production of PLP from dietary pyridoxal or PLP. Patients with PNPO mutations are responsive to pyridoxal phosphate but not to pyridoxine, and for this reason, as well clinical and biochemical differences, these patients are not classified as having PRS or PDE.

HPP is suggested on the basis of low/absent serum ALP, a constellation of clinical findings as noted previously and is supported by molecular testing of the TNSALP gene. Substrates of ALP are elevated and can be measured in the urine [phosphoethanolamine (PEA)] or serum [inorganic pyrophosphate (PPi) and pyridoxal-5′-phosphate (PLP)]. As previously noted, pyridoxine has been part of the treatment regimen in some children with seizures as a manifestation of HPP. We will discuss below these treatment options and how the advent of these treatments may change our consideration of HPP as a pyridoxine "responsive" rather than pyridoxine "dependent" entity.

Defective mineralization results in rickets and may lead to respiratory compromise and recurrent lung infections due to altered chest wall mechanics of the weakened thoracic cage in those with the most severe disease. Hypomineralized skull bones may be prone to premature fusion of the cranial sutures via an unknown mechanism. Craniosynostosis can lead to increased intracranial pressure and requires close monitoring (Nunes et al. 2002; Béthenod et al. 1967). HPP has also been found to be associated with increased intracranial pressure due to pseudotumor cerebri (Demirbilek et al. 2012). Varying degrees of hypercalcemia are present and may be accompanied by hypercalciuria and resultant nephrocalcinosis. Poor feeding and hypotonia are attributed to hypercalcemia. In severe disease, clinical features of small thorax, limb deformities, and blue sclera are seen.

Patient Presentation

Our patient was born at 35 weeks gestation to a 29-year-old G5P2→3 HSV-positive, previously opioid-dependent mother with a history of buprenorphine/naloxone use during pregnancy. All other serologies were negative. She received routine prenatal care and valacyclovir therapy during gestation. The antenatal course was complicated by preterm contractions beginning at 22 weeks which were treated with bed rest at home and oral terbutaline. Three prenatal ultrasounds were done with no noted fetal abnormalities. Delivery was spontaneous vaginal vertex. APGAR scores at 1 and 5 min were 9 and 10, respectively. Birth weight was 2,585 g (25th percentile), length 45.5 cm (25th–50th percentile), and head circumference 31 cm (5th–10th percentile). Physical examination was normal with no dysmorphic features. Father is of Caucasian-Japanese origin and mother is Caucasian.

At 18 h of life, the patient began to exhibit facial grimacing, flexion of upper extremities, and extension of the lower extremities lasting up to 5 min occurring multiple times a day. Complete blood count and serum electrolytes were normal, including serum calcium and magnesium levels (9.1 and 1.9 mg/dl, respectively). Serum phosphorous was just below the lower limit of normal (5.4 mg/dl, normal range 5.5–9.5 mg/dl). Blood, urine, and cerebrospinal fluid (CSF) cultures were negative. HSV PCR was negative from the CSF. Electroencephalogram (EEG) revealed multifocal sharp waves and mild discontinuity, but was without electrographic seizure activity. Head computerized tomography (CT) and brain magnetic resonance imaging (MRI) were unremarkable. Given maternal opioid use, neonatal abstinence syndrome was suspected and morphine therapy was instituted. He was then transferred to a tertiary care facility. Despite the EEG findings, the clinical suspicion for seizure was still high. These episodes persisted, and 5 mg/kg/day of phenobarbital was initiated. He was discharged at 4 weeks of life when he seemed to be free of further presumed seizures. Once home, he began to have 10–15 clusters of tonic activity, each lasting a few seconds, every two to three days. At 6 weeks of life, he was seen in the outpatient neurology clinic and was admitted for a 24-h video EEG, which showed an electroclinical seizure of right central onset, correlating with left arm jerking. Phenobarbital dose was increased to 7.5 mg/kg/day and he was discharged.

Within 2 weeks, on day of life 63, he presented in status epilepticus. Vitamin-responsive epilepsies were considered and empiric treatment was initiated with 100 mg IV pyridoxine followed by oral pyridoxine (25 mg/kg/day), pyridoxal-5-phosphate (30 mg/kg/day), and folinic acid (1 mg/kg/day) with resolution of seizures. Subsequently, poor feeding, hypercalcemia, and hypercalciuria were noted. Serum calcium was 11.2–12.3 mg/dl (normal range 8.8–10.8 mg/dl) and serum-ionized calcium 1.4 and 1.6 mmol/l (normal range 1.0–1.4 mmol/l). Serum phosphorous was normal. Parathyroid hormone (PTH) was appropriately suppressed for the degree of hypercalcemia at <3 pg/ml (normal range 10–65 pg/ml with a serum calcium in the normal range). Serum total 25-hydroxy vitamin D levels were normal. Renal ultrasound showed bilateral medullary nephrocalcinosis (Fig. 1). Skeletal radiography had changes typical of infantile hypophosphatasia: significant osteopenia of metaphyses with areas of fragmentation of the distal femurs; focal metaphyseal defects in the humeri; and cupping of the distal tibia,

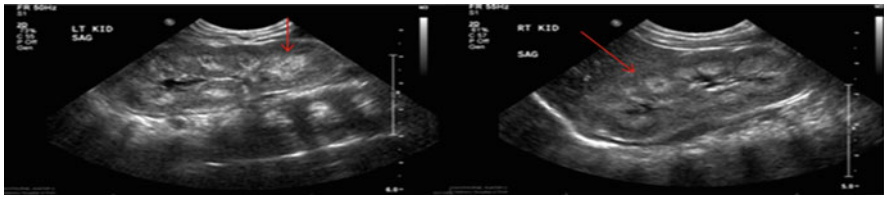

Fig. 1 Renal ultrasound showing bilateral medullary nephrocalcinosis at age 2 months (indicated by *red arrows*)

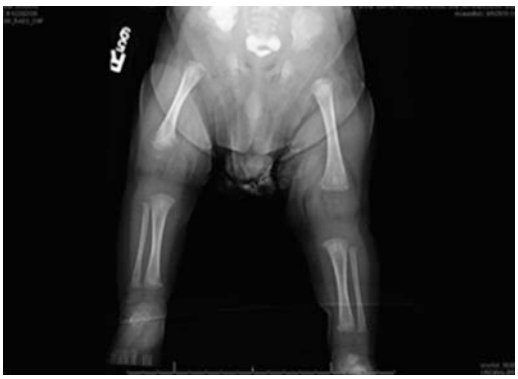

Fig. 2 Radiograph showing bilateral femoral metaphyseal defects and short right femur with slight curvature at age 2 months

radius, and ulna (Fig. 2). ALP was undetectable (<5 IU/l). On review of old records, ALP was undetectable on the first day of life but had gone unnoticed. In addition, demineralized areas of the skull were retrospectively noted on the bone window of the CT scan obtained at birth with marked worsening over 2 months (Figs. 3, 4).

Infantile HPP was therefore suspected. Supportive laboratory data showed elevated urine phosphoethanolamine (PEA) (9.31 mM/g creatinine; nl: <0.8 mM/g creatinine) and significantly elevated CSF PLP (1999 nmol/l; nl: 30–80) (Dr. Keith Hyland, Medical Neurogenetics, Atlanta, GA). Other forms of PRS and PDE were excluded based on normal testing of CSF neurotransmitters and serum pipecolic acid. PLP deficiency due to PNPO mutations would have shown increased CSF L-DOPA and decreased CSF homovanillic acid and 5-hydroxyindoleacetic acid (Gospe 2010). Serum pipecolic acid is elevated in PDE from antiquitin deficiency.

Definitive diagnosis of HPP was made by sequence analysis of the TNSALP gene (performed at Connective Tissue Gene Tests, Allentown, PA), which revealed compound heterozygosity for a novel mutation in exon 9, c.875_881delCAGGGGAinsT, and a previously reported mutation in exon 12, c.1559delT (Orimo et al. 1994).

Discussion

Our patient highlights the wide variability in clinical presentation of HPP. His presentation was dominated by intractable seizures and many of the common overt phenotypic features of HPP were absent. Given maternal history and clinical findings, the initial diagnosis of neonatal abstinence syndrome (NAS) was made. Treatment with morphine was instituted at appropriate dosing which should have extinguished the seizures, had they been due to NAS. Withdrawal symptoms from buprenorphine often manifest later in neonates than withdrawal from opiates or other opiate agonists such as methadone (Gaalema et al. 2012). The lack of response to appropriate opioid therapy and atypical timing of withdrawal from buprenorphine were indicators that this patient's presentation was not due to NAS.

Neonatal HSV meningitis was also considered until ruled out by evaluation. When seizures became refractory to anticonvulsant therapy, empiric therapy for vitamin-responsive epilepsies and systematic evaluation identified PRS. Subsequently, hypercalcemia, hypercalciuria, and diffuse metabolic bone disease were noted and HPP was suspected. It is important to appreciate that while our patient's initial presentation was apparently isolated PRS, an undetectable level of ALP recorded at the first hospitalization went unnoticed, delaying arrival at the correct diagnosis. In addition, this case highlights the importance of considering vitamin-responsive epilepsies in newborns with refractory seizures and empirically treating with a combination of pyridoxine, pyridoxal-5-phosphate, and folinic acid (Gospe 2010).

Our patient strongly resembles others reported in the literature, including a similar case by Baumgartner-Sigl et al. (2007). Their patient also presented first with PRS in

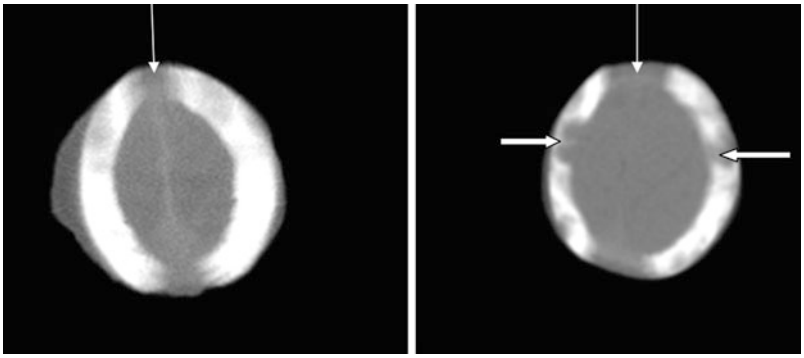

Fig. 3 Bone window of computerized tomography (CT) scans of the head show progressive widening of the sutures (*thin arrows*) and calvarial bone destruction (*thick arrows*). *Left*: first day of life. *Right*: age 2 months

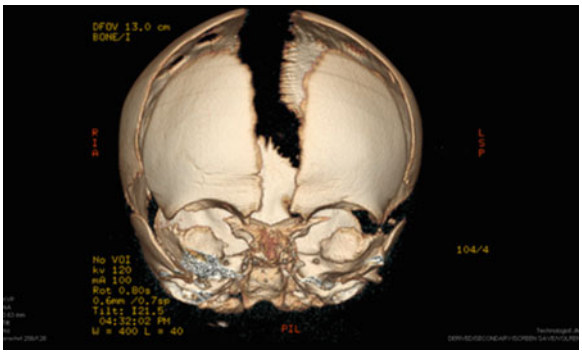

Fig. 4 Reconstructive head CT showing widened sutures due to poor mineralization at age 2 months

the neonatal period, followed later by skeletal demineralization, hypercalcemia, hypercalciuria, and nephrocalcinosis and died before one year. They suggested that the presence of PRS in HPP was a poor prognostic sign and encouraged the measurement of ALP activity in the evaluation of neonatal seizures. Litmanovitz et al. (2002) also reported a patient with pyridoxine-responsive neonatal seizures, a nearly nondetectable serum ALP and skeletal hypomineralization with early demise due to pneumonia.

Our patient was found to have a novel mutation of the TNSALP gene on exon 9 involving a large deletion predicted to lead to complete lack of protein production. The second mutation on exon 12 has previously been described in Japanese patients with severe infantile HPP (Orimo et al. 1994) and is consistent with his father's ethnicity. These two mutations confer severe disease ultimately observed in our patient. However, a delay in the development of overt phenotypic features of HPP implies that phenotype-genotype correlation could be variable even in severe disease. Ultimately, the patient manifested findings typically associated with HPP, including hypotonia, poor feeding, craniosynostosis, limb shortening, and several episodes of respiratory failure associated with viral illnesses.

Infantile HPP carries a mortality rate of perhaps 50% which further increases in the presence of PRS (Fraser 1957). PRS in general has a good prognosis. Prior to the introduction of new treatment options for HPP, which will be discussed below, PRS had been associated with increased disease severity of HPP (Baumgartner-Sigl et al. 2007; Litmanovitz et al. 2002).

Variability in severity of disease in patients with HPP is not easily explained. The mutant TNSALP enzymes produced have variable and possibly preferential catalytic activity of substrates, inorganic pyrophosphate, and PLP (Di Mauro et al. 2002). The mutant enzymes are also often found as heterodimers which have variable catalytic activity depending on the combination of monomers composing that particular heterodimeric enzyme (Di Mauro et al. 2002; Zhanhua et al. 2005). These characteristics of TNSALP produced in those with mutations of the TNSALP gene help explain some of the phenotypic variability of disease in HPP. Mouse models of TNSALP deficiency demonstrate a similar seizure phenotype due to defective metabolism of PLP, which can be rescued with pyridoxal (Waymire et al. 1995). Knockout mice have elucidated the role of TNSALP in the central nervous system. TNSALP not only has a role in GABA neurotransmission but was found in the synaptic cleft of primary sensory areas (Fonta et al. 2004).

Several potential therapies to treat HPP have been explored. In 1956, treatment with cortisone showed limited

success (Fraser and Laidlaw 1956). Early enzyme replacement with infusion of ALP-rich plasma from patients with Paget's disease was attempted in the 1980s (Whyte et al. 1982). Calcitonin and chlorothiazide have been used to treat hypercalcemia and hypercalciuria (Barcia et al. 1997). Allogenic mesenchymal stem cell, donor bone fragment, and isolated osteoblast transplantations have been attempted (Tadokoro et al. 2010; Cahill et al. 2007). In adults, teriparatide, a recombinant parathyroid hormone, has had limited success (Gagnon et al. 2010). Adeno-associated virus-8 vectors expressing TNSALP have been used to rescue mice with HPP (Matsumoto et al. 2011). Similarly, treatment using lentiviral vectors expressing a bone-targeted form of TNSALP has led to improved survival in mice with HPP (Yamamoto et al. 2011).

Most recently, enzyme replacement therapy for HPP has been explored with success in animal models and human clinical trials (MillÄn et al. 2008; McKee et al. 2011; Whyte et al. 2012). This investigational therapy, asfotase alfa (Alexion Pharmaceuticals), a bone-targeted, recombinant TNSALP, has recently been reported to improve survival and clinical outcome in HPP (Whyte et al. 2010). The results of the open-labeled study on 11 patients was recently reported showing improvement in rickets on skeletal radiographs, as well as improved pulmonary function and motor milestones in life-threatening hypophosphatasia (Whyte et al. 2012).

Our patient was started on asfotase alfa at nearly 5 months of age and remains alive at 31 months of age. Once his molecular diagnosis was confirmed and specific treatment with enzyme replacement therapy was made available, our patient was able to wean not only anticonvulsant therapy, but also pyridoxine supplementation, without the return of his seizures. Thus, HPP is truly a pyridoxine-responsive (but not pyridoxine-dependent) form of seizures. Further details on this therapy and long-term outcomes will be reported.

In conclusion, this case illustrates that careful scrutiny of laboratory data and continual revision of differential diagnosis is crucial in reaching the diagnosis of rare entities such as HPP. HPP must be considered in the differential diagnosis of any patient presenting with PRS, and alkaline phosphatase level must be measured. Therapy requires a multidisciplinary approach addressing feeding disorders, respiratory compromise, craniosynostosis and increased intracranial pressure, hypercalcemia, and developmental delay. Close follow-up is critical.

Acknowledgements The authors would like to thank Dr. Keith Hyland at Medical Neurogenetics, Atlanta, Georgia, for processing the CSF samples for neurotransmitters and PLP level.

Synopsis

This case reminds the physician that severe infantile HPP can present with PRS as its major initial manifestation and should alert clinicians to consider HPP in their differential of PRS.

Conflict of Interest

Drs. Belachew, Kazmerski, Libman, Goldstein, Stevens, Sperling, Balest and Ms. DeWard have no financial disclosures. Dr. Vockley discloses research support from Alexion Pharmaceuticals.

The authors attest that this is an original manuscript, and it has never been published. At this time, this manuscript is being submitted only to Journal of Inherited Metabolic Disease Reports and will not be submitted elsewhere while under consideration by this journal.

Contributor's Statement Page

Dr. Belachew drafted the initial draft of this case report and has made a substantial contribution towards the content, outline, background, discussion, revision, editing, and finalization of this manuscript. Dr. Kazmerski has made a substantial contribution to the content, revision, editing, and finalization of this manuscript. Dr. Libman has made a substantial contribution to the diagnosis of this case, revision and editing of this manuscript. Dr. Goldstein has contributed significantly to the neurological aspect of the manuscript content and to revision and editing of this manuscript. Drs. Vockley and Stevens and Ms. Deward have contributed to the genetic discussion of this manuscript and revision and editing. Dr. Sperling has contributed to the background, content, and editing of this manuscript. Dr. Balest has made substantial contributions to the background, revision, editing, and finalization of this manuscript. All of the authors have reviewed this manuscript and given final approval for its submission for publication.

References

Balasubramaniam S, Bowling F, Carpenter K et al (2010) Perinatal hypophosphatasia presenting as neonatal epileptic encephalopathy with abnormal neurotransmitter metabolism secondary to reduced co-factor pyridoxal-5'-phosphate availability. J Inherit Metab Dis. 2010 January 5

Barcia JP, Strife CF, Langman CB (1997) Infantile hypophosphatasia: treatment options to control hypercalcemia, hypercalciuria, and chronic bone demineralization. J Pediatr 130(5):825–828

Basura GJ, Hagland SP, Wiltse AM, Gospe SM Jr (2009) Clinical features and the management of pyridoxine-dependent and pyridoxine-responsive seizures: review of 63 North American cases submitted to a patient registry. Eur J Pediatr 168 (6):697–704

Baumgartner-Sigl S, Haberlandt E, Mumm S et al (2007) Pyridoxine-responsive seizures as the first symptom of infantile hypophosphatasia caused by two novel missense mutations (c.677T>C, p.M226T; c.1112C>T, p.T371I) of the tissue-nonspecific alkaline phosphatase gene. Bone 40(6):1655–1661

Baxter P (1999) Epidemiology of pyridoxine dependent and pyridoxine responsive seizures in the UK. Arch Dis Child 81:431–433

Béthenod M, Cotte MF, Collombel C, Fréderich A, Cotte J (1967) Neonatal discovery of hypophosphatasia. Bone improvement. Fatal convulsant encephalopathy. Ann Pediatr (Paris) 14(12):835–4

Cahill RA, Wenkert D, Perlman SA et al (2007) Infantile hypophosphatasia: transplantation therapy trail using bone fragments and cultured osteoblasts. J Clin Endocrinol Metab 92(8):2923–2930

Demirbilek H, Alanay Y, Alikaşifoğlu A et al (2012) Hypophosphatasia presenting with pyridoxine-responsive seizures, hypercalcemia, and pseudotumor cerebri: case report. J Clin Res Pediatr Endocrinol 4(1):34–38

Di Mauro S, Manes T, Hessle L et al (2002) Kinetic characterization of hypophosphatasia mutations with physiological substrates. J Bone Miner Res 17(8):1383–1391

Fleisch H, Russell RG, Straumann F (1966) Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature 212:901–903

Fonta C, Négyessy L, Renaud L, Barone P (2004) Areal and subcellular localization of the ubiquitous alkaline phosphatase in the primate cerebral cortex: evidence for a role in neurotransmission. Cereb Cortex 14(6):595–609, Epub 2004 Mar 28

Fraser D (1957) Hypophosphatasia. Am J Med 22:730–746

Fraser D, Laidlaw J (1956) Treatment of hypophosphatasia with cortisone. Lancet 270(6922):553

Gaalema D, Scott T, Heil S et al (2012) Differences in the profile of neonatal abstinence syndrome signs in methadone- versus buprenorphine-exposed neonates. Addiction 107(S1):53–62

Gagnon C, Sima NA, Mumm S et al (2010) Lack of sustained response to teriparatide in a patient with adult hypophosphatasia. J Clin Endocrinol Metab 95(3):1007–1012

Gospe SM Jr (2010) Neonatal vitamin responsive epileptic encephalopathies. Chang Gung Med J 33:1–12

Greenberg CR, Evans JA, McKendry-Smith S et al (1990) Infantile hypophosphatasia localization within chromosome region 1p36.1.1-34 and prenatal diagnosis using linked DNA markers. Am J Hum Genet 46:286–292

Greenberg CR, Taylor CL, Haworth JC et al (1993) A homoallelic Gly317Asp mutation in ALPL causes the perinatal (lethal) form of hypophosphatasia in Canadian mennonites. Genomics 17 (1):215–217

Harris H (1990) The human alkaline phosphatases: what we know and what we don't know. Clin Chim Acta 186:133–150

Hoffmann GF, Schmitt B, Windfuhr M et al (2007) Pyridoxal 5'-phosphate may be curative in early-onset epileptic encephalopathy. J Inherit Metab Dis 30(1):96–99

Litmanovitz RO, Dolfin T et al (2002) Glu274Lys/Gly309Arg mutation of the tissue-nonspecific alkaline phosphatase gene in neonatal hypophosphatasia associated with convulsions. J Inherit Metab Dis 25(1):35–40

Matsumoto T, Miyake K, Yamamoto S et al (2011) Rescue of severe infantile hypophosphatasia mice by AAV-mediated sustained expression of soluble alkaline phosphatase. Human Gene Ther 22(11):1355–1364

McComb RB, Bowers GN, Posen S (1979) Alkaline phosphatase. Plenum Press, New York

McKee MD, Nakano Y, Masica DL, Gray JJ, Lemire I, Heft R (2011) Enzyme replacement therapy prevents dental defects in a model of hypophosphatasia. J Dent Res 90(4):470–476

MillÄn JL, Narisawa S, Lemire I et al (2008) Enzyme replacement therapy for murine hypophosphatasia. J Bone Miner Res 23 (6):777–787

Mills PB, Surtees RAH, Champion MP et al (2005) Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox(am)ine 5'-phosphate oxidase. Hum Mol Genet 14 (8):1077–1086

Moore CM, Curry C, Henthorn PS et al (1999) Mild autosomal dominant hypophosphatasia: in utero presentation in two families. Am J Med Genetics 86(5):410–415

Mornet E (2008) Hypophosphatasia. Best Pract Res Clin Rheumatol 22:113–127

Mornet E, Yvard A, Taillandier A, Fauvert D, Simon-Bouv B (2011) A molecular-based estimation of the prevalence of hypophosphatasia in the European population. Ann Hum Genet 75 (3):1469–1809

Moss DW, Eaton RH, Smith JK, Whitby LG (1967) Association of inorganic-pyrophosphatase activity with human alkaline-phosphatase preparations. Biochem J 102:53–57

Narisawa S, Wennberg C, MillÄn JL (2001) Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. J Pathol 193(1):125–133

Négyessy L, Xiao J, KÄntor O et al (2011) Layer-specific activity of tissue non-specific alkaline phosphatase in the human neocortex. Neuroscience 172:406–418

Nunes ML, Mugnol F, Bica I, Fiori RM (2002) Pyridoxine-dependent seizures associated with hypophosphatasia in a newborn. J Child Neurol 17(3):222–224

Orimo H, Hayashi Z, Watanabe A, Hirayama T, Hirayama T, Shimada T (1994) Novel missense and frameshift mutations in the tissue-nonspecific alkaline phosphatase gene in a Japanese patient with hypophosphatasia. Hum Mol Genet 3(9):1683–1684

Plecko B, Stöckler S (2009) Vitamin B6 dependent seizures. Can J Neurol Sci 36(Suppl 2):S73–S77

Plecko B, Paul K, Paschke E et al (2007) Biochemical and molecular characterization of 18 patients with pyridoxine-dependent epilepsy and mutations of the antiquitin (ALDH7A1) gene. Hum Mutat 28(1):19–26

Rathbun JC (1948) Hypophosphatasia: a new developmental anomaly. Am J Dis Child 75(6):822–834

Robison R (1923) The possible significance of hexosephosphoric esters in ossification. Biochem J 17:286–293

Smilari P, Romeo DM, Palazzo P, Meli C, Sorge G (2005) Neonatal hypophosphatasia and seizures. A case report. Minerva Pediatr 57(5):319–323

Spentchian M, Merrien Y, Herasse M et al (2003) Severe hypophosphatasia: characterization of fifteen novel mutations in the ALPL gene. Hum Mutat 22(1):105–106

Stockler S, Plecko B, Gospe SM Jr et al (2011) Pyridoxine dependent epilepsy and antiquitin. Clinical and molecular characteristics and recommendations for diagnosis, treatment and follow-up. Mol Genet Metab 104(1–2):48–60

Tadokoro M, Machida H, Ohgushi H (2010) Genetic basis for skeletal disease: stem cell therapy for genetic bone disorders. Clin Calcium 20(8):1228–1235

The Tissue Nonspecific Alkaline Phosphatase Gene Mutations Database, last updated July 2011. Retrieved 2012, available from: http://www.sesep.uvsq.fr/03_hypo_mutations.php#presentation

Watanabe A, Karasugi T, Sawai H et al (2011) Prevalance of c.1559delT in ALPL, a common mutation resulting in the perinatal (lethal) of hypophosphatasia in Japanese and effects of the mutation on heterozygous carriers. J Hum Genet 56(2):166–168

Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, MacGregor GR (1995) Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat Genet 11(1):45–51

Whyte MP (2010) Physiological role of alkaline phosphatase explored in hypophosphatasia. Ann N Y Acad Sci 1192:190–200

Whyte MP (2012) Hypophosphatasia. In: Thakker RV, Whyte MP, Eisman J, Igarashi T (eds) Genetics of bone biology and skeletal disease. Academic Press, London, pp 337–360

Whyte MP, Valdes R, Ryan L, McAlister W (1982) Infantile hypophosphatasia: enzyme replacment therapy by intravenous infusion of alkaline phosphatase-rich plasma from patients with Paget bone disease. J Pediatr 101(3):379–386

Whyte MP, Greenberg CR, Wenkert D et al (2010) Hypophosphatasia in children: enzyme replacement therapy using bone-targeted tissue-nonspecific alkaline phosphatase. ASBMR 2010 Annual Meeting, Toronto

Whyte MP, Greenberg CR, Salman NJ et al (2012) Enzyme-replacement therapy in life-threatening hypophosphatasia. N Engl J Med 366(10):904–913

Yamamoto S, Orimo H, Matsumoto T et al (2011) Prolonged survival and phenotypic correction of AKp2 (−1-) hypophosphatasia mice by lentiviral gene therapy. J Bone Miner Res 26 (1):135–142

Zhanhua C, Gan JG, Lei L et al (2005) Protein subunit interfaces: heterodimers versus homodimers. Bioinformation 1(2):28–39

JIMD Reports
DOI 10.1007/8904_2013_218

CASE REPORT

Liver Transplantation Prevents Progressive Neurological Impairment in Argininemia

E. Santos Silva • M.L. Cardoso • L. Vilarinho • M. Medina • C. Barbot • E. Martins

Received: 07 December 2012 / Revised: 08 February 2013 / Accepted: 08 February 2013 / Published online: 5 April 2013

Abstract Argininemia is a rare hereditary disease due to a deficiency of hepatic arginase, which is the last enzyme of the urea cycle and hydrolyzes arginine to ornithine and urea. The onset of the disease is usually in childhood, and clinical manifestations include progressive spastic paraparesis and mental retardation. Liver involvement is less frequent and usually not as severe as observed in other UCDs. For this reason, and because usually there is a major neurological disease at diagnosis, patients with argininemia are rarely considered as candidates for OLT despite its capacity to replace the deficient enzyme by an active one. We report on long-term follow-up of two patients with argininemia. Patient 1 was diagnosed by the age of 20 months and despite appropriate conventional treatment progressed to spastic paraparesis with marked limp. OLT was performed at 10 years of age with normalization of plasmatic arginine levels and guanidino compounds. Ten years post-OLT, under free diet, there is no progression of neurological lesions. The second patient (previously reported by our group) was diagnosed at 2 months of age, during a neonatal cholestasis workup study. OLT was performed at the age of 7 years, due to liver cirrhosis with portal hypertension, in the absence of neurological lesions and an almost-normal brain MRI. After OLT, under free diet, there was normalization of plasmatic arginine levels and guanidino compounds. Twelve years post-OLT, she presents a normal neurological examination. We conclude that OLT prevents progressive neurological impairment in argininemia and should be considered when appropriate conventional treatment fails.

Communicated by: Jean-Marie Saudubray

Competing interests: None declared

E. Santos Silva (✉)
Gastrenterology Unit, Department of Child and Adolescent, Centro Hospitalar do Porto, Largo Abel Salazar, 4099-001, Porto, Portugal
e-mail: ermelinda.rss@gmail.com

M.L. Cardoso
Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal

L. Vilarinho
Neonatal Screening Unit, National Health Institute INSA, Porto, Portugal

M. Medina
Pediatric Hepatology, Hospital de Crianças Maria Pia, Porto, Portugal

C. Barbot
Pediatric Neurology, Hospital de Crianças Maria Pia, Porto, Portugal

E. Martins
Metabolic Unit, Department of Child and Adolescent, Centro Hospitalar do Porto, Porto, Portugal

Abbreviations

CSF	Cerebrospinal fluid
MRI	Magnetic resonance imaging
OLT	Orthotopic liver transplantation
UCDs	Urea cycle disorders

Introduction

Argininemia (MIM #207800) is a rare inborn error of urea cycle, due to arginase A1 deficiency. This enzyme is codified by ARG1 gene and is also known as hepatic arginase because it is responsible for 98% of arginase activity in the liver. The clinical symptoms of argininemia become apparent in early childhood, usually between 2 and 4 years of age. They include progressive spastic paraparesis (with marked degree of spasticity of the four extremities) and severe mental deterioration, which are characteristic of

this disease (and are not observed in other UCDs) (Brusilow and Horwich 2005; Scaglia and Lee 2006). The neurological symptoms referred to here cannot be explained merely as a consequence of arginine levels and hyperammoniemia (which is observed intermittently), since neurological impairment sometimes progresses despite good compliance with treatment (presenting stable arginine and ammonia plasmatic levels). So it was suspected that other factors, such as the presence of guanidino compounds, might be implicated in the neurological impairment (Mizutani et al. 1987), since the endogenous production of these substances may remain significant even when arginine is normalized (Marescau et al. 1990).

In the past, we demonstrated that OLT can normalize arginine, ammonia, and guanidino compounds levels in patients with argininemia, allowing them to follow a free diet for life (Santos Silva et al. 2001). Nevertheless, it remains to be clarified if in patients with already existing neurological lesions, OTL can prevent its progression, and in the ones who have not yet developed such symptoms, whether OLT will be able to prevent them in the long term.

Here, we report on two patients with argininemia, with more than 10 years follow-up after OLT, which support the evidence that this therapeutic strategy can prevent long-term development and progression of neurological impairment in patients with such an inborn error of metabolism.

Material and Methods

Amino acids profile was determined by liquid ion-exchange chromatography.

Guanidino compounds in plasma and urine were determined by high-performance liquid chromatography.

Case Reports

Case 1 – The patient is the first daughter of a non-consanguineous couple with irrelevant family history. Pregnancy and delivery were unremarkable. Birth weight was 4,100 g and height 51 cm. No neonatal jaundice was reported. She was overweight since the second year of life, and the motor milestones and cognitive development were normal.

She was diagnosed with infectious mononucleosis by 20 months of age, followed by persistent rise of transaminases and asymptomatic coagulation abnormalities. Biochemical data included total bilirubin 12 μmol/L, conjugated bilirubin 2.5 μmol/L, AST 170 UI/L (n<56), ALT 200 UI/L (n<39), GGT 10 UI/L (n<45), total cholesterol 4.5 mmol/L (n:3.4–6.0), thromboplastin time 46.6 s (n<35s), and prothrombin time 31 s (n<12s). Amino acids profile showed an elevated arginine concentration (481 μmol/l, n<140). Blood ammonia and urinary orotic acid were both within the normal range. Argininemia diagnosis was confirmed by the absence of arginase A1 activity in red blood cells and by molecular analysis of ARG1 gene (homozygous for R21X mutation). Other diagnoses have been excluded by appropriate testing, namely, alpha-1-antitrypsin deficiency, hepatitis B and C, autoimmune hepatitis, Wilson's disease, myopathy, and other amino-acidopathies. After diagnosis, she started immediately on a low-protein and arginine-restricted diet and oral sodium benzoate 300 mg/kg/day.

Outcome was spastic paraparesis with marked limp, despite good treatment compliance and lowering of plasmatic arginine levels (200–300 μmol/L). She benefited from physiotherapy and Vulpius surgery at 7 years of age. Meanwhile brain MRI, performed 1 year later, showed increased T2 signal intensity within the peritrigonal white matter (Fig. 1a) and increased T2 signal intensity within the semioval center, more prominent in the left hemisphere (Fig. 1b).

School performance was satisfactory (Wechsler scale GIQ – 97), growth was appropriate (height p50), but overweight was always an unsolved problem. She had raised transaminases, mild coagulation abnormalities, and liver steatosis (ultrasound). By the age of 8 years, she started oral phenylbutyrate 250 mg/kg/day, without much success in decreasing more effectively the levels of blood arginine. By this time, guanidino compounds in plasma and urine were markedly elevated (Fig. 2).

As spastic paraparesis worsened, it was proposed to the family to perform a liver transplant and thus cure the hepatic enzymatic deficiency. OLT was performed on the patient when she was 10 years old (at the Liver Transplantation Center of Coimbra – Portugal) with complete normalization of plasmatic arginine levels 24 h after OLT, and since then she has remained on an unrestricted diet. After OLT, plasmatic and urinary guanidine compounds also became normal (Fig. 2) and progression of the neurological disease stopped immediately. Ten years later, there has been no progression of neurological features or new MRI abnormalities (Fig. 1c and d).

Case 2 – This patient was previously reported upon (Braga et al. 1997; Santos Silva et al. 2001; Martins et al. 2011). Diagnosis of argininemia was made by 2 months of age during the workup of a neonatal cholestasis (plasma arginine 1,756 μmol/L, n: 22–88), and was confirmed by the absence of arginase A1 activity in blood red cells and by molecular analysis of ARG1 gene (homozygous for R21X mutation) (Cardoso et al. 1999). She was put on a low-protein and arginine-restricted diet, and oral sodium benzoate. The outcome was liver cirrhosis with portal hypertension, marked hypercholesterolemia

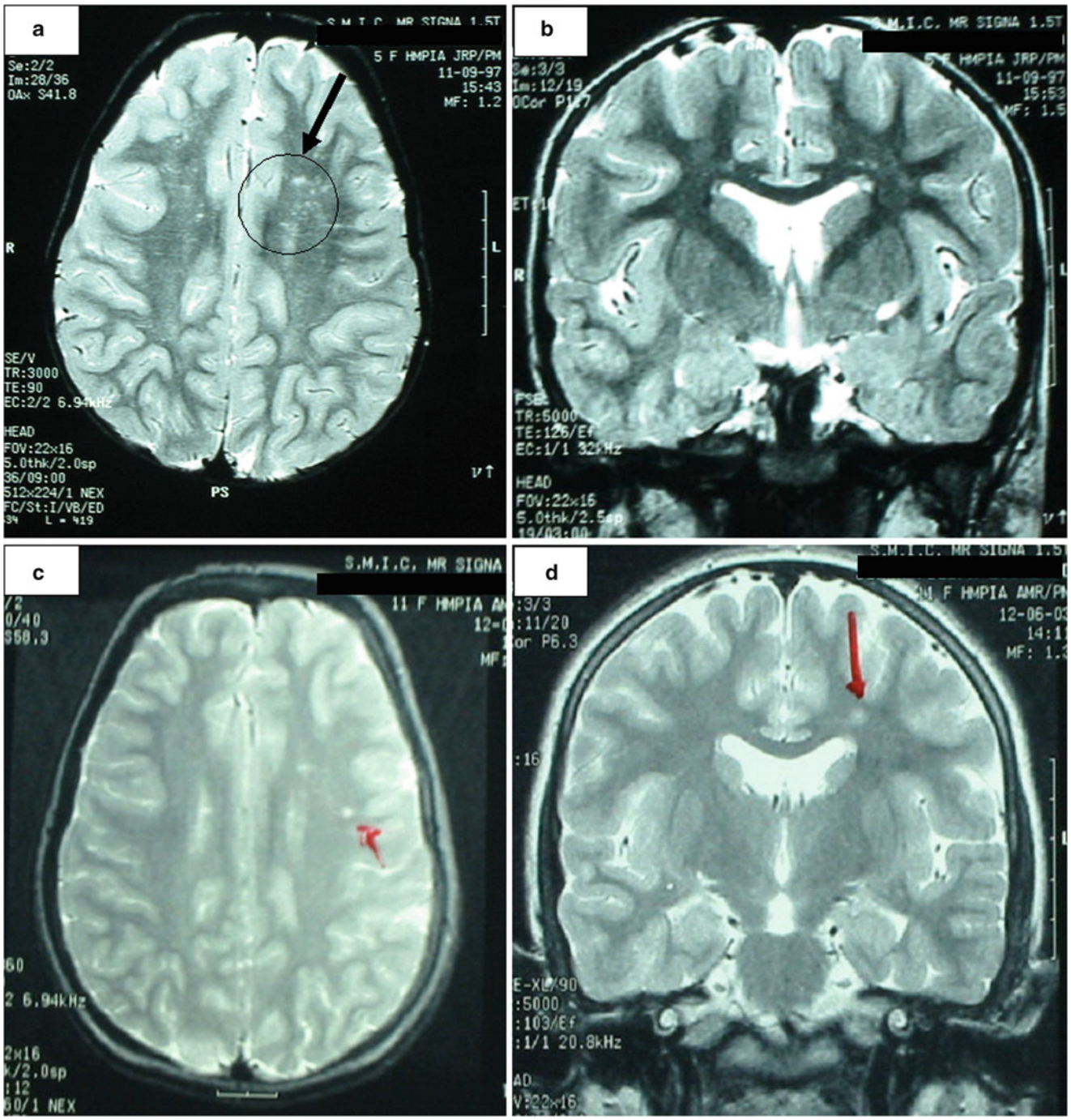

Fig. 1 Brain MRI studies in Patient 1. Before OLT we detected (**a**) an increased T2 signal intensity within the peritrigonal white matter (*black circle and arrow*) and (**b**) increased T2 signal intensity within the semioval center, more prominent in the left hemisphere. Later, 15 months post-OLT we found (**c**) an increased T2 signal intensity within the peritrigonal white matter, and (**d**) within the semioval center (more prominent in the left hemisphere), but less severe than before OLT (*red arrows*)

(18.0 mmol/L, N:3.4–6.0), xanthomatosis, and pruritus. Despite an extensive investigation, no other cause was established for this kind of liver involvement. By the age of 7 years, she had terminal liver cirrhosis, growth development delay ($P<3$), General Quotient Development = 80 (Griffiths method), and normal neurological features. Despite suitable dietary compliance, arginine plasmatic levels were around 300 μmol/l and guanidino compounds were markedly raised (Fig. 2). Brain MRI studies showed increased T1 signal intensity in the pallidum nucleus and no T2 signal abnormalities in the peritrigonal white matter. At this point, she underwent OLT at the Liver Transplantation

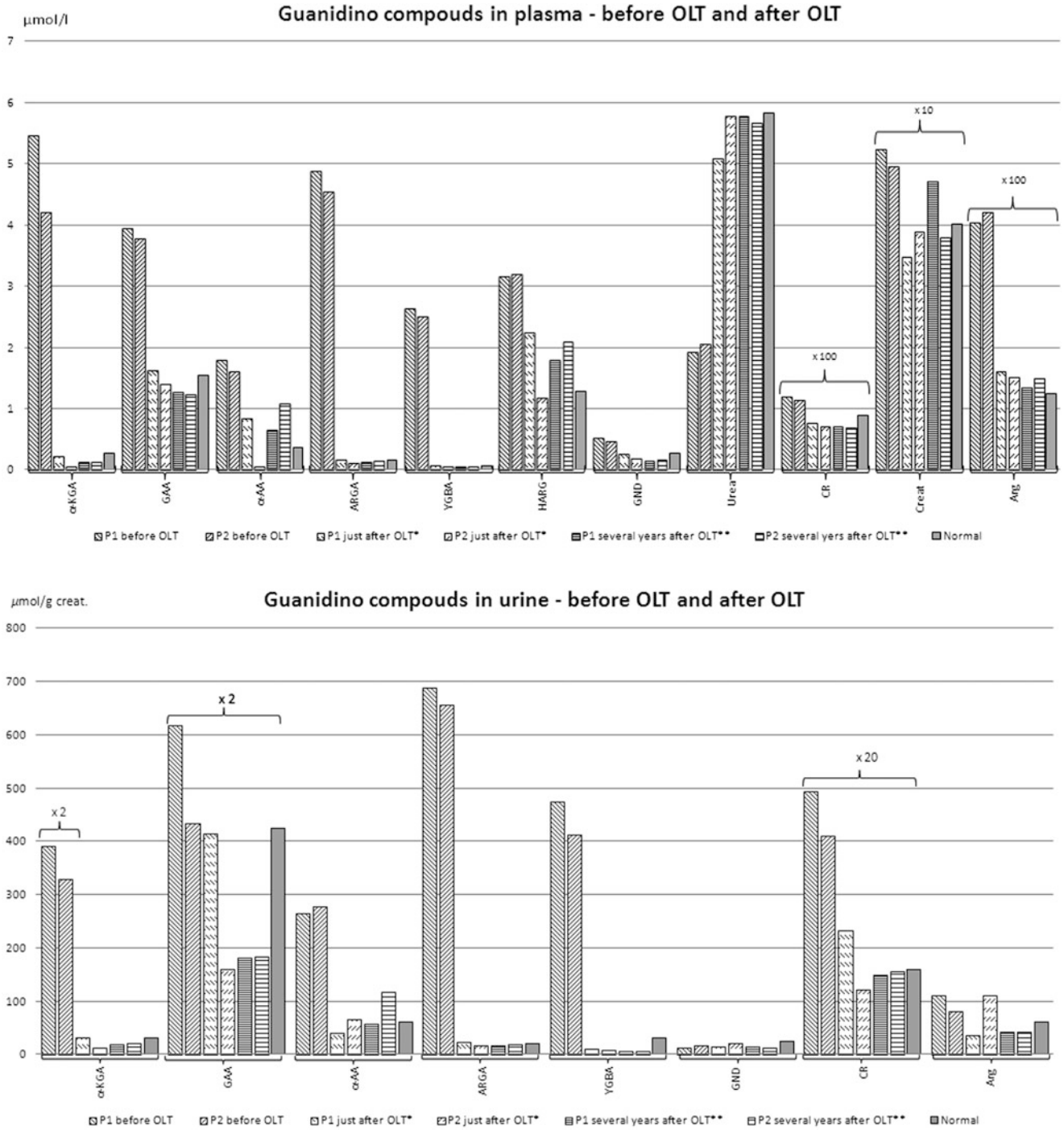

Fig. 2 Plasmatic and urinary levels of guanidino compounds in patients with argininemia. Patient 1 first control post-OLT at * 20 months and later ** at 8 years post-OLT. Patient 2 first control post-OLT at * 26 months and later ** at 4.5 years post-OLT. α-*KVA* α-keto-δ guanidinovaleric acid, *CR* creatine, *GAA* guanidinoacetic acid, α*AA* α-N-acetylarginine, *ARGA* argininic acid, *Creat* creatinine, *YGBA* γ-guanidinobutyric acid, *Arg* arginine, *HARG* homoarginine, *GND* guanidine

Center of Coimbra – Portugal. We observed a slight and transient hyperammoniemia with complete normalization of arginine and ammonia blood levels 24 h after OLT. When the patient started a free diet, blood arginine and ammonia levels remained normal and those of guanidino compounds normalized (Fig. 2). Twelve years after OLT, we note that neurological features, plasmatic arginine levels, and guanidino compounds remain normal. She has

a healthy and unaffected sibling, born July 2000, after molecular prenatal diagnosis.

Discussion

In several metabolic diseases, liver transplant, despite correcting the hepatic enzymatic deficiency, fails in stopping long-term consequences of the disease. As no data is available on the long-term surveillance of patients submitted to liver transplant for argininemia, this report is imperative.

Argininemia treatments that successfully lower arginine and guanidino compounds levels should prevent neurological symptoms, but although arginine plasmatic levels may normalize under conventional treatment, the arginine's catabolites can remain elevated (Marescau et al. 1990), and some of these guanidino compounds are neurotoxic (De Deyn et al. 2009; Hiramatzu, 2003). In both of our patients, arginine concentration decreased significantly under conventional treatment (but never normalized), and guanidine compounds were markedly elevated in plasma and urine. However, after OLT all these parameters normalized completely (Fig.2).

It would have been very interesting to evaluate the guanidino compounds also in CSF, however, that was not possible because Patient 1 never cooperated, and Patient 2 had terminal liver disease, with portal hypertension, thrombocytopenia, and coagulation abnormalities, which made the procedure unwise.

Patient 1 had a relatively early diagnosis (<2 years old) compared with the cohort of Portuguese symptomatic patients (12 patients, age of diagnosis > 3 years in the majority of cases). At diagnosis, she had subclinical liver disease (occasional finding) and was neurologically asymptomatic. Furthermore, she started conventional treatment immediately, with good compliance, and success in lowering the plasmatic levels of arginine. Despite everything, she developed progressive paraparesis and marked limp. Patient 2 had an uncommon form of presentation of argininemia (Braga et al. 1997; Martins et al. 2011) and benefited from an unusual early diagnosis and treatment (by 2 months of age). As in Patient 1, arginine plasmatic levels decreased significantly but never got completely normal. This patient never developed neurological impairment. Both patients had markedly elevated guanidino compounds pre-OLT. Perhaps the explanation for the different neurological outcome in these two patients is that vast brain growth with organizational changes takes place during the first two postnatal years (Knickmeyer et al. 2008). Thus, any toxic factor acting on the brain during this period of time will be more harmful and leave the worst possible sequels. Patient 1 was much more exposed to neurotoxicity in this crucial period for brain development than Patient 2.

When Patient 1 started to show signs of neurological impairment, we already had the experience of following other patients, under conventional treatment, with inexorable progression to spastic tetraparesis and severe mental retardation (unpublished data). But this time, we also had the experience of the first OLT performed in a patient with argininemia (Santos Silva et al. 2001). The encouraging result of OLT in Patient 2 prompted us to transplant Patient 1, in order to stop progression of neurological impairment. This aim was completely achieved. In Patient 1, progression of neurological disease stopped immediately, and there was even some regression in MRI features (Fig.1). This effect has been sustained 10 years after OLT. Patient 2 remains free of neurological disease 12 years post-OLT.

These two clinical observations also suggest that although the highest aggression for the brain may occur during the first 2 years of life, it may continue while no complete normalization of biochemical parameters is achieved, mainly of guanidino compounds levels. They also show that once normalization happens, the progression of the neurological disease stops. Based on such observations, it is recommended that patients with argininemia should be carefully monitored clinically (and biochemically) for early detection of the development of neurological symptoms.

Argininemia is the second most common urea cycle disease in Portugal, after ornithine transcarbamylase deficiency (1/215,000 live newborns) (Martins et al. 2011). Since 2004, it has been included in the Portuguese expanded newborn screening (3–6 day of life, arginine level in dried blood spot cutoff = 37 μM). If arginine is above the cutoff level, a second blood sample is requested. If the second blood analysis shows arginine plasmatic level > 140 μmol/L, the neonate is called for full assessment at a reference center. Based on this approach, we have, until now, diagnosed four cases of argininemia in newborns (1/181,550 live newborns) (Personal communication, E. Martins 2011), allowing the affected patients to start conventional treatment during the first month of life (median age 16 days), and thus protecting their brains during infancy and childhood from exposure to high amounts of neurotoxic compounds. The follow-up of such patients will further help to clarify the long-term outcome of the disease in ideally treated patients. Meanwhile, we know that OLT is able to prevent progressive neurological impairment in patients with argininemia, when appropriate conventional treatment fails, and should be offered to these patients before severe neurological impairment occurs.

Concise Sentence Take-Home Message

OLT prevents progressive neurological impairment in argininemia and should be considered when appropriate conventional treatment fails.

Reference to Electronic Databases

Urea cycle disorders, argininemia, liver transplantation, neurological impairment.

Conflict of Interest

There is no conflict of interest.

References

Braga AC, Vilarinho L, Ferreira E, Rocha H (1997) Hyperargininemia presenting as persistent neonatal jaundice and hepatic cirrhosis. J Pediatr Gastroenterol Nutr 24:218–221

Brusilow SW, Horwich AL (2005) Urea cycle enzymes. In: Scriver CR et al (eds) Metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, Chap 85

Cardoso ML, Martins E, Vasconcelos R et al (1999) Identification of a novel R21X mutation in the liver-type arginase gene (ARG1) in four Portuguese patients with argininemia. Hum Mutation 14:355–356

De Deyn PP, Vanholder R, Eloot S, Glorieux G (2009) Guanidino compounds as uremic (neuro)toxins. Semin Dial 22(4):340–5

Hiramatzu M (2003) A role for guanidine compounds in the brain. Mol Cell Biochem 244:57–62

Knickmeyer RC, Gouttard S, Kang C, Evans D, Wilber K, Smith JK et al (2008) A structural MRI study of human brain development from birth to 2 years. Journal of Neuroscience 28(47): 12176–12182

Marescau B, De Deyn PP, Lowenthal A, Qureshi IA, Antonozzi I, Bachmann C, Cederbaum SD, Cerone R, Chamoles N, Colombo JP et al (1990) Guanidino compound analysis as a complementary diagnostic parameter for hyperargininemia: follow-up of guanidino compound levels during therapy. Pediatr Res 27(3): 297–303

Martins E, Santos Silva E, Vilarinho S, Saudubray JM, Vilarinho L (2011) Neonatal cholestasis: an uncommon presentation of hyperargininemia. J Inherit Metab Dis. 2011; DOI 10.1007/s10545-010-9263-7.

Mizutani N, Hayakawa C, Ohya Y, Watanabe K, Watanabe Y, Mori A (1987) Guanidino compounds in hyperargininemia. Tohoku J Exp Med 153:197–205

Santos Silva E, Martins E, Cardoso ML, Barbot C, Vilarinho L, Medina M (2001) Liver transplantation in a case of argininemia. J inherit Metab Dis 24:885–887

Scaglia M, Lee B (2006) Clinical, biochemical and molecular spectrum of hyperargininemia due to arginase I deficiency. Am J Med Genet 142C:113–120

JIMD Reports
DOI 10.1007/8904_2013_219

RESEARCH REPORT

Motor and Speech Disorders in Classic Galactosemia

Nancy L. Potter • Yves Nievergelt • Lawrence D. Shriberg

Received: 31 December 2012 / Revised: 15 February 2013 / Accepted: 19 February 2013 / Published online: 2 April 2013

Abstract ***Purpose*** To test the hypothesis that children with classic galactosemia and speech disorders are at risk for co-occurring strength and coordination disorders.

Method This is a case–control study of 32 children (66% male) with galactosemia and neurologic speech disorders and 130 controls (50% male) ages 4–16 years. Speech was assessed using the Percentage of Consonants Correct (PCC) metric from responses to the Goldman-Fristoe Test of Articulation-2 and from a 5-min recorded speech sample, hand and tongue strength using the Iowa Oral Performance Instrument, and coordination using the Movement Assessment Battery for Children. The number of days on milk during the neonatal period was obtained by parent report. Analyses of covariance, distributions, and correlations were used to evaluate relationships among speech, strength, coordination, age, gender, and days on milk.

Results Children with galactosemia had weaker hand and tongue strength and most (66%) had significant coordination disorders, primarily affecting balance and manual dexterity. Among children with galactosemia, children with more speech errors and classified as childhood apraxia of speech ($n = 7$) and ataxic dysarthria ($n = 1$), had poorer balance and manual dexterity, but not weaker hand or tongue strength, compared to the children with fewer speech errors. The number of days on milk during the neonatal period was associated with more speech errors in males but not in females.

Conclusion Children with galactosemia have a high prevalence of co-occurring speech, coordination, and strength disorders, which may be evidence of a common underlying etiology, likely associated with diffuse cerebellar damage, rather than distinct disorders.

Abbreviations

CAS	Childhood Apraxia of Speech
kstest	Kolmogorov-Smirnov one sample one-tail test statistic
kstest2	Kolmogorov-Smirnov two sample one-tail test statistic
MABC	Movement Assessment Battery for Children
MSD-NOS	Motor Speech Disorder-Not Otherwise Specified
PCC:AT	Percentage of Consonants Correct from an Articulation Test
PCC:CS	Percentage of Consonants Correct from a 5-minute Conversational Speech Sample

Communicated by: Gerard T. Berry

Competing interests: None declared

N.L. Potter (✉)
Department of Speech and Hearing Sciences, Washington State University Spokane, 412 E. Spokane Falls Blvd.,
Spokane, WA 99202-2131, USA
e-mail: nlpotter@wsu.edu

Y. Nievergelt
Department of Mathematics, Eastern Washington University, Cheney, WA 99004, USA

L.D. Shriberg
Waisman Center, University of Wisconsin-Madison, Madison, WI 53706, USA

Classic galactosemia (OMIM 230400; referred to as galactosemia in this article) is a rare recessive autosomal inborn error of metabolism that prevents individuals from metabolizing galactose, a sugar present in breast milk and milk-based formula (Berry and Elsas 2011). During the newborn period, galactosemia may affect multiple organs and can be life threatening in infants ingesting lactose in

breast milk and milk-based formulas. In the United States, galactosemia is diagnosed through newborn screening with an incidence of 1 in 40,000–60,000. With early diagnosis and dietary galactose restriction through the elimination of breast milk or milk-based formula, children survive but are at risk for long-term complications including language (90 %), speech (60 %), cognitive (50 %), motor disorders (18 %; Waggoner et al. 1990), and gonadal failure in females (>80–90 %; Fridovich-Keil et al. 2011). Most studies report that the severity of long-term complications has minimal or no association with the number of days milk is consumed during the neonatal period prior to the galactosemia diagnosis (Berry and Elsas 2011).

Speech disorders in children with galactosemia, which have a neurologic origin, are classified as one of the following three subtypes of motor speech disorders: (1) childhood apraxia of speech (CAS), a deficit in motor planning or programming, (2) dysarthria, a deficit in neuromuscular control, or (3) motor speech disorder-not otherwise specified (MSD-NOS), a cover term for speech, prosody, and voice behaviors that are consistent with a motor speech disorder, but not specific for CAS or dysarthria (Shriberg et al. 2011).

Children with galactosemia and speech disorders have a high co-occurrence of motor disorders (Waggoner et al. 1990). Motor disorders include deficits in strength and coordination (Gaines and Missiuna 2007; Gallup et al. 2007; Pieters et al. 2012; Raynor 2001). Speech and coordination disorders frequently co-occur in the general population (Gaines and Missiuna 2007; Pieters et al. 2012). In a recent study of more than 3,000 children referred for an assessment of developmental delays, one-third (33.7 %) of children with speech disorders had co-occurring coordination disorders as opposed to <10 % in the general population (Pieters et al. 2012). Investigators have proposed that co-occurring coordination and speech disorders should not be considered two distinct disorders but rather the result of a common underlying etiology affecting a number of motor domains including speech (Gaines and Missiuna 2007; Pieters et al. 2012). Males are at greater risk for developmental disorders, with approximately twice the prevalence of coordination (1.8:1) and speech disorders (2:1) compared to females (Pieters et al. 2012). While speech disorders have been reported to affect 60 % of children with galactosemia (Waggoner et al. 1990), the ratio of males to females with speech or coordination disorders in galactosemia is not known.

Motor disorders have been reported in children with galactosemia (Waggoner et al. 1990) but the term "motor" has not been defined nor have the components of motor disorders been systematically assessed. The present study examined strength and coordination, two components of motor development that contribute to a motor disorder (Pieters et al. 2012; Raynor 2001) and their relationships to measures of speech production. Males and females were analyzed separately as males are more at risk for developmental disorders (Raynor 2001). Days on milk during the neonatal period was included in the analyses as the possibility of an association has not been definitively ruled out (Waggoner et al. 1990). Thus, the objectives of the present study were to: (1) determine if children with galactosemia and speech disorders differ from controls in strength and coordination skills, (2) examine relationships among speech, strength, and coordination skills, and (3) ascertain if there are relationships among the severity of motor or speech disorders and early ingestion of milk in children with galactosemia.

Methods

A total of 163 children between 4 and 16 years of age participated in this case–control study, which was part of a larger study of CAS (Potter et al. 2008; Potter 2011; Shriberg et al. 2011). There were 32 children with galactosemia, 21 males and 11 females, and 130 control children, 5 males and 5 females from each 6-month age group from 4–16 years of age (Potter et al. 2012). Children with galactosemia met the following criteria: (a) a confirmed diagnosis of classic galactosemia, (b) a history of treatment for speech disorders, (c) English as a first language, (d) no significant hearing loss as measured by a pure tone screening test, and (e) no craniofacial anomalies. Half (49 %) of the children with galactosemia had IQs in the normal range (85–115), 39 % in the borderline range (70–84), and 12 % in the low range (below 70; Potter et al. 2008). One male with galactosemia was assessed but excluded from the present study because of a diagnosis of cerebral palsy and an inability to complete the motor assessment. Controls met the following criteria: (a) academic performance at grade level with no history of referral for special educational services, (b) articulation within normal limits on a standardized articulation test, (c) English as a first language, (d) hearing within normal limits on a pure tone screening test, and (e) no craniofacial anomalies. Normal cognitive development in the controls was documented by teacher and parent questionnaire, with both groups indicating that the participant was functioning at or above grade level in academic subjects and physical education and had never been referred for special education.

The children with galactosemia were recruited through website, e-mail, and postal announcements to two support groups, Galactosemia Foundation and Galactosemic Families of Minnesota, and to metabolic clinics across the United States. All children with galactosemia were tested in their homes in 17 different states across the United States. Parents completed a written health history form. As reported by parents, all children with galactosemia adhered to a lactose-restricted diet. The control

participants were recruited from and tested at preschools and public schools in Washington State. A speech-language pathologist with advanced training in kinesiology tested all the participants.

The Madison Speech Assessment Protocol and the Speech Disorders Classification System (Shriberg et al. 2010) was used to classify speech as typically developing (all controls) or as meeting the criteria for one of the three motor speech disorder subtypes (all participants with galactosemia): (1) CAS ($n = 7$), (2) ataxic dysarthria ($n = 1$, referred to as dysarthria in this paper), and (3) MSD-NOS ($n = 24$; Shriberg et al. 2011). Speech findings were obtained by phonetically transcribing the children's responses to the Goldman-Fristoe Test of Articulation-2 (PCC:AT; Goldman and Fristoe 2000) and from a 5-min conversational speech sample (PCC:CS) and dividing the number of consonants produced correctly by the total number of consonants to obtain the *percentage of consonants correct* (PCC).

Tongue and hand strength were assessed using the Iowa Oral Performance Instrument (IOPI) with the standard silicon tongue bulb and the air-and-silicone-filled hand bulb. To measure tongue strength, the tongue bulb was positioned on the participant's alveolar ridge, immediately posterior to the central incisors (IOPI Northwest 2005). The children were asked to raise their tongues and squeeze the bulb against the palate as hard as they could for 2–3 s. To measure dominant and nondominant hand strength, the hand bulb was positioned in the center of the children's palms. The participants were asked to curl their fingers around the bulb and squeeze as hard as they could for 2–3 s. For each strength measurement, the children performed three trials with a 30-s rest between trials. The highest value from the three trials was defined as maximum strength. Ninety-one percent of the children with galactosemia, including all the children with CAS or dysarthria, and 85 % of the controls were right hand dominant.

Coordination in the children with galactosemia was assessed using the Movement Assessment Battery for Children (MABC) with scores compared to the published norms (Henderson and Sugden 1992). The MABC has three subtests, manual dexterity, ball skills, and balance, and a total impairment score, which is a sum of the three subtests. Higher scores indicate greater impairment. The first edition of the MABC, used in the present study, included reference data for participants ages 4–12 years. The comparative data for age 12 years was used for children ages 13–16 ($n = 3$).

The above assessments were conducted among children with galactosemia in their homes using a protocol that included measures in addition to those discussed in the present study (Potter et al. 2008; Potter 2011; Shriberg et al. 2011). Controls were assessed in a quiet schoolroom during the school day.

The Institutional Review Boards of the University of Wisconsin-Madison and Washington State University approved this study. A parent of each participant provided written consent, children age 12 years and older provided written consent, and children 11 years and younger provided written or verbal informed assent.

Statistical Analysis

Normality tests (Lilliefors and Jarque-Bera; MATLAB 2012) were done on all variables and residuals from their ordinary least-squares regressions on age, for all groups and subgroups. Statistically significant differences from two-sample one-tail *t*-tests, analyses of variance or analyses of covariance are reported for variables or residuals that passed at least one normality test in each group or subgroup. One-sample or two-sample, one-tail Kolmogorov-Smirnov tests (*kstest* or *kstest2*) were also performed on all variables, groups, and subgroups and statistically significant differences reported. Within groups, pairs of variables were subjected to tests of Pearson's and Spearman's correlation coefficients (with partial correlations to adjust for age). An $\alpha = 0.05$ was used for all analyses.

Results

Genders were combined where relationships among variables did not differ by gender (comparison between controls and galactosemia on the speech measures PCC:AT and PCC:CS) or no information was available by gender (MABC subtest and total scores). Analyses were done separately by gender for all other variables.

Speech

Males and females with galactosemia had more articulation errors (fewer consonants correct) than the controls on the articulation test and during conversation (PCC:AT, $kstest2 = 0.70$, $P = 2.93 \times 10^{-12}$ and PCC:CS, $kstest2 = 0.73$, $P = 2.69 \times 10^{-13}$). Within the galactosemia group, the children diagnosed with CAS or dysarthria had more speech errors on the articulation test and during conversation (PCC:AT, $kstest2 = 0.58$, $P = 0.0095$ and PCC:CS, $kstest2 = 0.58$, $P = 0.0095$) compared to the children classified as MSD-NOS.

Strength

As shown in Fig. 1, males with galactosemia had weaker tongue strength compared to the control males ($kstest2 = 0.7355$, $P = 9.52 \times 10^{-9}$) and females with galactosemia had weaker tongue strength compared to the control females ($kstest2 = 0.8615$, $P = 2.01 \times 10^{-7}$).

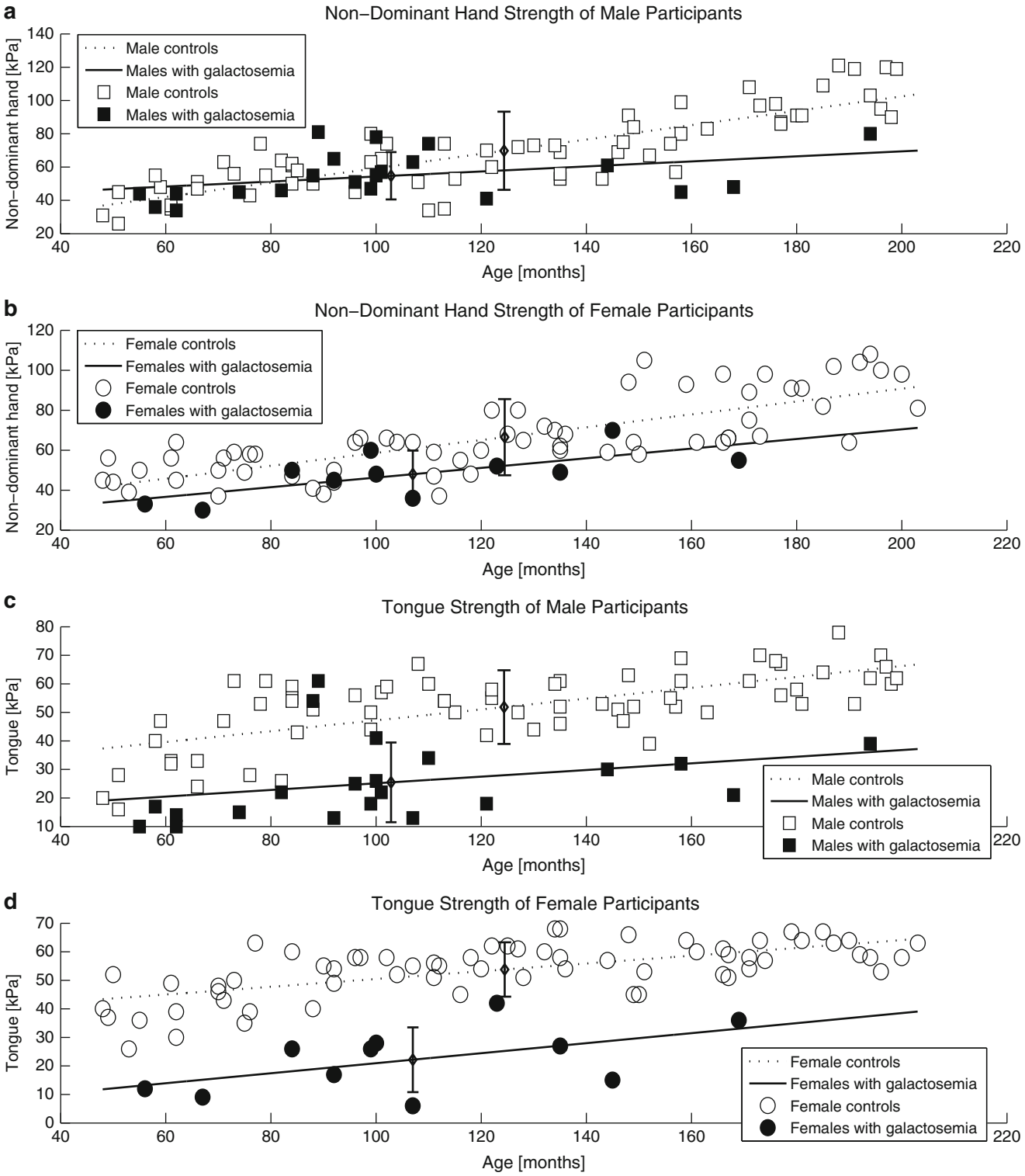

Fig. 1 Nondominant and dominant hand strength as a function of age. Means and standard deviations shown by diamonds and whiskers

Within the galactosemia group, tongue strength was equivalent across diagnoses (CAS, dysarthria, and MSD-NOS) and genders.

Comparison across groups showed that males with galactosemia had weaker dominant and nondominant hand strength (lower means for dominant hand, $t = 3.20$,

$P = 0.0012$ and nondominant hand, $t = 3.53$, $P = 4.09 \times 10^{-4}$). However, at the younger ages, males with galactosemia had greater hand strength (larger intercepts for dominant hand, $t = 2.76$, $P = 0.0072$ and nondominant hand, $t = 2.34$, $P = 0.022$) but then increased at a slower rate (shallower slopes for dominant hand, $t = 3.61$, $P = 5.32$ x 10^{-4} and nondominant hand, $t = 3.22$, $P = 0.0018$) compared to the male controls.

Females with galactosemia also had weaker dominant and nondominant hand strength (lower means for dominant, $t = 3.80$, $P = 6.04 \times 10^{-4}$ and nondominant hand, $t = 4.32$, $P = 1.64 \times 10^{-4}$). Unlike the males at the younger ages, they had weaker dominant and nondominant hand strength (smaller intercepts) and remained weaker across age (equivalent slopes) compared to the female controls. Within the galactosemia group, children with apraxia or dysarthria had equivalent hand strength compared to children with MSD-NOS.

Dominant hand strength was 2 % greater for male controls, 4 % greater for female controls, 0.3 % greater for males with galactosemia, and 4 % greater for females with galactosemia than nondominant hand strength, but these differences were not statistically significant. In the general population, there is no difference in hand strength in left-hand-dominant individuals. Dominant hand strength may be up to 10 % stronger in right-hand-dominant individuals (Gallup et al. 2007; Häger-Ross and Rösblad 2002).

Coordination

The performances of the children with galactosemia on the MABC were compared to the general population norms published in the test manual (Fig. 3; Henderson and Sugden 1992, p. 109), which are partitioned into two mutually disjoint age groups: "Ages 4 and 5 years" and "ages 6 and above". For comparison, the scores of the children in the present study were partitioned into the corresponding age groups, with 4 males and 2 females under 6 years of age, and 17 males and 9 females age 6 years and above. Typical tests of whether a sample was drawn randomly from a general population use the population cumulative distribution function (CDF), not percentiles as provided in the MABC test manual. Therefore to compare each age group of participants with the corresponding age group from the general population, the percentiles for each age group were converted into a by the formula: $\text{CDF} = \frac{\text{100-percentile}}{100}$. As shown in Fig. 2, a one-tailed, one sample Kolmogorov-Smirnov test (computed with MATLAB's *kstest*; MATLAB 2012) showed that the combined scores for coordination skills, as measured by the MABC total score, of the 4- and 5-year-old children with galactosemia were markedly below that of the general population (ksstat = 0.84, $P = 1.68 \times 10^{-5}$) as were the coordination skills of the children 6 years and older (ksstat = 0.66308, $P = 4.56 \times 10^{-12}$).

Fifty-three percent (17/32) of the children with galactosemia and speech disorders scored at or below the 5th percentile and 66 % (21/32) scored below the 10th percentile on the total score of the MABC, which are two of the frequently used cutoff scores for a coordination disorder diagnosis (Gaines and Missiuna 2007; Pieters et al. 2012). Using the MABC total impairment score, children with galactosemia and speech disorders have increased odds of 3.5 (odds ratio) of a co-occurring coordination disorder as compared to the general population with speech and language disorders (Pieters et al. 2012). Subtest scores (for manual dexterity, ball skills, and balance) could not be compared, as data for the general population was not published in the MABC manual or in subsequent publications. As shown in Fig. 3, the children with galactosemia who were diagnosed with CAS or dysarthria had poorer manual dexterity ($F = 4.55$, $P = 0.04$), markedly poorer balance ($F = 23.20$, $P = 4.0 \times 10^{-5}$), and poorer total scores ($F = 11.62$, $P = 0.0019$) on the MABC compared to the children diagnosed with MSD-NOS.

As shown in Table 1, poor coordination, measured by balance, ball skills, manual dexterity, and the total score on the MABC, was associated with weak dominant and nondominant hand and tongue strength in males, but not in females, with galactosemia. With genders collapsed, poor balance and manual dexterity were associated with weak tongue strength but not hand strength in children with galactosemia and MSD-NOS. Coordination was not associated with strength in children with galactosemia and CAS or dysarthria.

Days on Milk

The number of days on milk was associated with poorer articulation (PCC:AT, $\rho = -0.49$, $P = 0.03$; PCC:CS, $\rho = -0.45$, $P = 0.045$) for males with galactosemia but not for females with galactosemia ($P = 0.06$). The number of days on milk was not associated with measures of strength (range for $P = 0.78 - 0.82$) or coordination (range for $P = 0.26 - 0.63$).

Discussion

This is the first study to examine relationships among motor speech, strength, and coordination disorders in any pediatric population. It is also the first study to relate motor skills to speech disorders by gender in children with galactosemia. Compared to the controls, (1) males and females with galactosemia had weaker tongue strength across ages, (2) females with galactosemia had weaker hand strength

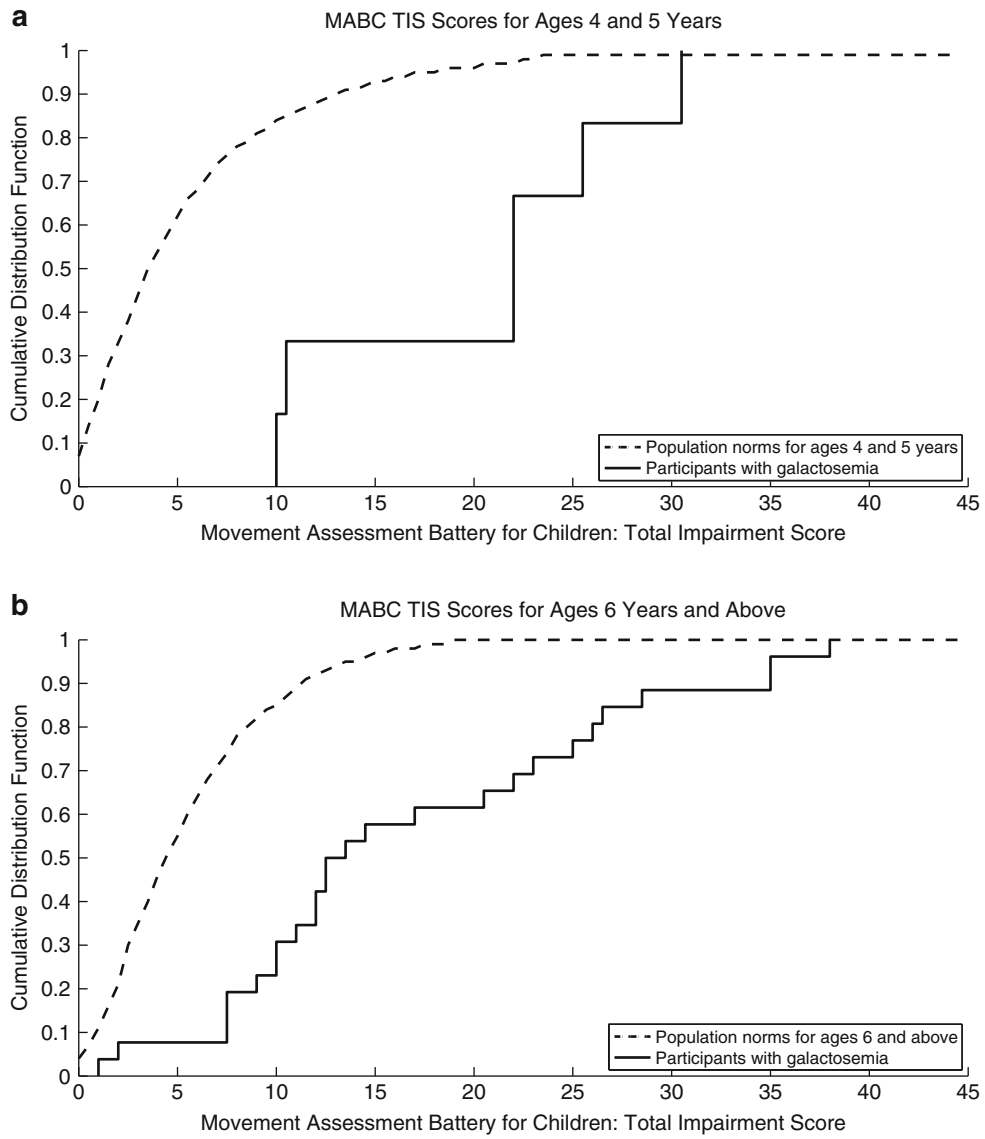

Fig. 2 Cumulative distribution functions of the MABC total impairment scores. Greater MABC total impairment scores correspond to greater impairment

across ages, (3) males with galactosemia had equivalent or slightly stronger hand strength at the younger ages but did not show the typical increase in hand strength expected of males during adolescence, (4) 66 % of children with galactosemia and speech disorders had co-occurring coordination disorders, (5) children with galactosemia and CAS or dysarthria had poorer balance and manual dexterity, and (6) the number of days on milk during the neonatal period was associated with worse speech outcomes for males, but not females, with galactosemia.

Speech

All children with galactosemia showed evidence of a neurological origin for their speech disorder, which was classified as one of the three subtypes of motor speech disorders: (1) CAS, (2) dysarthria, and (3) MSD-NOS (Shriberg et al. 2011). The children with CAS or dysarthria exhibited more speech errors and were less intelligible than the children with MSD-NOS.

Strength

We predicted that tongue strength would be reduced in males and females with galactosemia and speech disorders based on the results of two small-scale studies that examined tongue strength in CAS. Together these studies reported that eight of 10 children (nine males and one female) with CAS had weaker tongue strength compared to controls, suggesting that weak tongue strength may be

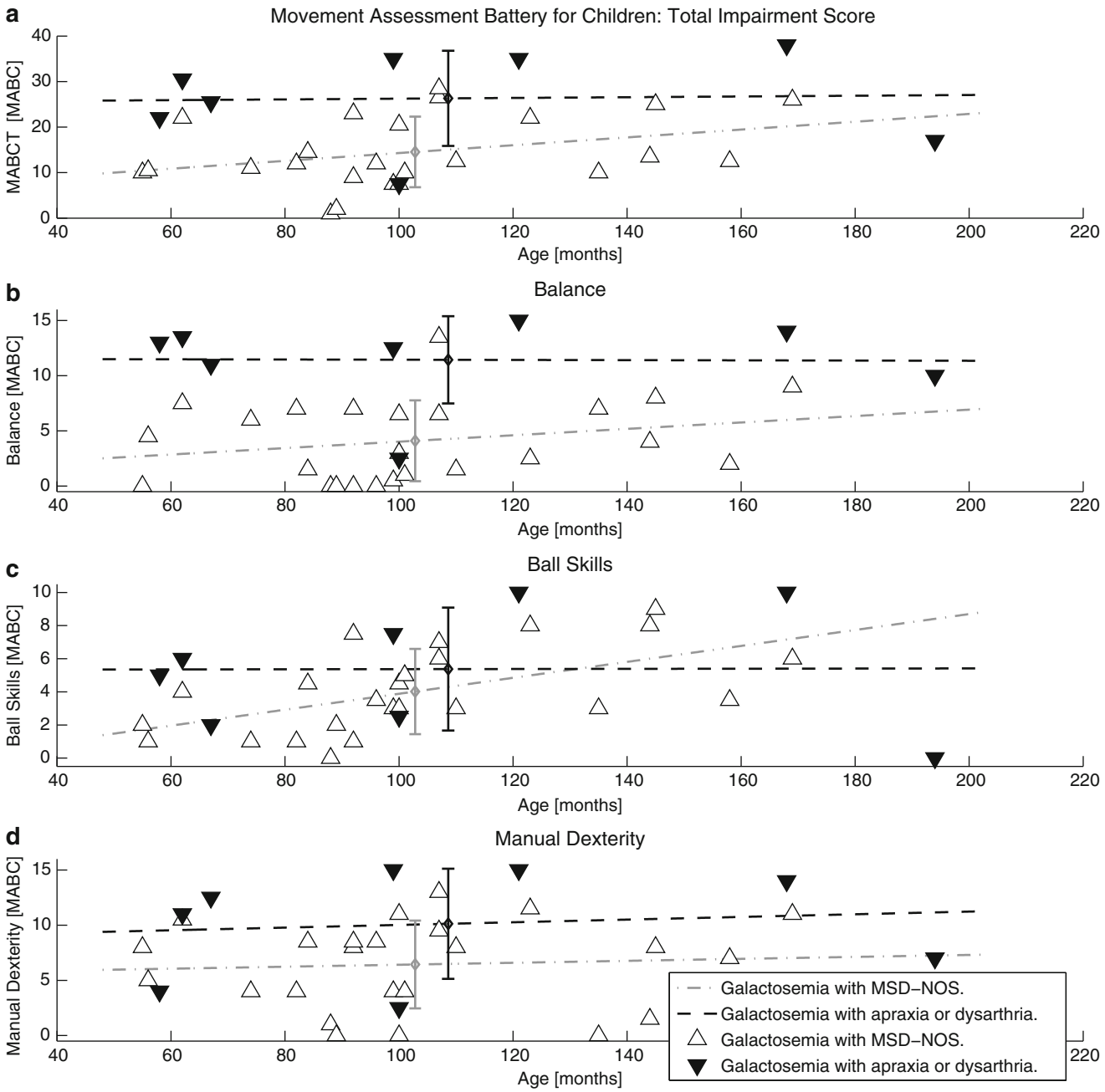

Fig. 3 MABC total and subtests scores for children with galactosemia. Greater MABC total impairment scores correspond to greater impairment. Means and standard deviations shown by diamonds and whiskers

associated with neurologic speech disorders (Murdoch et al. 1995; Robin et al. 1991). This premise was supported by the findings of the present study as all the children with galactosemia had neurologic speech disorders and, on average, had weaker tongues when compared to the controls. Although the mean tongue strength was decreased in children with galactosemia, the rate of increase in tongue strength across ages was equivalent for males and females with galactosemia and the controls. If decreased tongue strength was a causative factor for speech disorders, we would expect that least intelligible children, those with CAS or dysarthria, would have the weakest tongues. Our findings showed that the children with CAS or dysarthria had equivalent tongue strength when compared to the children with MSD-NOS. The relationship of tongue strength to speech competence is controversial in the field of speech-language pathology. Although there is little empirical evidence supporting this practice, most speech-language pathologists (up to 85 %) use nonspeech exercises to attempt to strengthen tongues with the goal of improving

Table 1 Relationships among measures of strength and coordination by gender (left) and by motor speech disorder classification (right)

	n	Dominant hand strength ρ (P)	Nondominant hand strength ρ (P)	Tongue strength ρ (P)	n	Dominant hand strength ρ (P)	Nondominant hand strength ρ (P)	Tongue strength ρ (P)
	Males				**Motor speech disorders-not otherwise specified (MSD-NOS)**			
Balance	21	−.59 (0.007)**	−.60 (0.005)**	−.51 (0.01)*	24	−.37 (0.08)	−.37 (0.09)	−.46 (0.03)*
Ball skills	21	−.49 (0.03)*	−.53 (0.02)*	−.46 (0.04)*	24	−.07 (0.76)	−.16 (0.46)	−.34 (0.11)
Manual dexterity	21	−.66 (0.002)**	−.56 (0.01)*	−.68 (0.0009)***	24	−.38 (0.08)	−.40 (0.06)	−.46 (0.03)*
MABC total score	21	−.67 (0.001)**	−.69 (0.0008)***	−.62 (0.004)**	24	−.37 (0.08)	−.46 (0.03)*	−.56 (0.006)**
	Females				**Childhood apraxia of speech (CAS) or dysarthria**			
Balance	11	−.46 (0.18)	−.56 (0.10)	−.49 (0.15)	8	−.56 (0.19)	−.43 (0.34)	−.47 (0.29)
Ball skills	11	.19 (0.59)	.25 (0.48)	−.18 (0.62)	8	−.36 (0.43)	−.18 (0.74)	−.22 (0.64)
Manual dexterity	11	−.32 (0.37)	−.32 (0.37)	−.34 (0.34)	8	−.51 (0.24)	−.56 (0.18)	−.67 (0.10)
MABC total score	11	−.39 (0.26)	−.44 (0.21)	−.60 (0.06)	8	−.62 (0.14)	−.42 (0.34)	−.56 (0.18)

Higher scores on the MABC subtests and total score indicate more difficulties. Higher scores on strength indicate greater strength

*Difference at $P < 0.05$ according to Two-Sample Spearman rank correlation

**Difference at $P < 0.01$ according to Two-Sample Spearman rank correlation

***Difference at $P < 0.001$ according to Two-Sample Spearman rank correlation

speech (Lof and Watson 2008). Findings from the present study of children with galactosemia suggest that tongue strength is not related to the type or severity of their motor speech disorder.

Hand strength is a strong indicator of disability and was included in the protocol to look for possible evidence of unilateral versus bilateral involvement and differences across genders (Gallup et al. 2007). In the present study, there was no evidence of unilateral involvement as both participants with galactosemia and control participants showed little variability in strength between their dominant and nondominant hands. Therefore, handedness is discussed next without differentiating between dominant and nondominant laterality. Males and females showed different patterns in hand strength development. Similar to previous studies of normal hand strength development, it increased in parallel in the male and female controls until 10 years of age, after which it increased faster in the males than the females (Häger-Ross and Rösblad 2002). Unlike the controls, males and females with galactosemia had similar hand strength across all ages. At 4–6 years of age, the males with galactosemia had slightly greater hand strength compared to the male controls, but then increased slowly across age, resulting in significantly weaker hand strength throughout adolescence. Hand strength in males is closely related to testosterone levels prenatally and during adulthood (Gallup et al. 2007). Small studies of adolescent males with galactosemia have reported that pubertal development may be delayed in up to 20 % of males although testosterone levels are near normal pre- and post-puberty (Gubbels et al. 2012). This area needs further study as adult males with galactosemia have slightly lower testosterone levels. Females with galactosemia had weaker hand strength across all ages but increased at approximately the same rate as the female controls. Females have varying levels of testosterone and these levels are not related to hand strength in females (Gallup et al. 2007). The decreased tongue and hand strength observed in males and females with galactosemia may have different contributing factors including galactose-1-phosphate levels during the prenatal period, central nervous system white matter deficits (Dubroff et al. 2008; Hughes et al. 2009), or hormone levels during childhood or adolescence (Gallup et al. 2007; Gubbels et al. 2012). Further study examining the relationship between hormone levels and strength is warranted as >80-90 % of females with galactosemia experience gonadal failure (Fridovich-Keil et al. 2011). The prevalence of decreased strength may be inflated due to the inclusionary criteria specifying that children with galactosemia must have a speech disorder to participate in the present study, thereby increasing their risk of other co-occurring disorders.

Coordination

In our study, two-thirds (66 %) of the children with galactosemia had a co-occurring coordination disorder, compared to only one-third (33.7 %) of the general pediatric population who have developmental disorders (Pieters et al. 2012). As a large retrospective survey reported that 60 % of individuals with galactosemia had speech disorders and 18 % had motor disorders, with no mention of co-occurrence (Waggoner et al. 1990), we predicted that approximately one third of the children with galactosemia would have co-occurring speech and coordination disorders. Our data indicates that children with galactosemia have 3.5 times the odds of having a co-occurring coordination disorder compared to the general population with developmental disorders. Of the three domains assessed by the MABC, manual dexterity (speed and accuracy), ball skills (eye-hand coordination), and balance (Henderson and Sugden 1992), balance was severely affected and manual dexterity was moderately affected in children with galactosemia. Children with CAS or dysarthria had poorer balance and manual dexterity than most of the children with MSD-NOS, providing further evidence that a common etiology may underlie motor speech and coordination disorders. As poor balance is characteristic of cerebellar involvement, it was not surprising that the single participant with ataxic dysarthria had significant balance difficulties; however, we did not expect the children with CAS to have equally poor balance, consistent with cerebellar involvement in CAS.

Determining potential predictors of outcomes and a progression of outcome severity in galactosemia has been challenging as each individual is uniquely affected (Waisbren et al. 2012). This lack of a predictable progression of outcomes was evident in the puzzling finding that tongue strength, but not hand strength, was related to poor balance and manual dexterity in participants with galactosemia and MSD-NOS and the lack of association between strength and coordination in participants most severely affected by disordered speech, strength, and coordination. The difference in relationships among coordination and strength across genders provides more evidence that galactosemia differentially affects individuals. For males with galactosemia, poor coordination was related to decreased strength, but the same association was not apparent in females.

Days on Milk

Previous studies (Berry and Elsas 2011; Jumbo-Lucioni et al. 2012) have not found clear associations among long-term complications in galactosemia and the number of days of lactose ingestion during the neonatal period. In the present study, strength and balance were not associated with early dietary lactose ingestion in the children with galactosemia; however, the days of milk ingestion was related to a mild decrease in speech articulation (PCC:AT and PCC:CS) in males only. There was no association between days on milk and percentage of speech errors in females. The association between speech errors and days on milk may be a result of the increased vulnerability of males to neurodevelopmental disorders, a well-documented but poorly understood finding (Pieters et al. 2012). Our findings support the importance of early notification of galactosemia during the neonatal period (Berry 2012), especially for males, as delays in notification and adoption of a lactose-restricted diet may have long-term adverse effects on speech development. Galactosemia is autosomal recessive and occurs equally in males and females. Interestingly in our sample, which had speech disorders as inclusionary criteria, twice as many families with boys volunteered compared to family with girls. This coincides with the 2:1 male–female incidence of speech and coordination disorders observed in the general population (Pieters et al. 2012; Johnson and Breslau 2000) raising the question for future study of a possible higher incidence of these disorders in males vs. females with galactosemia.

Common Underlying Etiology

The high co-occurrence of motor disorders, the decrease in tongue and hand strength in children with galactosemia and speech disorders, and the poorer balance and manual dexterity observed in children with the most speech errors, support the proposal that motor and speech disorders may be due to a common underlying etiology rather than two distinct disorders (Gaines and Missiuna 2007; Pieters et al. 2012). Imaging studies of individuals with CAS (Belton et al. 2003) and individuals with galactosemia (Dubroff et al. 2008; Hughes et al. 2009) both cite cerebellar deficits (in addition to basal ganglia (striatum) and cerebral left-hemisphere pre- and primary motor areas). Diverse areas of the cerebellum are involved in maintaining balance, refining motor movements, motor learning, and speech production (Doya 2000; Penhune and Steele 2012). Balance is mediated primarily near midline of the cerebellum (Stoodley and Schmahmann 2009), speech in the superior lateral area of the right cerebellar hemisphere, and manual dexterity and motor planning in the right and left lateral cerebellar hemispheres, ipsilateral to the affected hand. Since areas of the cerebellum associated with motor planning, motor movements, and refining speech are remote from the cerebellar areas associated with balance control, the association between severity of the speech deficits and balance suggests a common underlying etiology associated with diffuse damage to the cerebellum rather than distinct focal areas of damage. In future imaging

studies, the superior lateral and midline areas of the cerebellum, in addition to basal ganglia (striatum) and cerebral left hemisphere pre- and primary motor areas, should be considered regions of interest.

Limitations and Recommendations

The findings of this study are limited by the criteria used for participant recruitment. The present study was part of a larger study to delineate the speech characteristics associated with CAS in rare disorders, so only children with galactosemia who had received or were currently receiving speech therapy services and only control participants with no history of speech disorders were included. There is a second possible recruitment bias as parents of children with galactosemia and severe speech disorders may have been more likely to volunteer to participate. In addition, the differences between groups on measures of speech and strength may be inflated, as the controls had not received special education services.

Based on our findings, some suggestions for future research and practice can be made: (1) a motor skills assessment should be included in studies examining long-term outcomes in classic galactosemia, (2) males and females should be analyzed separately as they are differentially affected by galactosemia, and (3) healthcare professionals following children with galactosemia should screen for motor as well as speech disorders.

Acknowledgments This research was supported by National Institute on Deafness and Other Communication Disorders Grant DC000496 and by a core grant to the Waisman Center from the National Institute of Child Health and Development (Grant HD03352). We thank the following colleagues for their contributions to this study: Heather Karlsson, Heather Lohmeier, Jane McSweeny, Leslie Power, Lola Rickey, Sue Siemsen, Christie Tilkens, The Galactosemia Foundation, Galactosemic Families of Minnesota, and the children and parents who participated in this study.

One Sentence Synopsis

Children with classic galactosemia and speech disorders are at risk for co-occurring strength and coordination disorders.

Details of the Contributions of Individual Authors

Nancy L. Potter conducted the study and drafted the manuscript, Yves Nievergelt performed the data analyses and assisted in drafting the manuscript, Lawrence D. Shriberg was the PI on the grant that funded the present study, assisted in planning the study, analyzed the speech samples, and edited the manuscript.

Name of One Author Who Serves as Guarantor

Nancy L. Potter accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

Details of Funding

This research was supported by National Institute on Deafness and Other Communication Disorders Grant DC000496 and by a core grant to the Waisman Center from the National Institute of Child Health and Development (Grant HD03352). The authors confirm independence from the sponsors; the sponsors have not influenced the content of the article.

Details of Ethics Approval

The Institutional Review Boards of the University of Wisconsin-Madison and Washington State University approved this study.

Patient Consent Statement

A parent of each participant provided written consent, children age 12 years and older provided written consent, and children 11 years and younger provided written or verbal informed assent.

References

Belton E, Salmond CH, Watkins KE, Vargha-Khadem F, Gadian DG (2003) Bilateral brain abnormalities associated with dominantly inherited verbal orofacial dyspraxia. Hum Brain Mapp 18:194–200

Berry GT (2012) Galactosemia: when is it a newborn screening emergency? Mol Genet Metab 106:7–11

Berry GT, Elsas LJ (2011) Introduction to the Maastricht workshop: lessons from the past and new directions in galactosemia. J Inherit Metab Dis 34:249–255

Doya K (2000) Complementary roles of basal ganglia and cerebellum in learning and motor control. Curr Op Neurobiol 10:732–739

Dubroff JG, Ficicioglu C, Segal S, Wintering NA, Alavi A, Newberg AB (2008) FDG-PET findings in patients with galactosaemia. J Inherit Metab Dis 31:533–539

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

Gaines R, Missiuna C (2007) Early identification: are speech/language-impaired toddlers at increased risk for developmental coordination disorder? Child Care Health Dev 33:325–332

Gallup AC, White DD, Gallup GG Jr (2007) Handgrip strength predicts sexual behavior, body morphology, and aggression in male college students. Evolution Human Behav 28:423–429

Goldman R, Fristoe M (2000) Goldman Fristoe test of articulation, 2nd edn. MN, AGS Publishing, Circle Pines

Gubbels CS, Welt CK, Dumoulin JC, Robben SG et al (2012) The male reproductive system in classic galactosemia: cryptorchidism and low semen volume. J Inherit Metab Dis [Epub ahead of print].

Häger-Ross C, Rösblad B (2002) Norms for grip strength in children aged 4–16 years. Acta Paediatr 91:617–625

Henderson SD, Sugden DA (1992) The movement assessment battery for children. The Psychological Corporation, London

Hughes J, Ryan S, Lambert D, Geoghegan O, Clark A, Rogers Y, Hendroff U, Monavari A, Twomey E, Treacy EP (2009) Outcomes of siblings with classical galactosemia. J Pediatr 5:721–726

Northwest IOPI (2005) Iowa oral performance instrument: user's manual. IOPI Medical LLC, Carnation, WA

Johnson EO, Breslau N (2000) Increased risk of learning disabilities in low birth weight boys at age 11 years. Biol Psychiatry 47:490–500

Jumbo-Lucioni PP, Garber K, Kiel J et al (2012) Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. J Inherit Metab Dis 35:1037–49.

Lof GL, Watson MM (2008) A nationwide survey of nonspeech oral motor exercise use: implications for evidence-based practice. Lang Speech Hear Serv Sch 39:392–407

MATLAB version R2012a (7.14.0.739) (2012) Natick, Massachusetts, The MathWorks Inc.

Murdoch BE, Attard MD, Ozanne AE, Stokes PD (1995) Impaired tongue strength and endurance in developmental verbal dyspraxia: a physiological analysis. Int J Lang Comm Dis 30:51–64

Penhune VB, Steele CJ (2012) Parallel contributions of cerebellar, striatal and M1 mechanisms to motor sequence learning. Behav Brain Res 226:579–591

Pieters S, De Block K, Scheiris J et al (2012) How common are motor problems in children with a developmental disorder: rule or exception? Child Care Health Dev 38:139–145

Potter NL (2011) Voice disorders in children with classic galactosemia. J Inherit Metab Dis 34:377–385

Potter NL, Hall S, Karlsson HB et al (2012) Reference data for the Madison Speech Assessment Protocol (MSAP): A Database of 150 Participants 3-to-18 Years of Age with Typical Speech (Tech. Rep. No. 18). Phonology Project, Waisman Center, University of Wisconsin-Madison

Potter NL, Lazarus JA, Johnson JM, Steiner RD, Shriberg LD (2008) Correlates of language impairment in children with galactosaemia. J Inherit Metab Dis 31:524–532

Raynor AJ (2001) Strength, power, and coactivation in children with developmental coordination disorder. Dev Med Child Neurol 43:676–684

Robin DA, Somodi LB, Luschei ES (1991) Measurement of tongue strength and endurance in normal and articulation disordered subjects. In: Moore CA, Yorkston KM, Beukelman DR (eds) Dysarthria and apraxia of speech: perspectives on management. Paul H Brookes Pub Co, Baltimore, pp 173–184

Shriberg LD, Fourakis M, Hall SD et al (2010) Extensions to the Speech Disorders Classification System (SDCS). Clin Linguist Phon 24:795–824

Shriberg LD, Potter NL, Strand EA (2011) Prevalence and phenotype of childhood apraxia of speech in youth with galactosemia. J Speech Lang Hear Res 54:487–519

Stoodley CJ, Schmahmann JD (2009) Functional topography in the human cerebellum: a meta-analysis of neuroimaging studies. Neuroimage 44:489–501

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

Waisbren SE, Potter NL, Gordon CM et al (2012) The adult galactosemic phenotype. J Inherit Metab Dis 35:279–286

JIMD Reports
DOI 10.1007/8904_2013_220

CASE REPORT

Defect of Cobalamin Intracellular Metabolism Presenting as Diabetic Ketoacidosis: A Rare Manifestation

Sheetal Sharda · Suresh Kumar Angurana · Mandeep Walia · Savita Attri

Received: 03 September 2012 / Revised: 27 February 2013 / Accepted: 28 February 2013 / Published online: 2 April 2013

Abstract Hypoglycemia is the usual feature of commonly occurring organic acidemias. Organic acidemias manifesting as hyperglycemia or diabetic ketoacidosis are rare and only a few cases have been reported. We report a 13-month-old boy who presented with vomiting, dehydration, coma, hyperglycemia, high anion gap metabolic acidosis and ketosis, mimicking diabetic ketoacidosis (DKA). Treatment with parenteral fluid, electrolytes, and insulin infusion resulted in an improvement in hyperglycemia, but persistence of metabolic acidosis and lack of improvement of neurologic status led us to suspect an organic acidemia. Urinary organic acid analysis revealed increased methylmalonic acid levels. In addition, hyperhomocysteinemia and homocystinuria were also noted in presence of normal vitamin B_{12} levels. This confirmed the diagnosis of cobalamin metabolism defect leading to combined methylmalonic aciduria and homocystinuria. There was some improvement in neurologic status and metabolic parameters after treatment with low-protein diet, vitamin B_{12}, folic acid, and L-carnitine, but he ultimately succumbed to polymicrobial nosocomial sepsis. The entire MMACHC gene of the patient was sequenced and no mutations were identified. This is probably the first case report of cobalamin intracellular metabolism defect (CblC/CblD/CblF/CblJ or ABCD4) presenting as diabetic ketoacidosis.

Communicated by: Verena Peters

Competing interests: None declared

S. Sharda · S.K. Angurana · M. Walia · S. Attri (✉)
Dept of Pediatrics, Post Graduate Institute of Medical Education and Research, 160012, Chandigarh, India
e-mail: attrisavi@yahoo.co.in

Introduction

Metabolic acidosis and ketosis are the hallmark features of organic acidemias. Hypoglycemia is frequently associated with acute episodes of decompensation in most commonly encountered organic aciduria like methylmalonic aciduria, propionic aciduria, isovaleric aciduria, multiple carboxylase deficiency, and 3-oxothiolase deficiency. Hypoglycemia is possibly due to inhibition of gluconeogenesis through various mechanisms: inhibition of malate shuttle by methylmalonic acid and inhibition of pyruvate carboxylase by methylmalonyl-CoA, inhibition of multiple enzymes by propionyl-CoA and accumulation of organic acid-CoA esters through acylcarnitine formation (Worthen et al. 1994). However, metabolic acidosis and ketosis with hyperglycemia is a rare feature of methylmalonic aciduria (Guven et al. 2012), propionic aciduria (Dweikat et al. 2011), and biotinidase deficiency (Hou 2004). We present a presumed case of cobalamin metabolism defect presenting as diabetic ketoacidosis (DKA).

Derivatives of cobalamin (vitamin B_{12}) are required for activity of two enzymes. Adenosylcobalamin (AdoCbl) is required for activity of mitochondrial enzyme methylmalonyl CoA mutase (MUT) which converts methylmalonyl-CoA to succinyl-CoA, and methylcobalamin (MeCbl) is required for activity of cytoplasmic enzyme methionine synthase (MS) that catalyzes methylation of homocysteine to form methionine (Froese and Gravel 2010; Watkins and Rosenblatt 2011). Deficiency in cobalamin or impaired absorption of cobalamin can result in accumulation of methylmalonic acid and homocysteine in blood and serum homocysteine and urine homocystine. Inborn errors affecting cobalamin absorption (inherited intrinsic factor deficiency, Imerslund–Gräsbeck syndrome) and transport (transcobalamin deficiency) have been described

(Watkins and Rosenblatt 2011). A series of inborn errors of intracellular cobalamin metabolism, designated as cblA, cblB, cblC, cblD, cblE, cblF, cblG, and cblJ, have been differentiated by complementation analysis (Fowler et al. 2008; Coelho et al. 2012; Kim et al. 2012). These can give rise to isolated methylmalonic acidemia (cblA, cblB, cblD variant 2), isolated hyperhomocysteinemia (cblD variant 1, cblE, cblG), or combined methylmalonic acidemia and hyperhomocysteinemia (cblC, cblD, cblF, cblJ). Transcobalamin II deficiency and transcobalamin receptor deficiency have also been described with inborn errors of cobalamin absorption and metabolism, but it is not clear that they have any consistent clinical phenotype (Watkins and Rosenblatt 2011).

All these disorders are inherited as autosomal recessive traits. The genes underlying each of these disorders have been identified (Froese and Gravel 2010; Watkins and Rosenblatt 2011). Approximately 400 patients have been described with cblC, making it the most common disorder of intracellular vitamin B_{12} metabolism (Lerner-Ellis et al. 2009). The gene responsible for the cblC group is MMACHC (Lerner-Ellis et al. 2006). More than 50 different disease-causing mutations have been identified (Lerner-Ellis et al. 2009). It was recognized that cblF represents a defect in the export of cobalamin out of the lysosome into the cytosol (Rosenblatt et al. 1985). The gene responsible for cblF was very recently cloned and found to correspond to LMBRD1, which encodes the lysosomal membrane protein LMBD1 (Rutsch et al. 2009). The gene responsible for cblD is MMADHC (Coelho et al. 2008).

Combined methylmalonic acidemia and homocysteinemia can present with a wide spectrum of clinical manifestations ranging from intrauterine period to late infantile and also in adults. Here, we describe a patient with a form of combined methylmalonic aciduria and homocystinuria who presented with diabetic ketoacidosis. To the best of our knowledge, this is the first case of a combined cobalamin metabolism defect, most possibly due to cblC or cblD or cblF or cblJ deficiency, who presented clinically with diabetic ketoacidosis (DKA). Physicians should be aware of these diverse clinical presentations in order to provide an early diagnosis and to guide management of affected individuals and to screen other family members.

Case Report

A 13-month-old boy presented with a short history of fever, cough, vomiting, deep sighing breathing, and lethargy. His condition soon deteriorated to respiratory distress and coma requiring hospitalization in the pediatric emergency. He was the first child born to a non-consanguineous couple, delivered normally with a birth weight of 2,700 g, and had no adverse perinatal events. The child had no previous history of episodes of vomiting, seizures, lethargy, respiratory distress that required hospitalization, polyuria, polydipsia, or abnormal weight loss. He was developmentally normal for age. The past medical history was unremarkable; he was on mixed vegetarian and nonvegetarian diet with daily calorie and protein deficit of 200 Kcal and 5 g, respectively; and he was not on any vitamin supplements prior to this illness. Physical examination on admission showed an afebrile and comatose child with a Glasgow Coma Scale (GCS) of 7/15. His pulse rate was 150 beats/min, respiration was deep and sighing (Kussmaul respiration) with rate of 34/min, and blood pressure of 100/64 mm Hg. His weight, length, and head circumference was 8 kg (3rd percentile), 76 cm (75th percentile), and 46 cm (70th percentile) for age, respectively. The initial laboratory tests showed hyperglycemia (random blood glucose – 284 mg/dL), metabolic acidosis (pH – 7.09, bicarbonate – 8 mmol/L, and base excess – 20 mmol/L), high anion gap (AG – 29 mmol/L), and presence of ketones and glucose in urine. His hemoglobin was 10.2 g/dL, total leukocyte count 16,000/mm^3 (neutrophils 70%, lymphocytes 30%), platelets 1, 56, 000/mm^3, and calcium 9.5 mg/dL. Liver and renal function tests and other serum electrolytes were normal as were chest X-ray and abdominal ultrasound. No focus of sepsis was found. Based on clinical presentation and investigations, a provisional diagnosis of diabetic ketoacidosis was made. The child was intubated for low a GCS and mechanical ventilation was initiated. Intravenous (IV) fluids with recommended concentration of electrolytes were started along with insulin infusion at a rate of 0.1 unit per kg per hour (Milwaueke regimen protocol of the unit). With this therapy, there was improvement in hyperglycemia within 24 h. Despite the adequate oxygenation and tissue perfusion and correction of hyperglycemia, the metabolic acidosis, anion gap, and clinical condition failed to improve and his coma persisted. The lack of clinical response and persisting metabolic acidosis prompted us to investigate for an alternative diagnosis. An inborn error of metabolism, most likely an organic acidemia, was suspected and screening tests were sent. The child was found to have hyperammonemia [181μmol/L; normal <74 μmol/L] and elevated lactate [5.4 mmol/L; normal <1.5mmol/L]. High performance liquid chromatography (HPLC) showed increased plasma homocysteine concentration [40.1 μmol/L; normal range: 5–15 μmol/L], low methionine [5.6 μmol/L; normal range: 15–54 μmol/L], normal glycine [140 μmol/L; normal range: 100–300 μmol/L], and increased excretion of homocystine in urine [16.4 mmol/mol creatinine; normal range: 0.2–4 mmol/mol creatinine]. Urine gas chromatography–mass spectrometry (GCMS) analysis revealed increased excretion of methylmalonic acid

[141mmol/mol creatinine; normal: <9 mmol/mol creatinine]. Both vitamin B_{12} [455 pg/mL; normal range: 200–900 pg/mL] and folate levels (14 ng/mL) were normal. The diagnosis of methylmalonic aciduria with homocystinuria was made. Subsequently, insulin was discontinued and the baby was started on L-carnitine, pyridoxine, vitamin B_{12}, and folic acid along with a low-protein diet. After the fifth day of hospitalization, the general condition and neurologic status gradually improved along with some improvement in biochemical parameters: random blood glucose – 178 mg/dL; pH – 7.25; bicarbonate – 16 mmol/L; AG – 18 mmol/L; plasma ammonia – 94 μmol/L; and lactate – 2.2 mmol/L. He remained admitted in the hospital for 10 days and succumbed to polymicrobial sepsis (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*), in presence of multiple risk factors such as long hospital stay, presence of invasive lines, and mechanical ventilation. Since the glucose intolerance improved with insulin (for short duration) and metabolic therapy, further evaluation for type 1 diabetes mellitus was not undertaken. Sequencing of the entire MMACHC gene of the patient revealed no mutations, while sequencing of MMACHC gene of parents was not done (planned on follow-up).

Discussion

Many disorders of inborn errors of metabolism (IEMs) lead to hypoglycemia and ketoacidosis, including methylmalonic acidemia, propionic acidemia, isovaleric acidemia, maple syrup urine disease, congenital lactic acidosis, defects of gluconeogenesis and glycogen synthesis, and ketolytic defects (Henriquez et al. 1994; Worthen et al. 1994; Marles and Casiro 1998; Ozand 2000). Inborn errors of metabolism must always be considered as a possible diagnosis when an infant presents with a severe metabolic acidosis accompanied by an increased anion gap. When we consider severe metabolic acidosis with hyperglycemia and ketosis, DKA is always highest on the list of diagnosis. However, in case of lack of improvement in the clinical or metabolic profile even after appropriate therapy, one should always have a high index of suspicion for IEMs, and further investigational workup should be done.

Our patient was admitted with severe metabolic acidosis precipitated by an acute febrile illness. Severe ketoacidosis and hyperammonemia are typical features of acute forms of aminoacidopathies, including MMA, that typically present in the first weeks of life with complaints of poor feeding and lethargy, progressing to coma. The older infants or children may present with lethargy, seizures, muscular hypotonia, and hypoglycemia during an episode of metabolic decompensation that is often associated with an intercurrent illness (Guven et al. 2012). Although hyperglycemia is an unusual presentation for MMA, Boeckx and Hicks for the first time in 1982 reported a newborn female with severe and persistent metabolic acidosis and hyperglycemia resistant to large doses of insulin, who excreted large amounts of methylmalonic acid in urine, but the patient died before any further investigations (Boeckx and Hicks 1982). Mathew et al. also reported a newborn girl with transient diabetes mellitus in association with MMA. She died at the age of 6 months (Mathew and Hamdan 1988). Filippi et al. reported a newborn suffering from acute neonatal-onset MMA who presented acutely with dehydration, ketoacidosis, hyperammonemia, and insulin-resistant hyperglycemia (Filippi et al. 2009). Ciani et al. reported a case of late-onset cbl-B MMA in a 12-year-old female who presented acutely with vomiting, fever, bronchopneumonia, and coma associated with hyperglycemia, ketoacidosis, and hyperammonemia. She was misdiagnosed as a case of insulin-dependent diabetes mellitus (IDDM) and died 3 days later despite receiving IV insulin (Ciani et al. 2000).Two of the most recent cases of diagnosed MMA presenting as DKA include a 13-month-old female child (Guven et al. 2012) and a 14-month-old male child who presented with fever, vomiting, acute generalized dystonia, and lethargy (Imen et al. 2012). Investigations showed hyperglycemia, lactic acidosis, and hyperammonemia. Urinary organic acid analysis showed accumulation of methylmalonic acid, tiglylglycine, and methyl citrate which led to the diagnosis of MMA. He survived the metabolic crises (Imen et al. 2012). All these case reports concluded that hyperglycemia was a rare manifestation of MMA and could be a marker for worse prognosis (Table 1).

The index patient presented at 13 months of age with respiratory distress, progressive loss of consciousness, hyperglycemia, and metabolic ketoacidosis, mimicking DKA. Only after poor clinical response to appropriate management, the diagnosis of organic acidemia was considered and confirmed by appropriate tests. The administration of intravenous insulin and fluids led to improvement in blood glucose. Further evaluation revealed elevated levels of methylmalonic acid and homocystine in urine as well as raised homocysteine in the plasma. A defect in the intracellular metabolism of cobalamin could explain this combined metabolic abnormality in the index child. A low-protein diet, L-carnitine, pyridoxine, vitamin B_{12}, and folic acid could have contributed to some recovery in our patient though he later on succumbed to nosocomial sepsis. Although we were unable to pursue a complete evaluation for a cobalamin disorder, which should include obtaining a skin biopsy for fibroblast cellular biochemical studies and the procurement of patient and parental DNA for mutation studies (Fowler et al. 2008), our limited

Table 1 Summary of reported cases of methylmalonic acidemia that presented with hyperglycemia/diabetic ketoacidosis along with details of index case

S. No.	Reported patients	Age at onset, sex	Clinical features	Laboratory features[a]	Outcome
1	**Index patient**	13 months, male	Fever, cough, vomiting, dehydration, coma	Hyperglycemia, HAGMA, ketosis, elevated ammonia and lactate, methylmalonic aciduria, hyperhomocysteinemia, and homocystinuria	Died
2	**Boeckx et al. 1982**	3 weeks, female	Recurrent episodes of projectile vomiting, poor feeding, rapid respirations, and pallor	Hyperglycemia which was insulin resistant, HAGMA, ketonuria, hematuria, hypocalcemia, increased excretion of 3-hydroxybutyric acid, methylmalonic acid, 3-hydroxyvaleric acid, and 3-keto-n-valeric acid in urine	Died
3	**Mathew et al. 1986**	11 days, female	Poor feeding, drowsiness, rapid breathing, dehydration	Transient hyperglycemia, HAGMA, ketonemia, ketonuria, hypocalcemia, pancytopenia, high urine MMA	Died at 6 months
4	**Abramowicz et al.** 1994	Shortly after birth, female	Small for gestation age	Persisting hyperglycemia, acidosis, high urine methylmalonic acid, isodiosomy of chromosome 6, and agenesis of pancreatic beta cells (postmortem examination)	Died on day 16
5	**Federica et al. 2000**	12 years, female	Fever, vomiting, bronchopneumonia, coma	Hyperglycemia, ketosis, HAGMA, hyperammonemia, lactic acidosis, large amount of lactic acid, MMA, and methylcitric acid in aqueous humor (postmortem)	Died
6	**Filippi et al.** 2009	Newborn, male	Dehydration	Insulin-resistant hyperglycemia, ketoacidosis, hyperammonemia, high urine MMA	Died
7	**Guven et al. 2011**	13 months, female	Polyuria, polydipsia, weight loss, dehydration, acidotic breathing, lethargy, vomiting, loss of sensorium	Hyperglycemia, HAGMA, ketosis, hyperammonemia, high lactic acid, high excretion of MMA in urine	Survived
8	**Imen et al.** 2012	13 months, male	Fever, lethargy, vomiting, acute generalized dystonia	Hyperglycemia, lactic acidosis, hyperammonemia, increased urinary MMA, tiglylglycine, and methylcitrate	Survived

HAGMA high anion gap metabolic acidosis, *MMA* methylmalonic acid, *GCMS* gas chromatography–mass spectrometry

[a] None of the reported patients except the index case had hyperhomocysteinemia or homocystinuria

evaluation suggests the patient did not have cblC deficiency, but did appear to have a distinct inborn error of cobalamin metabolism.

After internalization, vitamin B_{12} enters a metabolic cascade to generate Mecbl in the cytsol and Adocbl in the mitochondria. At least eight different defects in the intracellular metabolism of cobalamin have been identified (Watkins and Rosenblatt 2011). These are designated cblA to cblJ (except cblH) (Fowler et al. 2008; Coelho et al. 2012; Kim et al. 2012). Defects of cblA, cblB, and cblD (variant II) cause MMA only. The cblE and cblG defect result in homocystinuria without MMA. In patients with cblC or cblD or cblF or cblJ defects, synthesis of both adenosylcobalamin and methylcobalamin is impaired, causing hyperhomocysteinemia in addition to MMA (Watkins and Rosenblatt 2011). Clinicians should be aware of diverse clinical presentations of combined methylmalonic aciduria and homocystinuria in order to provide an early diagnosis and to guide management of affected individuals. The workup in all patients with suspected cobalamin IEMs should include a skin biopsy for cellular biochemical studies and collection of parental samples for future genomic studies (after the proper consent) (Fowler et al. 2008). Unfortunately, we were unable to perform these tests before our patient expired, which are must for proper evaluation of cobalamin IEMs.

Isolated MMA masquerading as DKA or in combination with hyperglycemia has been reported in literature (Boeckx and Hicks 1982; Mathew and Hamdan 1988; Abramowicz et al. 1994; Ciani et al. 2000; Filippi et al. 2009; Guven et al. 2012; Imen et al. 2012) (Table 1). The mechanism of hyperglycemia in the setting of organic acidemias remains poorly understood and is possibly multifactorial. Unlike in previously described cases, the hyperglycemia in our patient was not insulin resistant (Boeckx and Hicks 1982; Ciani et al. 2000; Filippi et al. 2009). Other rare but possible differential diagnoses to be considered in an acute presentation with severe metabolic acidosis presenting with hyperglycemia instead of hypoglycemia includes other IEMs, e.g., fructose-1,6-diphosphatase enzyme deficiency

(Paksu et al. 2011) and also mitochondrial disorder such as Kearns–Sayre syndrome (Bachynski et al. 1986). The cobalamin intracellular metabolism defect [possibly cblC, cblD, cblF, and cblJ] presenting as DKA has not been described previously.

Conclusion

The unusual presentation in the index patient of cobalamin metabolism defect as DKA reminds us of the wide clinical spectrum of IEMs. Though diabetes mellitus is the commonest cause of hyperglycemia and metabolic ketoacidosis, IEMs should be suspected, especially when clinical deterioration occurs despite appropriate therapy. The etiology and impact of hyperglycemia on morbidity and mortality in patients with cobalamin metabolism defects need further evaluation.

Acknowledgments We acknowledge Dr Lisa Kratz, Director, Biochemical Genetics Laboratory, Kennedy Krieger Institute, Baltimore (USA) for GCMS analysis.

Conflicts of Interest

None

Funding

None

Take-Home Message

Inborn errors of cobalamin metabolism can present with hyperglycemia and ketoacidosis, mimicking classical diabetic ketoacidosis. Testing for metabolic disorders in new onset diabetes patients should be considered, especially when response to traditional therapy is incomplete.

References

Abramowicz MJ, Andrien M et al (1994) Isodisomy of chromosome 6 in a newborn with methylmalonic acidemia and agenesis of pancreatic beta cells causing diabetes mellitus. J Clin Invest 94(1):418–421

Bachynski BN, Flynn JT et al (1986) Hyperglycemic acidotic coma and death in Kearns-Sayre syndrome. Ophthalmology 93(3): 391–396

Boeckx RL, Hicks JM (1982) Methylmalonic acidemia with the unusual complication of severe hyperglycemia. Clin Chem 28(8): 1801–1803

Ciani F, Donati MA et al (2000) Lethal late onset cblB methylmalonic aciduria. Crit Care Med 28(6):2119–2121

Coelho D, Suormala T et al (2008) Gene identification for the cblD defect of vitamin B12 metabolism. N Engl J Med 358(14): 1454–1464

Coelho D, Kim JC et al (2012) Mutations in ABCD4 cause a new inborn error of vitamin B12 metabolism. Nat Genet 44(10): 1152–1155

Dweikat IM, Naser EN et al (2011) Propionic acidemia mimicking diabetic ketoacidosis. Brain Dev 33(5):428–431

Filippi L, Gozzini E, et al (2009). Insulin-resistant hyperglycaemia complicating neonatal onset of methylmalonic and propionic acidaemias. J Inherit Metab Dis 32 Suppl 1:S179–86

Fowler B, Leonard JV et al (2008) Causes of and diagnostic approach to methylmalonic acidurias. J Inherit Metab Dis 31(3):350–360

Froese DS, Gravel RA (2010) Genetic disorders of vitamin B(1)(2) metabolism: eight complementation groups–eight genes. Expert Rev Mol Med 12:e37

Guven A, Cebeci N et al (2012) Methylmalonic acidemia mimicking diabetic ketoacidosis in an infant. Pediatr Diabetes 13(6):e22–25

Henriquez H, el Din A, et al (1994) Emergency presentations of patients with methylmalonic acidemia, propionic acidemia and branched chain amino acidemia (MSUD). Brain Dev 16 Suppl: 86–93

Hou JW (2004) Biotin responsive multiple carboxylase deficiency presenting as diabetic ketoacidosis. Chang Gung Med J 27(2): 129–133

Imen M, Hanene B et al (2012) Methylmalonic acidemia and hyperglycemia: an unusual association. Brain Dev 34(2): 113–114

Kim JC, Lee NC et al (2012) Late onset of symptoms in an atypical patient with the cblJ inborn error of vitamin B12 metabolism: diagnosis and novel mutation revealed by exome sequencing. Mol Genet Metab 107(4):664–668

Lerner-Ellis JP, Tirone JC et al (2006) Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Nat Genet 38(1):93–100

Lerner-Ellis JP, Anastasio N et al (2009) Spectrum of mutations in MMACHC, allelic expression, and evidence for genotype-phenotype correlations. Hum Mutat 30(7):1072–1081

Marles SL, Casiro OG (1998) Persistent neonatal hypoglycemia: diagnosis and management. Paediatr Child Health 3(1):16–19

Mathew PM, Hamdan JA (1988) Transient diabetes mellitus in neonatal methylmalonic aciduria. J Inherit Metab Dis 11(2): 218–219

Ozand PT (2000) Hypoglycemia in association with various organic and amino acid disorders. Semin Perinatol 24(2): 172–193

Paksu MS, Kalkan G et al (2011) Gluconeogenesis defect presenting with resistant hyperglycemia and acidosis mimicking diabetic ketoacidosis. Pediatr Emerg Care 27(12):1180–1181

Rosenblatt DS, Hosack A et al (1985) Defect in vitamin B12 release from lysosomes: newly described inborn error of vitamin B12 metabolism. Science 228(4705):1319–1321

Rutsch F, Gailus S et al (2009) Identification of a putative lysosomal cobalamin exporter altered in the cblF defect of vitamin B12 metabolism. Nat Genet 41(2):234–239

Watkins D, Rosenblatt DS (2011) Inborn errors of cobalamin absorption and metabolism. Am J Med Genet C Semin Med Genet 157(1):33–44

Worthen HG, al Ashwal A et al (1994) Comparative frequency and severity of hypoglycemia in selected organic acidemias, branched chain amino acidemia, and disorders of fructose metabolism. Brain Dev 16 Suppl: 81–85

JIMD Reports
DOI 10.1007/8904_2013_221

CASE REPORT

Cerebral Magnetic Resonance Spectroscopy Demonstrates Long-Term Effect of Bone Marrow Transplantation in α-Mannosidosis

Else R. Danielsen • Allan M. Lund • Carsten Thomsen

Received: 07 August 2012 / Revised: 27 February 2013 / Accepted: 01 March 2013 / Published online: 24 April 2013

Abstract α-Mannosidosis, OMIM #248500, is an autosomal recessive lysosomal storage disease caused by acidic α-mannosidase deficiency. Treatment options include bone marrow transplantation (BMT) and, possibly in the future, enzyme replacement therapy. Brain magnetic resonance spectroscopy (MRS) enables non-invasive monitoring of cerebral treatment effect. Accumulated cerebral mannose-containing oligosaccharides were demonstrated by MRS in a patient who at age 2 years and 11 months received a BMT from a haploidentical non-carrier sibling. The cerebral mannose-containing oligosaccharides had disappeared as early as 9½ months after BMT. MRS furthermore demonstrated the persistent treatment effect at regular intervals up to 5½ years after BMT. MRS is a non-invasive tool that can demonstrate the effect of BMT treatment. Likewise, MRS may be used to demonstrate the cerebral effect of other potential treatments such as enzyme replacement therapy.

Communicated by: Frits Wijburg, MD, PhD

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2013_221) contains supplementary material, which is available to authorized users.

E.R. Danielsen (✉) · C. Thomsen
9, Blegdamsvej, Section X3023, DK-2100, Copenhagen Ø, Denmark
e-mail: else.rubaek.danielsen@regionh.dk

A.M. Lund
9, Blegdamsvej, Section 4062, DK-2100, Copenhagen Ø, Denmark

Introduction

α-Mannosidosis, OMIM #248500, is an autosomal recessive lysosomal storage disease caused by acidic α-mannosidase deficiency. Patients accumulate mannose-rich oligosaccharides within the lysosomes. Three forms of the disease are described, though the phenotypes of the patients represent a continuum with vast clinical variation: There is mild adult onset (type 1) at one end of the spectrum, at the other end a severe form (type 3), and finally an intermediate, moderate form (type 2) (Chester et al. 1982; Malm and Nilssen 2008).

Findings on neuroimaging include periventricular white matter changes, enlarged Virchow-Robin spaces, enlarged suprasellar cistern, narrow foramen magnum, and small posterior fossa (Ara et al. 1999; Dietemann et al. 1990; Niemann et al. 1996; Gutschalk et al. 2004).

Results of magnetic resonance spectroscopy (MRS) have not previously been reported in severe α-mannosidosis. In a report of three patients with adult-onset α-mannosidosis, MRS was normal (Gutschalk et al. 2004). Avenarius et al. (2011) performed MRS on three patients with moderate type 2 α-mannosidosis, one of whom had received a bone marrow transplantation 6 years prior to MRS; they found an abnormally broad peak representing oligosaccharides in the untreated patients, and this peak was not present in the patient, who had undergone bone marrow transplantation.

Feline models of α-mannosidosis showed abnormal apparent diffusion coefficients, hyperintensities in white matter on T_2-weighted magnetic resonance images (Vite et al. 2001, 2008), and MRS showed a broad signal in the 3.4–4.3 ppm region thought to represent mannose-rich oligosaccharides (Magnitsky et al. 2010).

The mannose-rich oligosaccharides accumulating in α-mannosidosis contain mostly two to four mannose

groups, but also branched or linear variants with up to nine mannose groups are found (Van Halbeek et al. 1980; Strecker et al. 1976; Abraham et al. 1986; Malm and Nilssen 2008). In vivo human MRS does not allow for individual characterization of the mannose-rich oligosaccharides, but it may complement urine analysis and provide in vivo measurements of the oligosaccharides in the brain where the composition and amounts may not necessarily reflect those found in urine. Hård et al. showed that the relative amounts of the different mannose-rich oligosaccharides differ in urine and brain (Hård et al. 1991) in a feline model of α-mannosidosis.

The purpose of this report is to demonstrate abnormal mannose-rich oligosaccharides by in vivo cerebral MRS in a patient with type 2 α-mannosidosis in the severe end of the spectrum, and to show the immediate and long-term effect of allogenous bone marrow transplantation (BMT) on presence of brain mannose-rich oligosaccharides and on normal cerebral metabolites seen on MRS in the same individual patient. Similarly, Takahashi et al. demonstrated the effect of BMT in patients with mucopolysaccharidoses using MRS (Takahashi et al. 2001).

Patient

The patient was a girl with severe type 2 α-mannosidosis in the severe end of the spectrum with early presenting signs of skeletal and CNS disease from about 5 months of age. She was compound heterozygous for c.2248C>T and c.2426T>C in *MAN2B1*. At age 2 years and 11 months, she received a BMT from a haploidentical non-carrier sibling. Psychomotor development was judged 6 to 9 months delayed at age 2½ years. Six months after BMT, biochemical analyses showed normalized excretion of urine oligosaccharides and normal leucocyte α-mannosidase activity, and both remained normal hereafter. Psychomotor development judged at age 6 years remained delayed with motor skills 2 years delayed and language skills about 1 year delayed.

Methods

The patient was evaluated by magnetic resonance imaging and spectroscopy, in general anaesthesia. Examinations were performed 8½ months before bone marrow transplantation, and 9½ months, 22 months, 3½ years, and 5½ years after. Brain magnetic resonance imaging used standard T_1-, T_2-, and diffusion-weighted imaging protocols. The last examination was supplemented by fluid attenuated inversion recovery (FLAIR). The short echo time magnetic resonance spectra were sampled from three volumes of interest located in the occipital grey matter, parieto-occipital white matter, and centrum semiovale, using methods as suggested by Elberling et al. (2003). The first three examinations were performed on a 1.5 Tesla Siemens Vision scanner (STEAM, TE 20 ms, TR 3000 ms, 86 acquisitions), the remaining on a 3 Tesla Siemens Trio scanner (PRESS, TE 30 ms, TR 3000 ms, 80 acquisitions). Magnetic resonance spectra were analysed by the manufacturers' post-processing software for the purpose of spectral display. Post-processing of the spectra included eddy-current correction, zero filling, minimal Gaussian apodization (time constant 512 ms), fast Fourier transform, phase correction, and filtration of the residual water signal, but no corrections of the baseline was employed. The fully automated and user-input-independent quantitative post-processing analysis LCModel (Provencher 1993) was used for quantitation of cerebral metabolites. The mannose-containing oligosaccharide resonance complex (MC) was quantified using the LCModel basis-set with an additional simulated basis accounting for the broad resonance detected at baseline. Due to the broad nature of the MC, absence of MC was defined by the following three requirements: (1) the ratio MC/Cr gave a value similar to values obtained in normal controls, when MC was included in the basis-set; (2) excluding MC from the basis-set gave fitting results with normal baselines and residuals; (3) the broad hump between 3.5 and 3.9 ppm was undetectable by visual inspection. Routinely used neurometabolic markers of disease development included N-acetylaspartate (NAA), myo-inositol (mI), choline containing compounds (Cho), and glutamine+glutamate (Glx). They were calculated as ratios of metabolites related to total creatine (Cr) and compared to the normal controls used in the clinic for the relevant scanners. Values outside ± 2 SD were considered abnormal.

Results

Brain magnetic resonance imaging showed mild, diffuse hyperintense signals on T_2-weighted images and increased apparent diffusion coefficients in white matter near the lateral horns in locations not overlapping with MRS volumes of interest. The apparent diffusion coefficient was normal in normal-appearing tissue. The imaging findings were stable throughout the observation period. FLAIR images obtained only from the last examination showed discrete signal abnormalities, also only at the lateral horns.

Brain MRS prior to bone marrow transplantation showed abnormally broad resonances in the 3.5–3.9 ppm region. It was clearly visible in the baseline spectrum from centrum semiovale and parieto-occipital white matter, but in the baseline spectrum from occipital grey matter, it was at the

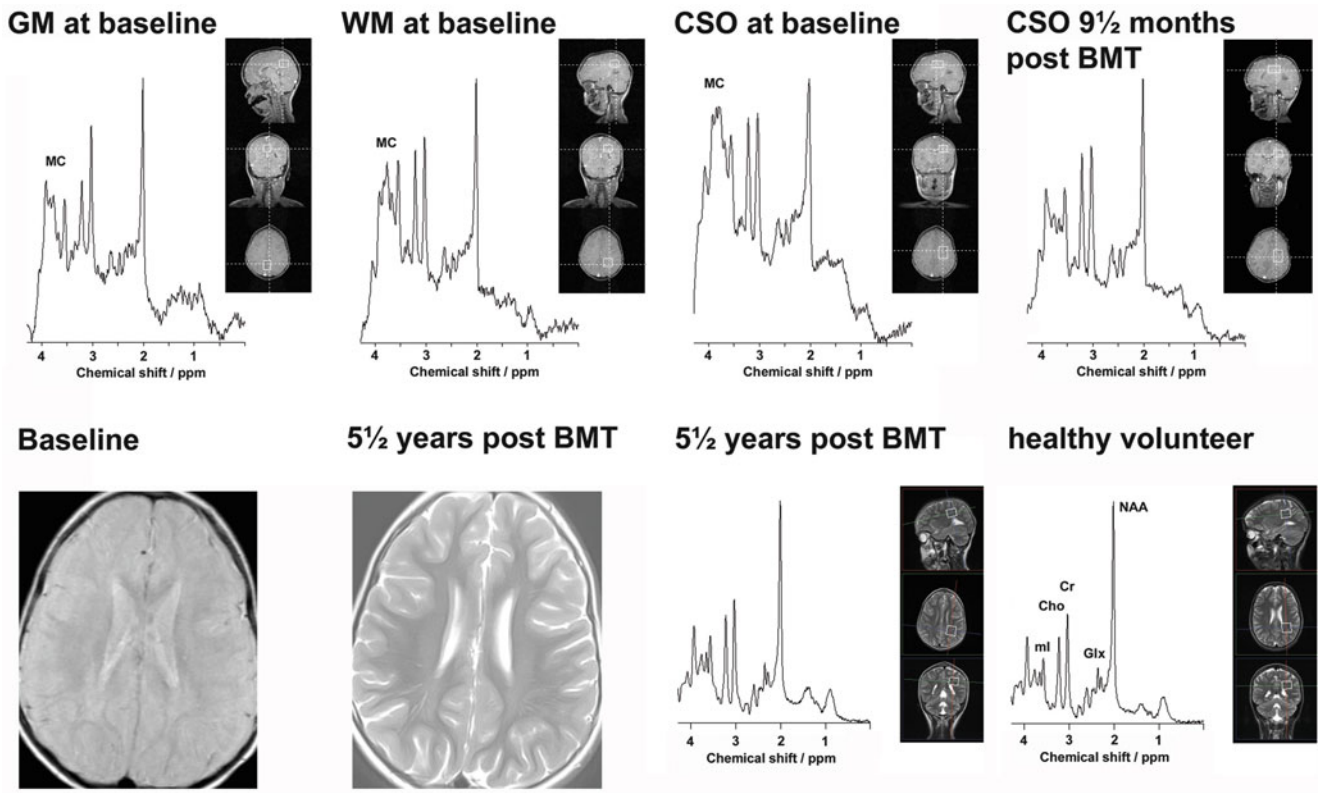

Fig. 1 The short echo time magnetic resonance spectra (MRS) from grey matter (GM), white matter (WM), and centrum semiovale (CSO) at baseline 8½ months before bone marrow transplantation and from CSO 9½ months after bone marrow transplantation demonstrate disappearance of the mannose-containing oligosaccharide resonance complex (MC) after treatment (MRS from GM and WM also had normalized, not shown). These four spectra were sampled at 1.5 T using the pulse sequence STEAM. Brain magnetic resonance imaging showed mild, diffuse hyperintense signals on T_2-weighted images without temporal evolution; images shown here are MRI at baseline (1.5 T) and MRI 5½ years after BMT (3 T). The persistent absence of the mannose-containing oligosaccharide resonance complex (MC) is illustrated by MRS from WM in the patient 5½ years after BMT in comparison to MRS from a healthy volunteer measured using identical conditions. These two spectra were sampled at 3 T using the pulse sequence PRESS

detection limit only. The broad resonance complex could be identified as a combination of mannose-rich oligosaccharides. Quantitation of the individual mannose-containing oligosaccharides was not attempted, because the information in the detected broad resonance was insufficiently detailed. The abnormal pretreatment MC signal however was quite prominent compared to findings done at the first follow-up 9½ months after BMT, when it had disappeared (Fig. 1). All the following MRS studies demonstrated continued absence of the MC in the spectra (Fig. 1).

Analysis of the NAA/Cr, Cho/Cr, mI/Cr, and Glx/Cr ratios in grey and white matter measured during the five examinations showed normal values compared to normal controls with a few exceptions, Cho/Cr in grey matter and mI/Cr in white matter: In the grey matter, Cho/Cr was 30% decreased at the fourth examination and 25% decreased at the fifth examination. In the white matter, the mI/Cr ratio was 40% increased at baseline, at the second examination it was 40% increased, at the third it was 30% increased, at the fourth it was 35% increased, and finally it was 20% increased indicating a tendency towards normalization of mI/Cr over time.

Discussion

Information on the natural course of early presenting type 2 α-mannosidosis is limited. As our patient progressed relatively quickly, BMT was offered and this successfully stopped further progression. The metabolic benefit of the intervention was demonstrated by brain MRS that showed the disappearance of MC within 9½ months after the BMT. Future studies of BMT treated α-mannosidosis patients should include earlier follow-up to find the earliest time for intracerebral removal of MC.

This study has for the first time demonstrated the effect of BMT in the same individual. Avenarius et al. (2011) demonstrated MC in two untreated patients and absence of MC in a BMT treated patient, but this patient may not have had increased MC in the brain before BMT. Gutschalk et al. (2004) reported three untreated cases, all without MC.

Including this study, a total of six untreated patients with α-mannosidosis have been studied, and only half showed elevated MC. The frequency and correlation to symptoms of elevated cerebral MC in untreated patients remains to be studied in a larger population.

Five examinations were carried out during a period of 6 years, monitoring the effect of BMT on neurometabolism in the patient presented here. The last two examinations showed a mild reduction of Cho/Cr ratio compared to normal grey matter; the significance of this is unknown, but it may reflect a minor abnormality of the composition of grey and white matter in the volume of interest.

All examinations showed elevated mI/Cr ratio compared to normal white matter. Over time, mI/Cr ratio moved towards normal values. Likewise, data from Gutschalk et al. (2004) showed a non-significant tendency towards elevated mI/Cr ratio. Elevated mI/Cr ratio in white matter was detected in MRI normal-appearing tissue and may be a result of gliosis or demyelination reflecting MRI invisible damage that has occurred before treatment became effective. Another possibility that cannot be excluded as part of the reason for the elevated mI/Cr ratio is residual underlying MC, too little to be recognized by in vivo MRS, but sufficient to affect mI/Cr.

Preclinical animal studies have shown efficacy of enzyme replacement therapy in mice and guinea pigs, including data to suggest that the enzyme crosses the blood-brain barrier (Blanz et al. 2008; Roces et al. 2004; Crawley et al. 2006). Whether the enzyme replacement therapy passes the blood-brain barrier in humans, however, remains to be demonstrated. Another possible future avenue of treatment may be gene therapy. Such developments of less-invasive treatments hopefully will replace BMT in the future. We have demonstrated that MRS may be a biomarker of disease activity after BMT. This opens the possibility that in vivo cerebral studies could be carried out in future studies of treatment alternatives to BMT in α-mannosidosis.

Conclusion

Brain MRS has detected MC at baseline and demonstrated its disappearance after successful BMT in a patient with α-mannosidosis. Future studies with evaluation of new treatment protocols including enzyme replacement therapies should include brain MRS to monitor the intracerebral efficiency of the treatments.

References

Abraham D, Daniel P, Dell A et al (1986) Structural analysis of the major urinary oligosaccharides in feline alpha-mannosidosis. Biochem J 233(3):899–904

Ara JR, Mayayo E, Marzo ME et al (1999) Neurological impairment in alpha-mannosidosis: a longitudinal clinical and MRI study of a brother and sister. Childs Nerv Syst 15(8):369–371

Avenarius DF, Svendsen JS, Malm D (2011) Proton nuclear magnetic resonance spectroscopic detection of oligomannosidic n glycans in alpha-mannosidosis: a method of monitoring treatment. J Inherit Metab Dis 34(5):1023–1027

Blanz J, Stroobants S, Lüllmann-Rauch R et al (2008) Reversal of peripheral and central neural storage and ataxia after recombinant enzyme replacement therapy in alpha-mannosidosis mice. Hum Mol Genet 17:3437–3445

Chester MA, Lundblad A, Öckerman PA et al (1982) Mannosidosis. In: Durand P, O'Brian J (eds) Genetic errors of glycoprotein metabolism. Edi-Ermes, Milan, Italy, pp 89–120

Crawley AC, King B, Berg T et al (2006) Enzyme replacement therapy in alpha-mannosidosis guinea-pigs. Mol Genet Metab 89(1–2):48–57

Dietemann JL, Filippi de la Palavesa MM, Tranchant C et al (1990) MR findings in mannosidosis. Neuroradiology 32(6): 485–487

Elberling TV, Danielsen ER, Rasmussen AK et al (2003) Reduced myo-inositol and total choline measured with cerebral MRS in acute thyrotoxic Graves' disease. Neurology 60(1):142–145

Gutschalk A, Harting I, Cantz M et al (2004) Adult alpha-mannosidosis: clinical progression in the absence of demyelination. Neurology 63:1744–1746

Hård K, Mekking A, Kamerling JP et al (1991) Different oligosaccharides accumulate in the brain and urine of a cat with alpha-mannosidosis: structure determination of five brain-derived and seventeen urinary oligosaccharides. Glycoconj J 8(1):17–28

Magnitsky S, Vite CH, Delikatny EJ et al (2010) Magnetic resonance spectroscopy of the occipital cortex and the cerebellar vermis distinguishes individual cats affected with alpha-mannosidosis from normal cats. NMR Biomed 23(1):74–79

Malm D, Nilssen Ø (2008) Alpha-mannosidosis. Orphanet J Rare Dis 3:21

Niemann S, Beck M, Seidel G et al (1996) Neurology of adult alpha-mannosidosis. J Neurol Neurosurg Psychiatry 61(1):116–117

Provencher SW (1993) Estimation of metabolite concentrations from localized in vivo proton NMR spectra. Magn Reson Med 30(6): 672–679

Roces DP, Lüllmann-Rauch R, Peng J et al (2004) Efficacy of enzyme replacement therapy in alpha-mannosidosis mice: a preclinical animal study. Hum Mol Genet 13(18):1979–1988

Strecker G, Fournet B, Bouquelet S et al (1976) Chemistry of urinary mannosides excreted in mannosidosis 58(5):579–586

Takahashi Y, Sukegawa K, Aoki M et al (2001) Evaluation of accumulated mucopolysaccharides in the brain of patients with mucopolysaccharidoses by 1H-magnetic resonance spectroscopy before and after bone marrow transplantation. Pediatr Res 49(3): 349–355

Van Halbeek H, Dorland L, Veldink GA et al (1980) A 500 MHz 1H NMR study of urinary oligosaccharides from patients with mannosidosis. FEBS Lett 121(1):71–77

Vite CH, Magnitsky S, Aleman D et al (2008) Apparent diffusion coefficient reveals gray and white matter disease, and T2 mapping detects white matter disease in the brain in feline alpha-mannosidosis. AJNR Am J Neuroradiol 29(2):308–313

Vite CH, McGowan JC, Braund KG et al (2001) Histopathology, electrodiagnostic testing, and magnetic resonance imaging show significant peripheral and central nervous system myelin abnormalities in the cat model of alpha-mannosidosis. J Neuropathol Exp Neurol 60(8):817–828

JIMD Reports
DOI 10.1007/8904_2013_222

RESEARCH REPORT

Early Cardiac Changes in Children with Anderson–Fabry Disease

Stepan Havranek • Ales Linhart • Zuzana Urbanova • Uma Ramaswami

Received: 03 October 2012 / Revised: 27 February 2013 / Accepted: 28 February 2013 / Published online: 2 April 2013

Abstract Objective: Limited evidence is available about the early cardiac manifestation of Fabry disease (FD) in children. We aimed to evaluate cardiac involvement in children with FD by analysing serial structural and electrocardiographic changes.

Methods: The data were acquired from 22 children with FD [11 males; median age 9.8 (ranging 2.5–16) years]. Seven patients (5 males) were on enzyme replacement therapy (ERT) with Agalasidase alpha. Echocardiography, ECG and 24-h ECG monitoring recordings were acquired during routine annual clinical controls. ECG data were compared to a group of age-and gender-matched controls.

Results: At baseline, ECG and ECHO parameters of left ventricular mass were similar in both males and females. Three boys (all were on ERT) developed left ventricular hypertrophy (LVH) during two-year follow-up. The progression to LVH was accompanied by the appearance of frequent ventricular premature beats in two cases and supraventricular premature beats (SPBs) with T wave inversion in one case. T wave inversion and SPBs were detected in two younger relatives of a patient with LVH, in the absence of detectable LVH. Seven out of 22 patients had T wave abnormalities. Five of them were males ($p = 0.03$) all carrying the N215S mutation ($p = 0.03$). At baseline, median PR intervals were prolonged in FD subjects compared to controls [143 (122–177) vs. 122 (82–165) ms; $p < 0.0001$].

Conclusions: Cardiac complications of FD become apparent in childhood as subtle changes with slow but detectable progression over time, with males more frequently affected than females. Progression of LVH was apparent in three children despite ERT.

Communicated by: Verena Peters

Competing interests: None declared

S. Havranek · A. Linhart
Department of Medicine – Department of Cardiovascular Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic

Z. Urbanova
Department of Paediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic

U. Ramaswami (✉)
Willink Biochemical Genetics Unit, Central Manchester University Hospitals, NHS Foundation Trust, Oxford Road, Manchester M13 9WL, UK
e-mail: uma.ramaswami@cmft.nhs.uk

Abbreviations

AVB	AV block
ECHO	Echocardiography
ERT	Enzyme replacement therapy
FD	Fabry disease
Holter ECG	24-h ECG monitoring
HR	Heart rate
LV	Left ventricle
LVH	Left ventricular hypertrophy
M	Month
SPBs	Supraventricular premature beats
UR	Uma Ramaswami
VPBs	Ventricular premature beats
Y	Year

Introduction

Anderson–Fabry disease (FD) (OMIM Number 301500) is an X-linked inherited lysosomal storage disease caused by a defect in the gene encoding lysosomal enzyme α-galactosidase A (Desnick et al. 2003). The resulting

absence or severe deficiency of enzymatic activity leads to intracellular storage of glycosphingolipids, in particular globotriaosylceramide (Gb_3). This accumulation can be detected in different organs and tissues.

The storage within the heart affects all type of cells including cardiomyocytes, conduction system tissue, vascular endothelial and smooth muscle cells and valvular fibroblasts (Brady and Schiffmann 2000). Cardiac involvement represents the major cause of premature morbidity and mortality in adult patients with FD (Cybulla et al. 2007; Patel et al. 2011). Left ventricular hypertrophy (LVH) is the most frequent and well-recognised cardiac manifestation in adults (Linhart et al. 2000; Kampmann et al. 2002; Sachdev et al. 2002; Elliott et al. 2011). Other frequent complications and changes in adult patients with FD include atrial fibrillation, non-sustained ventricular tachycardia, QRS broadening, shortening of PR interval or atrio-ventricular blocks (AVBs) and sinus node dysfunction (Mehta et al. 1977; Pochis et al. 1994; Shah et al. 2005). Several patients may develop mild to moderate valve insufficiencies, dilatation of the proximal aorta, arterial hypertension and coronary artery disease (Linhart et al. 2000; Kampmann et al. 2002; Nagueh 2003; Kleinert et al. 2006; Elliott et al. 2006; Barbey et al. 2010).

Symptoms of the disease typically start in childhood and subtle organ changes can be detected early (Ries et al. 2003; Ramaswami et al. 2006; Tøndel et al. 2008). However, life-threatening complications appear almost exclusively in adult patients. Severe arrhythmias, LVH and cardiac failure in children were not reported. Very little is known about cardiac changes in children. Only Kampmann et al. demonstrated that cardiac manifestations in FD are detectable already in paediatric population as well (Kampmann et al. 2008b).

Therefore, the aim of our study is to evaluate early onset cardiac manifestations in children with FD by analysing serial changes on electrocardiography (ECG), 24-h ECG monitoring (Holter ECG) and echocardiography.

Methods

Study Population

We performed a retrospective single-site observational analysis of available electrocardiographic (standard ECG and Holter ECG data) and echocardiographic recordings from 22 paediatric patients with FD acquired during systematic annual visits. All children were under the care of Uma Ramaswami (UR) in a tertiary centre in the Paediatric Metabolic Unit, Addenbrooke's Hospital, Cambridge, UK between the years 2005 and 2011. Patients were included to the clinical care program for patients with FD having either a newly established diagnosis of FD or being identified as affected relatives of patients with already known FD. All patients included to the study had the disease confirmed by the identification of the underlying mutation within the α-galactosidase A gene. Clinical, ECG, Holter ECG and echocardiography recordings were acquired during routine clinical visits performed on annual basis. Clinical examination was performed in all children by a single clinician (UR). Anonymised data provided by UR were compiled and processed by an independent observer during his short stay in Addenbrooke's Hospital. The research passport was obtained specifically to review link-anonymised data. No data were exported outside Addenbrooke's Hospital. The protocol of analysis was approved by the local institutional ethics board.

Seven patients (32%) received enzyme replacement therapy (ERT) based on fulfilling UK National Guidelines for Treatment of FD. All subjects were treated with Agalasidase alpha (Shire Human Genetic Therapies, Cambridge, MA) at the dose of 0.2 mg/kg delivered biweekly as a 40-min intravenous infusion.

Baseline ECG parameters were compared to a control population of age- and gender-matched healthy children without evidence of cardiovascular and metabolic diseases. The healthy controls were recruited prospectively from children investigated in a preventive program of the Department of Paediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague, Czech Republic. The anonymised data were acquired exclusively for the purpose of this study and analysed by the same observer as the data from FD patients.

Echocardiography

Echocardiography was done at the annual review for FD patients as per the National UK guidelines.

Complete echocardiographic examinations were performed according to the recommendations of American Society of Echocardiography (Douglas et al. 2011). For the purpose of the study, we calculated left ventricular (LV) mass by using the Devereux modified formula (Devereux et al. 1986). LV mass was then indexed to body height (in metres squared to 2.7). LVH was defined as LV mass > 95th percentile for the population of the given age (Khoury et al. 2009).

ECG Data and Holter Monitoring

Conventional 12-channel resting ECG analysis included measurement of following parameters: heart rhythm; heart rate (HR); conduction intervals: PR, QRS, QT/QTc; heart axis; T wave polarity in V5 and V6 and voltage or

Table 1 Baseline clinical and demographical characteristics

	FD patients (total) ($n = 22$)	Males ($n = 11$)	Females ($n = 11$)
Male	11 (50%)	–	–
Age at baseline (years)	9.8 (2.5–16)	7 (4–14)	9.5 (2.5–16.5)
Enzyme activity < 2 nmol/mg per hour	12 (55%)	9 (82%)	3 (27%)
Typical symptoms of FD	19 (86%)	11 (100%)	9 (82%)
ERT anytime	7 (32%)	5 (45.5%)	2 (18%)
ERT baseline	4 (18%)	3 (27%)	1 (9%)

FD Fabry disease, *ERT* enzyme replacement therapy with Agalasidase alpha

Value expressed as: No (%) or median (range)

voltage/duration markers of LVH: Sokolow–Lyon voltage, Cornell voltage, Cornell index. QTc was calculated according to Bazzet's formula. Sokolow–Lyon voltage was defined as sum of voltages of S in V1 and R in V5 or V6 (whichever was higher). The sum of voltages of S in V3 and R in aVL constituted Cornell voltage. Cornell index was calculated as the product of Cornell voltage multiplied by QRS duration. The measurements were performed on digitised paper tracings which are acquired as standard recordings at 25 mm/s paper speed with the amplitude of 1 mV/mm. Holter ECG monitoring was used to obtain following parameters: heart rhythm, HR (maximal, minimal and mean), occurrence and type of arrhythmias. Special concern was given about pauses and bradycardia episodes, conduction blocks; supraventricular (SPBs) and ventricular premature beats (VPBs) and both supraventricular and ventricular tachyarrhythmias. Frequently, both SPBs and VPBs were defined as occurrence of more than 10 beats per 24 h.

Statistical Analysis

Statistical analysis was carried out using Statistica 6.1 (StatSoft, Inc., Tulsa, OK, USA) statistical package. Continuous variables were expressed as means with standard deviations after testing for normality of distribution (Shapiro Wilk's test) and compared by the two-tailed *t*-test for independent samples. Non-normally distributed variables were expressed as medians with range and compared by Mann–Whitney *U* test. Categorical variables were expressed as percentages and compared by $\chi 2$-test. A P-value < 0.05 was considered significant.

Results

Study Population Characteristics

The data were obtained from 22 children with FD from 16 families [11 males; baseline mean age 9.6 ± 4.6 years; median 9.8 (ranging 2.5–16.0) years]. The most frequently represented mutation was N215S type in 7 (32%) cases. Seven (32%) patients [5 males, mean age 8.6 ± 3.0; median 7.8 (ranging 4.3–14.3) years] were on ERT (Agalasidase alpha in all cases) during the entire study period, none of them carrying the N215S mutation. Baseline clinical and demographic data are shown in Table 1. At baseline, no significant differences between males and females were detected. The list of mutations, occurrence of symptoms and signs of FD, treatment and summary of abnormal findings is shown in Table 2.

The serial ECG, Holter ECG and echocardiography data were available in 22, 20 and 16 subjects at baseline, first and second annual control, respectively. At baseline, both ECG and ECHO parameters were comparable between FD patients and controls and no gender differences in both ECG and ECHO parameters of LVH were detected in patients with FD, as shown in Table 3.

The control group for baseline ECG comparison consisted of 44 children [22 males, mean age 9.1 ± 1.2 years; median 9.1 (ranging 5.2–10.9) years] of comparable age and gender.

Progression of Left Ventricular Hypertrophy

Figure 1 shows the proportion of patients with LV mass above 75th percentile and LVH at baseline and during follow-up. The number of patients with LV mass in the upper percentiles at baseline (> 75th percentile at baseline) progressively increased during the follow-up. Whilst no patient met the criteria of LVH at baseline, one developed LVH after the first year (patient #13) and two additional patients (#10 and 17) after the second year of follow-up. The characteristics of these patients are shown in Table 4. All three patients were males with very low or undetectable levels of enzyme activity, and all were symptomatic and receiving ERT with Agalasidase alpha already at baseline. All three had mutations in the α-galactosidase A gene known to be associated with the classical phenotype of FD. In these three patients, at least two baseline LVH

Table 2 List of mutation and case profiles

Mutation type	Patient# ($n = 22$)	Family# ($n = 16$)	Baseline age (Y/M)	Gender	Typical symptoms of FD	ERT	Detected abnormality
Gln107X	1	1	4/0	Male	Yes	No	No
N215S	2	2	13/6	Male	Yes	No	T wave inversion
	3	2	14/4	Male	Yes	No	No
	4	3	15/4	Female	Yes	No	T wave inversion
	5	4	14/1	Female	Yes	No	Prolonged PR interval
	6	5	4/5	Male	Yes	No	T wave inversion
	7	6	4/10	Male	Yes	No	T wave inversion
	8	7	15/1	Female	Yes	No	T wave inversion
N298H	9	8	10/1	Female	Yes	No	No
A143T	10	9	7/10	Male	Yes	Yes	LVH + T wave inversion + SPBs
	11	9	6/5	Male	Yes	Yes	T wave inversion
p.N33D	12	10	9/6	Male	Yes	No	No
c.950T>C	13	11	6/9	Male	Yes	Yes	LVH + VPBs
c.802-3_802-2delCA	14	12	11/6	Female	Yes	No	No
R301X	15	13	16/0	Female	No	No	No
	16	13	7/7	Female	Yes	No	No
R49S	17	14	14/4	Male	Yes	Yes	LVH + VPBs + sinus bradycardia
	18	14	10/9	Female	No	No	SPBs
P2005T	19	15	7/0	Male	Yes	Yes	No
W209X	20	16	10/5	Female	Yes	Yes	No
	21	16	7/5	Female	Yes	No	SPBs
	22	16	2/6	Female	No	No	No

ERT enzyme replacement therapy with Agalasidase alpha, *LVH* left ventricular hypertrophy, *SPBs* supraventricular beats, *VPBs* ventricular beats, *Y* year, *M* month

Table 3 ECHO and ECG parameters of left ventricular hypertrophy at baseline

	FD patients (total) ($n = 22$)	FD patients males ($n = 11$)	FD patients females ($n = 11$)	ECG controls (total) ($n = 44$)	ECG controls males ($n = 22$)	ECG controls females ($n = 22$)
ECG						
Sokolow–Lyon voltage (mV)	2.7 (1.1–4.8)	2.8 (1.9–4.6)	2.7 (1.1–4.8)	2.9 (0.6–5.8)	2.9 (1.2–5.8)	2.8 (0.6–3.9)
Cornel voltage (mV)	1.1 (0.3–3.0)	1.8 (0.9–2.7)	0.9 (0.3–3.0)	1.6 (0.4–2.3)	1.7 (0.8–2.3)	1.6 (0.4–2.1)
Cornel index (mV * ms)	888 (267–2808)	1484 (753–2808)	781 (267–2808)	1050 (320–2070)	1123 (523–2070)	985 (320–1946)
Echocardiography						
LV mass index ($g/h^{2.7}$)	36 (24–46)	41 (36–45)	35 (24–46)	–	–	–

LV left ventricle, *FD* Fabry disease

Value expressed as: median (range)

parameters on ECG (Sokolow–Lyon voltage, Cornel voltage or Cornel index) were within the upper quartile of the values measured in FD male population. In contrast, their PR intervals were within interquartile range in male subgroup. Patient #10 manifested frequent SPBs before detection of LVH. In this patient, the repolarisation changes appeared at the same time as LVH. The remaining two patients demonstrated frequent VPBs detected within 1 year after the appearance of LVH. The last patient (#17) had asymptomatic sinus bradycardia.

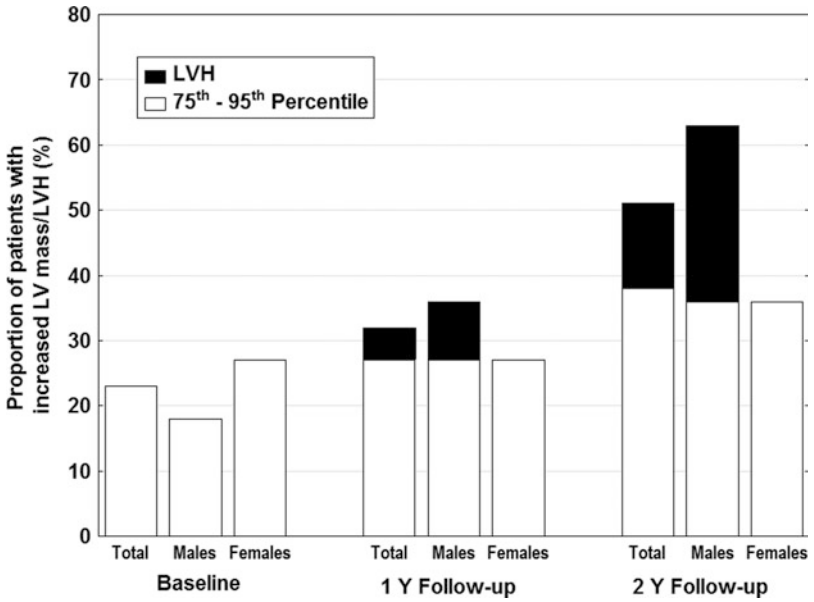

Fig. 1 *Proportion of patients with left ventricular mass ≥ 75th percentile and those with left ventricular hypertrophy at baseline and during follow-up.* LV mass ≥ 75th percentile – *white parts* of the column. LVH – *black parts* of the column. *LV* left ventricle, *LVH* left ventricular hypertrophy, *Y* year

Table 4 Profiles of three FD male patients with progression of left ventricular mass to left ventricular hypertrophy

Patient #	Mutation	ERT since baseline	Typical clinical signs of FD	Detected (year of follow-up)	Enzyme activity (nmol/mg per hour)	Baseline Sokolow–Lyon voltage (mV)	Baseline Cornel voltage (mV)	Baseline Cornel index (mV * ms)	Changes in Holter ECG	Repolarisation changes
10	A143T	Yes	Yes	3rd	UD	2.3	2.7	2808	SPBs in 2nd year	Yes
13	c.950T > C	Yes	Yes	2nd	<0.5	3.7	3.0	2467	VPBs in 3rd year	No
17	R49S	Yes	Yes	3rd	<0.5	3.9	2.2	1936	Sinus bradycardia + VPBs in 3rd year	No

FD Fabry disease, *M* male, *ERT* enzyme replacement therapy with Agalasidase alpha, *UD* undetectable, *SPBs* supraventricular premature beats, *VPBs* ventricular premature beats

12-Leads ECG Parameters

As shown in Table 5, PR intervals at baseline were prolonged in patients with FD as compared to controls. Moreover, PR intervals after the first and second year of follow-up were significantly shorter in boys than in girls. This significance was lost when one female outlier was excluded. The outlier (patient # 5) is a symptomatic 14-year-old girl carrying the N215S mutation not receiving ERT, with a reduced enzyme activity. She progressively exceeded the normal value ranges of PR at the second year of follow-up, Fig. 2.

Mean HR, median QRS, median QT and QTc intervals in FD patients were not different from healthy control group and did not change over time.

The T wave inversion indicating repolarisation changes in lateral leads were present in four (18%) patients with FD at baseline. In contrast, such finding was not found in the control group. The number of patients with T wave inversion increased over time. At the end of 2-year follow-up, seven FD patients (32%) had T wave abnormalities. Of note, five of the seven patients with T wave abnormalities were males ($p = 0.03$ vs. females) and five had the N215S mutation ($p = 0.03$ vs. other mutations).

Table 5 ECG parameters at baseline and during follow-up

	FD patients (total) ($n = 22$)	FD patients males ($n = 11$)	FD patients females ($n = 11$)	ECG controls (total) ($n = 44$)	ECG controls males ($n = 22$)	ECG controls females ($n = 22$)
HR (bpm)	77 (63–120)	80 (65–120)	78 (63–112)	85 (64–118)	82 (66–114)	92 (64–118)
1 Y follow-up	77 (59–112)	83 (69–112)	77 (59–112)	–	–	–
2 Y follow-up	79 (59–104)	86 (68–89)	81 (59–104)	–	–	–
PR (ms)	143 (122–177)*	145 (122–166)	143 (122–177)	122 (82–165)	121 (105–155)	133 (82–165)
1 Y follow-up	144 (127–185)	135 (127–148)	145 (127–185)***	–	–	–
2 Y follow-up	144 (118–186)	138 (118–151)	146 (118–186)***	–	–	–
QRS (ms)	79 (67–104)	81 (70–104)	79 (67–103)	81 (52–95)	82 (61–93)	77 (52–95)
1 Y follow-up	82 (68–89)	86 (69–89)	80 (68–87)	–	–	–
2 Y follow-up	86 (67–91)	89 (68–91)	86 (67–91)	–	–	–
QT (ms)	355 (288–408)	345 (302–408)	356 (288–408)	330 (295–405)	354 (312–405)	312 (295–340)
1 Y follow-up	357 (305–397)	349 (307–371)	357 (305–397)	–	–	–
2 Y follow-up	333 (315–390)	329 (315–365)	333 (315–390)	–	–	–
QTc (ms)	393 (372–459)	395 (384–459)	393 (372–459)	412 (362–444)	391 (362–433)	422 (368–444)
1 Y follow-up	393 (368–446)	405 (374–446)	393 (368–446)	–	–	–
2 Y follow-up	391 (368–426)	394 (380–426)	391 (368–426)	–	–	–
T wave inversion	4 (18%)*	4 (36%)	0 (0%)**	0 (0%)	0 (0%)	0 (0%)
New onset at 1 Y	1 (4.5%)	1 (9%)	0 (0%)	–	–	–
New onset at 2 Y	2 (9%)	0 (0%)	2 (18%)	–	–	–

HR heart rate, *PR, QRS, QT, QTc* conduction intervals on ECG, *FD* Fabry disease, *Y* year

Value expressed as: No (%) or median (range)

*$p < 0.05$ (compare FD vs. controls)

**$p < 0.05$ (compare males vs. females)

***$p < 0.05$ (compare males vs. females), see the text – the difference is lost when one female outlier is excluded from analysis

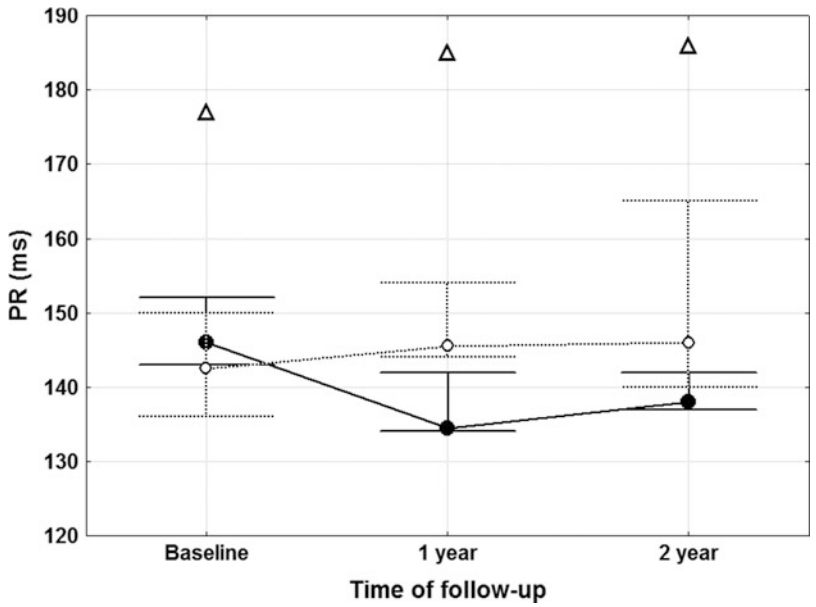

Fig. 2 *PR intervals at baseline and during follow-up in paediatric patients with Fabry disease*. For explanation, see the text. Male Fabry patients – *closed circles*, *solid line*. Female Fabry patients – *open circles*, *dotted line*. *Open triangle* – outlier female patient. *Points* and *whiskers* represent medians and quartile ranges values, respectively

Table 6 Holter ECG parameters at baseline and during follow-up

	FD patients (total) ($n = 22$)	FD males ($n = 11$)	FD females ($n = 11$)
Mean HR (bpm)	85 (72–109)	89 (78–98)	82 (72–109)
1 Y follow-up	84 (70–102)	87 (75–96)	83 (70–102)
2 Y follow-up	80 (71–103)	89 (79–103)	79 (71–88)
Min HR (bpm)	59 (50–77)	63 (50–77)	58 (53–70)
1 Y follow-up	59 (48–72)	60 (48–72)	58 (49–71)
2 Y follow-up	60 (47–75)	62 (54–75)	58 (47–65)
Max HR (bpm)	143 (114–188)	159 (114–188)	140 (118–175)
1 Y follow-up	141 (118–179)	149 (131–163)	136 (118–179)
2 Y follow-up	141 (120–163)	153 (131–163)	138 (120–143)
Supraventricular premature beats	8 (36.5%)	3 (27%)	5 (45%)
New onset in 1 Y	2 (9%)	0 (0%)	2 (18%)
New onset in 2 Y	2 (9%)	1 (9%)	1 (9%)
Ventricular premature beats	11 (50%)	7 (64%)	4 (36%)
New onset in 1 Y	2 (9%)	0 (0%)	2 (18%)
New onset in 2 Y	0 (0%)	0 (0%)	0 (0%)
Bradycardia episodes	1 (4.5%)	0 (0%)	1 (9%)
New onset in 1 Y	1 (4.5%)	1 (9%)	0 (0%)
New onset in 2 Y	0 (0%)	0 (0%)	0 (0%)
Sinus arrest	2 (9%)	1 (9%)	1 (9%)
New onset in 1 Y	2 (9%)	1 (9%)	1 (9%)
New onset in 2 Y	1 (4.5%)	1 (9%)	0 (0%)
AVB first degree	1 (4.5%)	0 (0%)	1 (9%)
New onset in 1 Y	0 (0%)	0 (0%)	0 (0%)
New onset in 2 Y	1 (4.5%)	0 (0%)	1 (9%)
AVB second degree (type 1)	4 (18%)	2 (18%)	2 (18%)
New onset in 1 Y	0 (0%)	0 (0%)	0 (0%)
New onset in 2 Y	1 (4.5%)	1 (9%)	0 (0%)
Junctional beats	9 (40%)	4 (36%)	5 (45%)
New onset in 1 Y	2 (9%)	1 (9%)	1 (9%)
New onset in 2 Y	0 (0%)	0 (0%)	0 (0%)
Ventricular escaped	1 (4.5%)	0 (0%)	1 (9%)
New onset in 1 Y	0 (0%)	0 (0%)	0 (0%)
New onset in 2 Y	0 (0%)	0 (0%)	0 (0%)

HR heart rate, *AVB* atrio-ventricular block, *FD* Fabry disease, *Y* year
Value expressed as: No (%) or mean ± SD; median (range)

Only one patient with isolated T wave inversion was on ERT with Agalasidase alpha. Of note, one of the boys with T wave inversion (patient #11) was the brother (aged 6 years at baseline) of a patient #10 with manifest LVH and T wave abnormality.

Holter ECG Parameters

No serious arrhythmias were detected on Holter ECG monitoring in any of the study subgroups, neither at baseline nor during follow-up. Only minor changes were present with increasing manifestation rate, Table 6. We detected only SPBs and VPBs which were considered clinically benign. Of note, two asymptomatic girls presented with frequent SPBs. The first patient (aged 11 years at baseline with mildly reduced enzyme activity) is the younger sister of patient # 17 with LVH. The second patient (# 21) was a girl (aged 7 years at baseline with mildly reduced enzyme activity). Her two sisters had manifest classical symptoms of FD without cardiac involvement.

There were no clinically important brady-arrhythmias including serious sinus arrests or AVB of advanced degree observed in this cohort of patients. Only two patients demonstrated intermittent first-degree AVB (both female)

and five patients presented with second-degree AVB type 1 during study period (3 boys). The incidence of all minor changes was not gender dependent.

Discussion

The major finding of our study is the early detection of subtle cardiac changes in children with FD. Although no patient met criteria for LVH at baseline, three males progressed to LVH during the follow-up. The progression towards LVH was accompanied with the appearance of abnormalities on ECG and Holter ECG (i.e. premature beats and T wave inversions). The progression of disease occurred in some cases despite established ERT with Agalasidase alpha. Some ECG abnormalities were detectable in younger siblings of these rapidly progression patients. In addition, we found a longer PR interval as compared to healthy gender- and age-matched controls in both males and females. Interestingly, detected conduction abnormalities were separated from occurrence of LVH in time.

Left Ventricular Mass and Left Ventricular Hypertrophy

LVH is the most frequent cardiac manifestation of FD, and thereby FD represents an important differential diagnosis in patients with unexplained hypertrophic cardiomyopathy (Kampmann et al. 2002; Sachdev et al. 2002; Monserrat et al. 2007; Elliott et al. 2011). In contrast to other hypertrophic cardiomyopathies, FD-associated hypertrophy of significant degree seldom develops in young patients (Kampmann et al. 2008a). In agreement with previous data (Kampmann et al. 2008b), we have found only mildly increased LV mass with its slight progression over time, more so in boys than in girls with FD. However, three boys had fulfilled the definition of LVH during the follow-up. Progression of LVH was apparent in three children on ERT, and therefore the timing of ERT in children requires attention. Moreover, the manifestation of LVH was accompanied with other ECG and Holter ECG abnormalities in all three cases: T wave inversion in the first case, frequent VPBs in the second case and frequent VPBs with sinus bradycardia in the third case. Some other abnormalities on Holter ECG (frequent SPBs) preceded the LVH by one year in first case (patient # 10) emphasising the importance of annual follow-up. As LVH seems to respond to the therapy only before it reaches a significant degree (Weidemann et al. 2009), its early detection appears to be of paramount importance since its appearance advocates early enzyme replacement initiation. The association of LVH with described ECG abnormalities underlines the significant role of at least annual ECG and Holter ECG monitoring.

T Wave Inversion

In our cohort, only one patient presented both T wave inversion and LVH. One younger brother to the patient with LVH manifested T wave inversion without detectable LVH. The ECG analysis of our patients shows relatively frequent changes in T wave polarity with progressive increase in incidence of these abnormalities. Our data is in concordance with previous literature in adult patients with FD, where the inversion of T wave was described as a frequent finding in combination with or without manifest LVH in adult patients with FD (Mehta et al. 1977; Linhart et al. 2002). We have found that the T wave inversion was predominantly expressed in male patients and those carrying N215S mutation. The progressively increasing polarity changes within lateral leads should be considered as abnormal and may reflect the impact of myocardial storage before the onset of manifest LVH.

It should be mentioned that T wave morphology, particularly in precordial leads, is progressively changing throughout childhood even in healthy subjects. The most stable pattern (upright polarity) of T wave in childhood is in leads V5–V6. Opposite to this T wave inversion is physiological in leads V1–V3 in children and normalises in relatively prolonged period during late childhood or early adolescence (Dickinson 2005). For this reason in our study, T wave polarity was evaluated in lateral leads only.

ECG LV Mass Indexes

The ECG analysis of our cohort has shown that at least two parameters of LVH (Sokolow–Lyon voltage, Cornel voltage or Cornel index) were within the upper quartile of all values measured in total FD male population at baseline. On the other hand, in two patients with LVH one out of three parameters was not symmetrically elevated. In addition, differences in QRS voltage and LVH indices between male and female patients were minimal.

The ECG voltage parameters in our population were lower than previously reported by Kampmann (Kampmann et al. 2008). We hypothesise that this difference might be due to the wide variation in QRS amplitudes seen in normal children and adolescents (Kampmann et al. 2002; Rijnbeek et al. 2008b) or may be attributable to ERT administration in our cohort. ERT has been shown to reduce LV mass (Ries et al. 2006) and also in potentially preventing its onset (Weidemann et al. 2005; Mehta et al. 2009; Ramaswami et al. 2012). The likely influence of ERT is supported by our finding of lower LV mass in our cohort in comparison to previously reported data by Kampmann (Kampmann et al. 2008b).

24-h ECG Monitors

In patients with LVH, frequent VPBs and SPBs were noticed to appear before the onset of LVH. One patient with frequent SPBs was the younger sister of the patient with manifest LVH. Therefore, we hypothesise that premature beats may be considered as a marker of disease progression. Considering the limited number of patients, we have no clear evidence whether all patients with frequent SPBs will develop LVH. Nevertheless, the simultaneous existence of LVH and premature beats emerges as a potentially clinically important observation of our study.

The comparison of Holter ECGs obtained in Fabry patients with previously published cohorts of healthy children aged 7–16 years (Southall et al. 1981; Scott et al. 1980; Dickinson and Scott 1984) revealed that most of the abnormalities may be present in otherwise healthy children as well. As in healthy children, our Fabry patients had SPBs or VPBs in the majority as isolated beats with uniform morphology and usually with low frequency per 24 h. The HR variations in our study are within previously described normal ranges. Bradycardia and junctional rhythm episodes, sino-atrial blocks, AVB of first degree and pauses, including incidental and AVB of second degree Mobitz type I, have been detected also in all healthy groups particularly during sleep.

Our data have not shown any serious rhythm disturbances in paediatric FD patients. This is in contrast with findings described in adults with FD who present with high incidence of paroxysmal atrial fibrillation, non-sustained ventricular tachycardia, AVB and sinus node dysfunction (Shah et al. 2005; Frustaci and Chimenti 2007).

Conduction Intervals

Short PR intervals and QRS broadening have been shown as frequent findings in adult patients with FD on ECG (Mehta et al. 1977; Pochis et al. 1994). Shortening of PR interval occurs by rapid AV conduction and is often observed in early stage of FD (Mehta et al. 1977). Invasive studies excluded the presence of accessory bypass pathways causing PR interval changes and confirmed that PR shortening is result of accelerated atrio-ventricular nodal conduction (Jastrzebski et al. 2006; Aryana et al. 2008) or by shortening of atrial conduction characterised by shorter P wave duration (Namdar et al. 2011). The PR interval prolongs during aging in normal paediatric population (Rijnbeek et al. 2001; O`Connor et al. 2008). Significant increase of PR duraton with age was described also in the adult population of FD (O'Mahony et al. 2011). Our data showed an unexpected observation in children and adolescents. Patients with FD had baseline PR interval prolonged compared to healthy population. On comparison of genders, in females the PR interval prolonged over time. One female patient (case # 5) had PR interval length above the 98th percentile of PR interval for a given age derived from the normal population (Rijnbeek et al. 2001). When this outlier is excluded from the analysis, the PR intervals appear comparable in both genders. In addition, this girl manifested the slowest HR in both first year and second year of follow-up. An important aspect is that conduction abnormalities were separated from occurrence of LVH. This temporal separation implies more likely different mechanisms of both changes.

Our finding of longer PR intervals among patients with FD is, in part, in agreement with the observation that short PR is less frequent than originally reported (Namdar et al. 2010). Moreover, FD is known to be associated not only with short PR interval, but also with significant increases in PR interval duration with age in adults with FD and with high rates of AV conduction impairments, occurring particularly in older patients (O'Mahony et al. 2011). Broadening of QRS interval along with decreasing heart rate was described in adult patients with FD (Mehta et al. 1977; Pochis et al. 1994). In our study, the QRS and QTc interval duration as well as HR were found within the ranges of normal values for paediatric patients (Rijnbeek et al. 2001).

N215S Mutation

To our knowledge, we are reporting about the largest cohort of N215S paediatric patients. The N215S mutation in the alpha-galctosidase A gene is known to be associated with late-onset cardiac variant of the disease. In our cohort, it was the most frequent mutation and the patients presented mostly only subtle ECG changes including prolongation of PR interval or T wave abnormalities. N215S as a cardiac variant has been described previously (Eng et al. 1993b; Sachdev et al. 2002). No patient in our cohort who progressed to LVH had the N215S mutation. The absence of detection of LVH in N215S patients confirms the typically delayed onset of symptoms in this variant (Eng et al. 1993b). The late onset of symptoms was shown to be associated with the presence of significant residual enzymatic activity in patients with N215S mutation. This activity seems to prevent the accumulation of ceramide trihexoside storage in organs with a high cellular renewal or with lesser α-galactosidase A substrate turnover. This may explain why other symptoms of the disease (renal, neurological, gastrointestinal or cutaneous) do not develop early or are significantly attenuated (Eng et al. 1993a, b; Desnick et al. 2003). Subjects with N215S mutation develop clinically relevant symptoms (including LVH, electrophysiological abnormalities and heart failure symptoms) relatively late (usually after the third decade of age).

Although the authors are aware of anecdotal unpublished cases of FD patients with N215S mutation who have a more severe phenotype (unpublished; personal communication), these appear very rare. Longer-term follow-up of patients is essential to understand the natural history and disease progression.

Limitations

We recognise several limitations of our study including the relatively limited number of patients and the retrospective character of the analysis. However, this is a single centre study with regular follow-up by one experienced clinician. The follow-up was based on pre-specified National Guidelines and the data collection was satisfactory with only few data missing. Although our patients are not covering the entire infancy age distribution, it should be underlined that our comparisons between genders are relevant since males and females were of comparable age and the control group was age matched. The potential impact of ERT is unfortunately unavoidable since ERT is part of the current standards of care in symptomatic children with FD and based on National Guidelines for Treatment of FD. In our cohort, five boys and two girls (total 32 %) were receiving ERT with Agalasidase alpha. This study addressed cardiac parameter changes in both ERT and non-ERT patients. ERT may certainly influence conduction abnormalities, repolarisation changes, LVH and arrhythmias progression rate. Our data therefore reflect in part a natural history of the disease in the current era with the availability of ERT and also the natural history of the untreated disease. Of note, patients with LVH progressed despite receiving ERT. This however does not allow any conclusions about the ERT efficacy. On the other hand, since the heart appears to be relatively unresponsive to ERT-induced lysosomal clearance (Thurberg et al. 2009), it may reflect the need of early initiation before the development of significant LVH. The subtle ECG changes noticed in these children are often overlooked and may prove to be important early indicators for initiating early ERT. However, to confirm this observation, a substantially larger cohort of patients should be studied.

Conclusion

Early changes in ECG/Holter ECG and ECHO related to cardiac involvement caused by FD can be demonstrated already in children of both genders. The progression of disease to LVH was more apparent in FD males. Interestingly, some changes on ECG and Holter ECG preceded LVH development and were noted also in the siblings of these rapidly progressing individuals. Although clinically relevant arrhythmias were not found in this cohort of children with FD, our findings justify regular sequential annual follow-up in all children (both males and females) with FD including a complex cardiac evaluation.

Acknowledgements We thank the metabolic nurses and the cardiology technicians at Addenbrooke's Hospital for their help with collating data and performing cardiac ECHO and Holter monitoring.

Synopsis

Early changes in ECG/Holter ECG and echocardiography related to cardiac involvement caused by Fabry disease can be observed in children, both males and females.

Details of the Contributions of Individual Authors

- Stepan Havranek: analysis and interpretation of data; drafting of manuscript
- Ales Linhart: analysis and interpretation of data; revising article critically for important intellectual content
- Zuzana Urbanova: analysis of data; revising article critically for important intellectual content
- Uma Ramaswami: Developing concept, design of study, patient follow-up, data collection; revising article critically for important intellectual content

Guarantor Author

Dr. Uma Ramaswami MD, FRCPCH.

Funding

Supported by Program for research development in Charles University: PRVOUK-P35/LF1/5.

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

Details of Ethics Approval

The study was approved by Norfolk Research Ethics Committee. Reference number: 11/H0310/4.

Disclosure

None declared.

References

Aryana A, Fifer MA, Ruskin JN, Mela T (2008) Short PR interval in the absence of preexcitation: a characteristic finding in a patient with Fabry disease. Pacing Clin Electrophysiol 31:782–783

Barbey F, Qanadli SD, Juli C et al (2010) Aortic remodelling in Fabry disease. Eur Heart J 31:347–353

Brady RO, Schiffmann R (2000) Clinical features of and recent advances in therapy for Fabry disease. JAMA 248:2771–2775

Cybulla M, Walter K, Neumann HP et al (2007) Fabry disease: demographic data since introduction of enzyme replacement therapy. Dtsch Med Wochenschr 132:1505–1509

Desnick RJ, Brady R, Barranger J et al (2003) Fabry disease, an under-recognized multisystemic disorder: expert recommendations for diagnosis, management, and enzyme replacement therapy. Ann Intern Med 138:338–346

Devereux RB, Alonso DR, Lutas EM et al (1986) Echocardiographic assessment of left ventricular hypertrophy: comparison to necropsy findings. Am J Cardiol 57:450–458

Dickinson DF (2005) The normal ECG in childhood and adolescence. Heart 91:1626–1630

Dickinson DF, Scott O (1984) Ambulatory electrocardiographic monitoring in 100 healthy teenage boys. Br Heart J 51:179–183

Douglas PS, Garcia MJ, Haines DE et al (2011) ACCF/ASE/AHA/ASNC/HFSA/HRS/SCAI/SCCM/SCCT/SCMR 2011 appropriate use criteria for echocardiography. J Am Soc Echocardiogr 24:229–267

Elliott PM, Kindler H, Shah JS et al (2006) Coronary microvascular dysfunction in male patients with Anderson-Fabry disease and the effect of treatment with alpha galactosidase A. Heart 92:357–360

Elliott P, Baker R, Pasquale F et al (2011) Prevalence of Anderson-Fabry disease in patients with hypertrophic cardiomyopathy: the European Anderson-Fabry Disease Survey. Heart 97:1957–1960

Eng CM, Niehaus DJ, Desnick RJ (1993a) Molecular analysis of classical and variant phenotypes. Pediatr Res 33:128A

Eng CM, Resnick-Silverman LA, Niehaus DJ, Astrin KH, Desnick RJ (1993b) Nature and frequency of mutations in the α-galactosidase A gene that cause Fabry disease. Am J Hum Genet 53:1186–1197

Frustaci A, Chimenti C (2007) Cryptogenic ventricular arrhythmias and sudden death by Fabry disease: prominent infiltration of cardiac conduction tissue. Circulation 116:350–351

Jastrzebski M, Bacior B, Dimitrow PP, Kawecka-Jaszcz K (2006) Electrophysiological study in a patient with Fabry disease and a short PQ interval. Europace 8:1045–1047

Kampmann C, Baehner F, Whybra C et al (2002) Cardiac manifestations of Anderson-Fabry disease in heterozygous females. J Am Coll Cardiol 40:1668–1674

Kampmann C, Linhart A, Baehner F et al (2008a) Onset and progression of the Anderson-Fabry disease related cardiomyopathy. Int J Cardiol 130:367–373

Kampmann C, Wiethoff CM, Whybra C, Baehner FA, Mengel E, Beck M (2008b) Cardiac manifestations of Anderson-Fabry disease in children and adolescents. Acta Paediatr 97:463–469

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

Kleinert J, Dehout F, Schwarting A et al (2006) Prevalence of uncontrolled hypertension in patients with Fabry disease. Am J Hypertens 19:782–787

Linhart A, Palecek T, Bultas J et al (2000) New insights in cardiac structural changes in patients with Fabry's disease. Am Heart J 139:1101–1108

Linhart A, Magage S, Palecek T, Bultas J (2002) Cardiac involvment in Fabry disease. Acta Paediatr Suppl 91:15–20

Mehta J, Tuna N, Moller JH, Desnick RJ (1977) Electrocardiographic and vectorcardiographic abnormalities in Fabry's disease. Am Heart J 93:699–705

Mehta A, Beck M, Elliott P et al (2009) Enzyme replacement therapy with agalsidase alfa in patients with Fabry's disease: an analysis of registry data. Lancet 374:1986–1996

Monserrat L, Gimeno-Blanes JR, Marin F et al (2007) Prevalence of Fabry disease in a cohort of 508 unrelated patients with hypertrophic cardiomyopathy. J Am Coll Cardiol 50:2399–2403

Nagueh SF (2003) Fabry disease. Heart 89:819–820

Namdar M, Kampmann C, Steffel J et al (2010) PQ interval in patients with Fabry disease. Am J Cardiol 105:753–756

Namdar M, Steffel J, Vidovic M et al (2011) Electrocardiographic changes in early recognition of Fabry disease. Heart 97:485–490

O`Connor M, McDaniel N, Brady W (2008) The pediatric electrocardiogram Part I. Am J Emerg Med 26:221–228

O'Mahony C, Coats C, Cardona M et al (2011) Incidence and predictors of anti-bradycardia pacing in patients with Anderson-Fabry disease. Europace 13:1781–1788

Patel MR, Cecchi F, Cizmarik M et al (2011) Cardiovascular events in patients with Fabry disease natural history data from the Fabry registry. J Am Coll Cardiol 57:1093–1099

Pochis WT, Litzow JT, King BG, Kenny D (1994) Electrophysiologic findings in Fabry's disease with a short PR interval. Am J Cardiol 74:203–204

Ramaswami U, Whybra C, Parini R et al (2006) Clinical manifestations of Fabry disease in children: data from the Fabry Outcome Survey. Acta Paediatr 95:86–92

Ramaswami U, Parini R, Pintos-Morell G, Kalkum G, Kampmann C, Beck M (2012) Fabry disease in children and response to enzyme replacement therapy: results from the Fabry Outcome Survey. Clin Genet 81:485–490

Ries M, Ramaswami U, Parini R et al (2003) The early clinical phenotype of Fabry disease: a study on 35 European children and adolescents. Eur J Pediatr 162:767–772

Ries M, Clarke JTR, Whybra C et al (2006) Enzyme-replacement therapy with agalsidase alfa in children with Fabry disease. Pediatrics 118:924–932

Rijnbeek PR, Witsenburg M, Schrama E, Hess J, Kors JA (2001) New normal limits for the paediatric electrocardiogram. Eur Heart J 22:702–711

Rijnbeek PR, Herpen G, Kapusta L, Harkel DJ, Witsenburg M, Kors JA (2008b) Electrocardiographic criteria for left ventricular hypertrophy in children. Pediatr Cardiol 29:923–928

Sachdev B, Takenaka T, Teraguchi H et al (2002) Prevalence of Anderson-Fabry disease in male patients with late onset hypertrophic cardiomyopathy. Circulation 105:1407–1411

Scott O, Williams GJ, Fiddler GI (1980) Results of 24 hour ambulatory monitoring of electrocardiogram in 131 healthy boys aged 10 to 13 years. Br Heart J 44:304–308

Shah JS, Hughes DA, Sachdev B et al (2005) Prevalence and clinical significance of cardiac arrhythmia in Anderson-Fabry disease. Am J Cardiol 96:842–846

Southall DP, Johnston F, Shinebourne EA, Johnston PGB (1981) 24-hour electrocardiographic study of heart rate and rhythm patterns in population of healthy children. Br Heart J 45: 281–291

Thurberg BL, Fallon JT, Mitchell R, Aretz T, Gordon RE, O'Callaghan MW (2009) Cardiac microvascular pathology in Fabry disease: evaluation of endomyocardial biopsies before and after enzyme replacement therapy. Circulation 119: 2561–2567

Tøndel C, Bostad L, Hirth A, Svarstad E (2008) Renal biopsy findings in children and adolescents with Fabry disease and minimal albuminuria. Am J Kidney Dis 51:767–776

Weidemann F, Breunig F, Beer M et al (2005) The variation of morphological and functional cardiac manifestation in Fabry disease: potential implications for the time course of the disease. Eur Heart J 26:1221–1227

Weidemann F, Niemann M, Breunig F et al (2009) Long-term effects of enzyme replacement therapy on Fabry cardiomyopathy: evidence for a better outcome with early treatment. Circulation 119:524–529

JIMD Reports
DOI 10.1007/8904_2013_223

RESEARCH REPORT

Development of a Scoring System to Evaluate the Severity of Craniocervical Spinal Cord Compression in Patients with Mucopolysaccharidosis IVA (Morquio A Syndrome)

Christian Möllmann · Christian G. Lampe · Wibke Müller-Forell · Maurizio Scarpa · Paul Harmatz · Manfred Schwarz · Michael Beck · Christina Lampe

Received: 31 August 2012 / Revised: 7 March 2013 / Accepted: 8 March 2013 / Published online: 12 April 2013

Abstract Background: As spinal cord compression at the craniocervical junction (CCJ) is a life-threatening manifestation in patients with mucopolysaccharidosis (MPS) IVA, surgical decompression should be performed before damage becomes irreversible. We evaluated the diagnostic value of several examinations for determining the need for decompression surgery.

Methods: We retrospectively analysed results of clinical neurological examination, somatosensory evoked potential (SEP) and magnetic resonance imaging (MRI) in 28 MPS IVA patients. A scoring system – based on the severity of findings – was used to compare results of patients with and without indication for decompression surgery. Individual test scores and two composite scores were evaluated for their potential to assess severity of CCJ impairment.

Results: Sixteen patients had an indication for surgery; 12 of them had undergone surgery. Twelve patients had no indication for surgery; none had received surgery. Neurological ($P = 0.004$), MRI ($P < 0.001$) and atlantoaxial subluxation ($P = 0.006$) scores, but not SEP and odontoid hypoplasia scores, differed significantly between patients with and without surgical indication. Both the abbreviated CCJ score, i.e. sum of neurological and MRI scores, and the extended CCJ score, i.e. sum of abbreviated CCJ and atlantoaxial subluxation score, discriminated between patients with and without surgical indication (abbreviated: 0–2 points vs 2–5 points, $P < 0.001$; extended: 0–3 points vs 3–7 points; $P < 0.001$). Although CCJ instability plays a major role in cervical cord pathology, decompression surgery without occipito-cervical stabilisation may yield good postoperative results.

Conclusions: The abbreviated and extended CCJ scores are objective, transparent and reproducible tools for assessing the CCJ pathology and the need for surgery.

Communicated by: Gregory M. Pastores, MD

Competing interests: Competing interests declared by authors can be found at the end of this chapter

C. Möllmann · M. Beck · C. Lampe (✉)
Department of Paediatric and Adolescent Medicine, Villa Metabolica, University Medical Centre of the Johannes Gutenberg-University Mainz, Langenbeckstr. 2, 55131, Mainz, Germany
e-mail: christina.lampe@unimedizin-mainz.de

C.G. Lampe
Department of Paediatrics, Horst-Schmidt-Hospital, Wiesbaden, Germany

W. Müller-Forell
Institute of Neuroradiology, University Medical Centre, Mainz, Germany

M. Scarpa
Department of Paediatrics, University of Padova, Padova, Italy

P. Harmatz
Children's Hospital & Research Center Oakland, Oakland, CA, USA

M. Schwarz
Department of Neurosurgery, Horst-Schmidt-Hospital, Wiesbaden, Germany

Introduction

Mucopolysaccharidosis IVA (MPS IVA, OMIM #253000) or Morquio A syndrome is a rare metabolic disease caused by deficiency of the lysosomal enzyme N-acetylgalactosamine 6-sulfatase (EC 3.1.6.4), which is involved in the degradation of the glycosaminoglycans (GAGs) chondroitin-6-sulfate and keratan sulfate. Consequently, partially degraded GAGs accumulate within lysosomes, resulting in

progressive cellular damage and dysfunction in multiple tissues and organs, mainly in bone (Muenzer 2011; Tomatsu et al. 2011). Given the excessive deposition of GAGs in bone, the major feature of MPS IVA is skeletal dysplasia, including short trunk dwarfism, odontoid hypoplasia, joint hypermobility/ligamentous laxity, etc. (Muenzer 2011). Odontoid hypoplasia in combination with ligamentous laxity often results in atlantoaxial subluxation/instability, causing narrowing of the spinal canal at the craniocervical junction (CCJ), which is further increased by extradural soft tissue deposition. This leads to compression of the lower brain stem and upper cervical cord. If left untreated, this can ultimately result in irreversible damage of the spinal cord, i.e. myelomalacia. Clinically, it manifests as reduced muscle strength, paresis and ultimately death (Montaño et al. 2007; Muenzer 2011).

As cervical cord compression in MPS IVA can be severely disabling and even life-threatening, it should be addressed before damage becomes irreversible. Surgical stabilisation of the CCJ and decompression are the only means to achieve this goal. However, the risks associated with surgery and anaesthesia should be weighed against the severity of spinal cord compression, the patient's progression rate and risk of significant permanent spinal cord injury (McLaughlin et al. 2010; Walker et al. 1994). Hence, determining the optimal indications and timing for surgery is crucial. Unfortunately, literature on this topic is scarce. Several diagnostic methods have been described to detect and assess the severity of cervical cord compression in MPS IVA patients, i.e. clinical neurological examination (Takeda et al. 1991), somatosensory evoked potentials (SEPs) (Boor et al. 2000; Takeda et al. 1991) and imaging of the cervical spine/cord – including assessment of atlantoaxial subluxation and odontoid hypoplasia – using X-ray radiography, magnetic resonance imaging (MRI) or computed tomography (CT) (Hughes et al. 1997; Roach et al. 1984; Stevens et al. 1991). However, there is neither consensus on the best diagnostic method, nor on the optimal indications or timing for neurosurgery at the CCJ. Thus, the final decision to perform surgery still depends on the surgeon's clinical judgement, which is subjective and prone to interobserver variability. Therefore, we aimed at quantifying the individual contribution of each of these diagnostic methods to the decision-making process, i.e. at identifying objective and reproducible criteria to assess the severity of cervical cord compression in MPS IVA patients.

Methods

Patients

Demographic and clinical data were retrospectively collected from all patients with a biochemically confirmed diagnosis of MPS IVA in our department (Villa Metabolica, Mainz), who received at least two of the following assessments, i.e. neurological examination, SEP of the median nerve or MRI of the CCJ. For patients who had undergone at least one surgical intervention at the CCJ in the past, findings from the last preoperative and first postoperative examination were used for evaluation of postoperative outcomes. For patients with a surgical indication who had not yet been operated, the test results that revealed the indication for surgery were used. For patients without indication for decompression surgery, the most recent available test results were used.

Patients were classified as having a rapidly or a slowly progressive phenotype based on body height, physical performance, mobility achieved with orthopaedic support and degree of independence regarding daily activities.

Diagnostic Tests

Clinical neurological examination, registration of SEP of the median nerve and MRI of the CCJ were the main methods used to evaluate the pathology of the CCJ. In addition, atlantoaxial subluxation and odontoid hypoplasia were evaluated using CT or MRI. For each test method, a new scoring system was developed to rate the degree of changes from 0 (normal findings) to 3 (most severe changes) (Table 1). The most severe pathological finding in any examination was used to assess the severity and to determine the score.

Clinical neurological examination included assessment of easily evaluable and reproducible signs caused by lesions of the pyramidal tract, i.e. deep tendon reflexes and pyramidal signs.

SEP of the median nerve was carried out as described previously (Boor et al. 1998, 2000). As patients with Morquio A syndrome generally have a shorter stature than age-related healthy individuals, not absolute latencies but interpeak latencies between the responses were evaluated and compared with age-specific reference values of our department. Examinations were judged to be pathological in case of significant variation from the age-specific reference values on either right or left side.

For the MRI evaluation, both T1- and T2-weighted images were created. The presence or absence of cerebrospinal fluid (CSF) around the spinal cord could be deduced from the sagittal and axial T2-weighted images. The T2-weighted images (both sagittal and axial) were also examined for increased signal intensity in the cord, suggesting myelomalacia (Lachman et al. 2010). For most patients, images were taken in neutral position of the head. If images were available in flexion and extension of the head, the most severe pathological finding was chosen.

Table 1 *Scoring system for pathology of the craniocervical junction and proximal cervical spine*. Scoring system for findings of the clinical neurological examination, somatosensory evoked potentials (SEPs) of the median nerve, magnetic resonance imaging (MRI) of the craniocervical junction (CCJ), assessment of atlantoaxial subluxation and development of the dens axis

Score	Test findings
	Clinical neurological examination
0	Normal neurological findings
1	Increased deep tendon reflexes, side differences of muscle reflexes
2	Presence of pyramidal tract signs: Babinski, Oppenheim reflex, clonus
3	Paresis or weakness of upper and/or lower extremities
	SEP of the median nerve
0	Normal SEP
1	Extension of at least one of the interpeak latencies N9/P13, N9/N13b or N13a/N20[a] (more than 2.5 SD)
2	Lack of P13 and/or N13b (subcortical)
3	Lack of N20 (cortical)
	MRI of CCJ (sagittal images)
0	No effacement of cerebrospinal fluid (CSF) around the spinal cord
1	Effacement of CSF on at least one side of the spinal cord
2	Effacement of CSF in all directions around the spinal cord
3	Myelomalacia
	Atlantoaxial subluxation
0	Normal position of the atlas
1	Obvious ventral dislocation of the atlas relative to the axis
2	Severe ventral dislocation of the atlas relative to the axis, making a regular position of the spinal cord unlikely, regardless of glycosaminoglycan accumulation
	Development of dens axis
0	(Possibly) hypoplastic bony part of dens axis extends completely into the atlas
1	Bony part of dens axis extends partially into the atlas
2	Bony part of dens axis does not extend into the atlas

[a] Anatomical correlations according to Stöhr et al. (2005):

N9/P13: plexus brachialis – nucleus cuneatus

N9/N13b: plexus brachialis – nucleus cuneatus

N13a/N20: dorsal horn (caudal cervical cord) – cortex

Surgery

The indication for decompression surgery was made on clinical grounds, focusing on signs and symptoms of upper motor neuron lesion and compromised lower brain stem function, especially central apnoea. MRI findings and SEP were also taken into account. Posterior decompression surgery consisted of partial laminectomy of C1-C3 in combination with enlargement of the foramen magnum (if indicated), and removal of the thickened ligaments without opening the dura. Additional stabilisation of the cervical spine was performed if needed.

Table 2 Demographic information at last follow-up ($N = 28$)

	N (%)
Gender	
Male	16 (57%)
Female	12 (43%)
Age	
$<$ 18 years (children)	21 (75%)
$\geq$ 18 years (adults)	7 (25%)
Phenotypical disease severity	
Slowly progressive (attenuated) phenotype	8 (28%)
Rapidly progressive (severe) phenotype	20 (72%)
Neurosurgery at the craniocervical junction (CCJ)	
History of neurosurgery at the CCJ ($\geq$1 intervention)	12 (43%)
Indication for neurosurgery at the CCJ, but not yet operated	4 (14%)
No indication for neurosurgery at the CCJ	12 (43%)

N number of patients

Statistics

Statistical analysis was done using IBM SPSS 19. The Fisher Exact, Mann-Whitney, Wilcoxon and sign tests were used for ordinal variables, continuous variables, ordinal data, and for comparison of pre- and postoperative outcomes for both ordinal and continuous variables, respectively. A P-value < 0.05 was considered statistically significant.

Results

Demographics and Baseline Characteristics

The study included 28 patients with a biochemically and/or genetically confirmed diagnosis of MPS IVA (16 males, 12 females) (Table 2). Median age at the time of analysis was 12.9 years (range 5–49). Twelve patients had undergone at least one neurosurgical intervention at the CCJ in the past. Three patients underwent posterior decompression surgery plus cervical spine stabilisation, while nine patients did not need stabilisation. Four patients had an indication for cervical cord surgery but had not yet been operated, either because of recent worsening of their situation or because of anaesthesia risks. Another 12 patients did not (yet) have an indication for decompression surgery.

All 16 patients with an indication for surgery were children ($<$ 18 years) at the time of surgery or at the time of finding the indication, except for one 39-year-old woman. Their median age at that time was 7.9 years (range 2–39), which is lower than the median age of 11.9 years of the 12 patients without surgical indication at last follow-up (range 5–20; 10 children, 2 adults). All 16 patients who

Table 3 Findings of diagnostic tests to evaluate the pathology of the craniocervical junction (CCJ) in patients without (−; results of last follow-up) and with (+; preoperative results) an indication for neurosurgery at the CCJ

Diagnostic test	Surgical indication	N	Percentage of patients with score				P (− vs +)
			0	1	2	3	
Clinical neurological examination	−	12	67	33	0	0	0.004
	+	14	7[a]	57	21	14	
Somatosensory evoked potentials of the median nerve	−	12	83	17	0	0	0.571
	+	10	90	10	0	0	
Magnetic resonance imaging	−	10	80	20	0	0	< 0.001
	+	14	7[a]	14	43	36	
Atlantoaxial subluxation assessment	−	11	82	18	0	–	0.006
	+	14	21	36	43	–	

Explanation of scoring system for each test: see Table 1

[a] Pathology scores 0 for clinical neurological examination and magnetic resonance imaging were not observed in a single patient

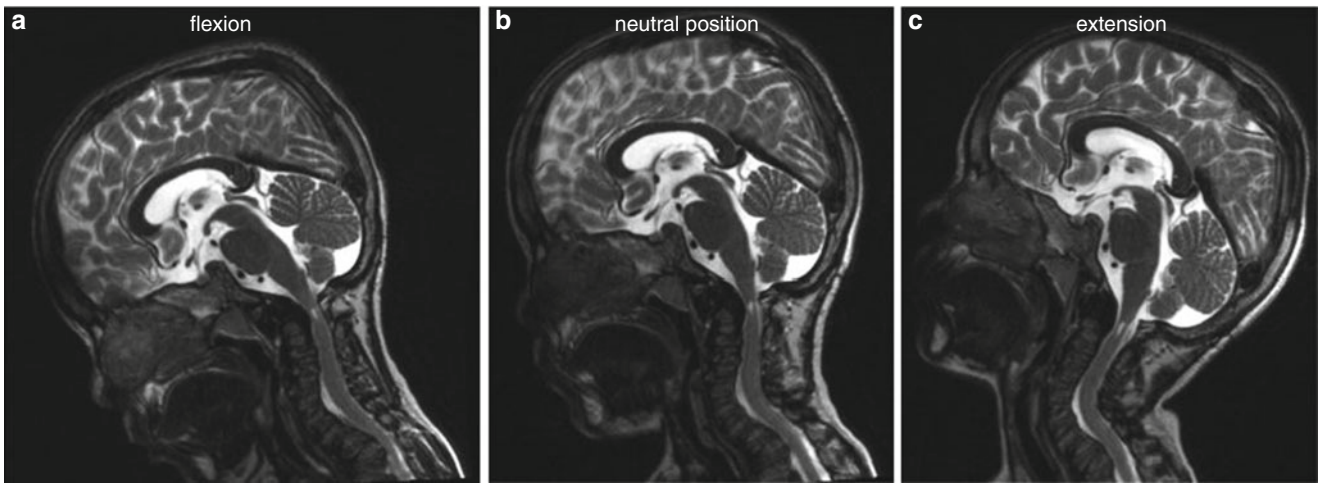

Figure 1 *Impact of position of the head on cervical cord compression.* T2-weighted magnetic resonance imaging of the craniocervical junction during (**a**) flexion, (**b**) neutral position and (**c**) extension of the head of an 8-year-old boy with severe MPS IVA, who underwent decompression surgery when he was 4 years old. Spinal cord compression depends on the position of the head and is the most obvious during flexion

needed surgery – except for the 39-year-old woman – had a rapidly progressive phenotype, compared to only 5/12 patients without surgical indication.

Comparison of Cervical Cord Pathology in Patients With and Without Surgical Indication

The scores of diagnostic tests to assess the severity of cervical cord compression were compared between patients with and without an indication for neurosurgery at the CCJ. The outcomes are summarised in Table 3. For SEP of the median nerve, results were comparable between patients with and without surgical indication. In contrast, for neurological examination and MRI of the CCJ, pathological findings were more common and more severe among patients who needed neurosurgery. As only few preoperative images were available for assessment of atlantoaxial subluxation and odontoid hypoplasia, some postoperative images were used as well (only for patients who did not undergo stabilisation). Although we could not establish a scoring system for atlantoaxial subluxation based on clear objective criteria, such as atlanto-dens interval, due to the different ages of our patients, subluxation was significantly more common in patients who needed spinal cord decompression. In contrast, development of the bony part of the dens axis (N = 27) did not differ significantly between both patient groups (data not shown; $P = 0.634$).

For some patients, MRI pictures of the CCJ were taken in several positions of the head, i.e. in neutral position, flexion and/or extension (Fig. 1). From these images, one can see that compression of the cervical cord depends on

the position of the head. During flexion of the head, compression is obvious, with no visible CSF around the spinal cord. In contrast, in neutral position of the head, the cervical cord is surrounded by a minimal amount of CSF. During extension, the space available for the spinal cord is even more increased.

Composite CCJ Scores for Defining the Need for Neurosurgery

Two composite CCJ scores were developed to evaluate whether combining the results of the individual examinations could improve the assessment of severity of cervical cord compression. The abbreviated CCJ score is the sum of the pathology scores for neurological examination and MRI and ranges from 0 to 6. The extended CCJ score is the sum of the abbreviated CCJ score and the atlantoaxial subluxation score and ranges from 0 to 8. SEP and odontoid hypoplasia scores were not included, as they did not differ significantly between both patient groups. Both scores were compared between patients with and without an indication for surgery.

Both scores could discriminate between patients with and without surgical indication (both $P < 0.001$). Indeed, in patients without surgical indication, abbreviated and extended CCJ scores ranged from 0 to 2 (median 0.5) and from 0 to 3 (median 1), respectively, while in patients with surgical indication, they ranged from 2 to 5 (median 4) and from 3 to 7 (median 5). Thus, in none of the patients requiring surgery, neurological examination and MRI findings were normal (score 0) at the same time.

Impact of Cervical Cord Decompression

To assess if surgery had an impact on the test results, we compared the pre- and postoperative scores of the three main diagnostic procedures and the abbreviated CCJ score. Differences between pre- and postoperative findings were not statistically significant. For neurological examination and for the abbreviated CCJ score, there was a trend towards improvement, with 45% ($P = 0.219$) and 78% ($P = 0.180$) of patients showing postoperative improvement, respectively.

Discussion

This retrospective study aimed at quantifying the relative utility of individual methods to assess the severity of CCJ impairment, in order to establish objective and reproducible criteria regarding the need for neurosurgery, allowing comparison between different centres. Clinical neurological examination, MRI and atlantoaxial subluxation appeared to be reliable methods to determine the indication for cervical cord surgery, with MRI being the most sensitive one, in line with a previous publication (Hughes et al. 1997). In contrast, SEP of the median nerve in neutral position of the head could not discriminate between patients with and without a surgical indication (Takeda et al. 1991), as opposed to a previous report (Boor et al. 2000). Similarly, the degree of odontoid hypoplasia appeared not to be a good criterion to determine the need for surgery in MPS IVA patients, as suggested previously (Hughes et al. 1997; Stevens et al. 1991). This is not surprising, as transition from cartilage to bone in the dens axis might not be completed before the age of 8 years, and 11/28 patients in our study were < 8 years old. Furthermore, cervical cord compression may also occur in the absence of bony odontoid hyperplasia (Shukla et al. 2011). The abbreviated and extended CCJ scores – combining MRI, neurological examination and atlantoaxial subluxation scores – appeared to have a higher discriminatory power than the individual tests.

When interpreting these results, it should be noted that the classification of patients into two groups, i.e. those with and without surgical indication, was dependent on the decision of a single neurosurgeon. Although this surgeon was very experienced in judging the need for surgery, this decision remained highly subjective and might have been taken too early or too late (i.e. in those patients who already had myelomalacia). However, it is currently the only available standard against which the new scoring system could be tested. Indeed, the only other existing scoring system for evaluating cervical cord involvement is solely based on MRI findings and was not developed to determine the optimal timing for surgery (Castro et al. 2008; Lachman et al. 2010). Future research will be needed to confirm the validity of our scoring system, by comparing the (long-term) outcomes of patients with and without a surgical indication according to the CCJ scores.

Based on our observations, we propose relative and absolute indications for neurosurgery at the CCJ (Table 4). Importantly, when interpreting this table, evolution of the severity scores over time, i.e. rapidity of progression, should also be taken into account. Thus, pyramidal tract signs and CSF effacement around the spinal cord might be considered as relative indications for surgery when stable over time – requiring close monitoring – but they might require immediate surgical intervention in case of rapid aggravation. This is a limitation of the current study. Therefore, it might be interesting for future studies to add a ‘longitudinal’ or ‘time’ component to the new scoring system, allowing intervention in earlier stages, when pathology is still reversible.

In addition, our study showed that flexion-extension imaging is indispensable to detect CCJ instability in all

Table 4 *Proposed absolute and relative indications for neurosurgery at the craniocervical junction (CCJ) in patients with MPS VIA.* Outcomes of diagnostic methods suggesting the need for decompression surgery are indicated

Diagnostic method[a]	Relative indication	Absolute indication
Clinical neurological examination	Score 2 (pyramidal tract signs)	Score 3 (paresis or limb weakness)
MRI of CCJ	Score 2 (absence of CSF around spinal cord)	Score 3 (myelomalacia)
Abbreviated CCJ score	Score 2	Score ≥ 3
Extended CCJ score	Score 3	Score ≥ 4

[a] The most extensive diagnostic method available for a single patient should be considered. Extended CCJ score is more extensive than abbreviated CCJ score, while abbreviated CCJ score is more extensive than MRI of CCJ or clinical neurological examination alone

MPS IVA patients – unless there is a severe risk of aggravating the patient's conditions – as cervical cord compression might only be noticed in flexion and not in neutral position of the head (Fig. 1) (Roach et al. 1984; Stevens et al. 1991). We preferred MRI over other methods for flexion-extension imaging such as CT and X-ray radiography, because it directly visualises the spinal cord and uses non-ionising radiation, which is especially advantageous in children. Moreover, plain flexion-extension X-ray films might be difficult to interpret. Indeed, overlap between the thickened skull and the very small C1-C2 vertebrae is common in MPS IVA patients and might lead to delayed detection of instability, when myelopathy is already present (Ransford et al. 1996). As many MPS IVA patients are children at the time of diagnosis, very short sequence MRI and limited flexion-extension movements were required to minimise the risks of general anaesthesia and damaging the spinal cord by prolonged flexion of the neck.

Finally, to our knowledge, this is the first study in MPS IVA patients showing that cervical laminectomy for cervical spine decompression is feasible without posterior occipito-cervical fixation/fusion, leading to improvement in abbreviated CCJ score in 78% of patients. Indeed, given the major role of instability/hypermobility of the CCJ in the pathophysiology of spinal cord damage (Ashraf et al. 1991), (prophylactic) cervical fusion has always been preferred as surgical intervention, either with or without posterior decompression, with variable success rates (Ain et al. 2006; Kopits et al. 1972; Lipson 1977; Northover et al. 1996; Ransford et al. 1996; White et al. 2009). However, metal stabilisation may cause artefacts on MRI or CT scan, thereby hindering future monitoring, while bone grafts may complicate revision surgery in case of relapse. In addition, stabilisation severely limits neck motion (White et al. 2009). Therefore, in our study, stabilisation was only performed in three patients. The (short-term) success of laminectomy-only suggests that, next to CCJ instability, permanent cervical cord compression due to extradural soft tissue thickening plays a role in spinal cord damage (Stevens et al. 1991), thereby confirming the mixed pathophysiology of CCJ lesions (White et al. 2009). However, comparison of our results with those of previous studies applying cervical stabilisation is very difficult, as the severity of neurological impairment before surgery might differ between studies. Therefore, a prospective long-term follow-up study comparing laminectomy with cervical stabilisation will be needed to confirm our findings.

Take-Home Message

Clinical neurological examination, magnetic resonance imaging and evaluation of atlantoaxial subluxation are the best diagnostic methods to assess the severity of cervical cord compression in patients with MPS IVA; they can be combined into two composite scores which can help to determine the need and optimal timing for decompression surgery.

Authors' Contributions

Christian Möllmann was involved in designing the study and collecting and interpreting the data. He was involved in drafting the manuscript and revising it critically for important intellectual content. This publication is part of Christian Möllmann's MD thesis, entitled 'Die kraniozervikale Stenose bei Mukopolysaccharidose IVA (M. Morquio A)', defended at the Johannes Gutenberg-Universität in Mainz in 2012.

Christian Lampe was involved in designing the study and in analysing and interpreting the data, especially SEP data. He was involved in drafting the manuscript and revising it critically for important intellectual content.

Wibke Müller-Forell was involved in performing and interpreting the MRI examinations and in revising the manuscript.

Maurizio Scarpa, Paul Harmatz and Michael Beck were involved in analysing and interpreting the data and in revising the manuscript for important intellectual content.

Manfred Schwarz has been involved in the interpretation of data, drafting the manuscript and revising it critically for important intellectual content.

Christina Lampe was involved in designing the study and in collecting, analysing and interpreting the data. She was involved in drafting the manuscript and revising it critically for important intellectual content. Christina Lampe accepts full responsibility for the work and the conduct of the study, had access to all data and controlled the decision to publish.

All authors have given final approval of this version to be published.

Competing Interests

Christian Möllman received travel grants from BioMarin Europe Ltd.

Christian Lampe received travel grants from BioMarin Europe Ltd.

Wibke Müller-Forell has no conflicts of interest.

Maurizio Scarpa provided consulting services to BioMarin Pharmaceutical Inc, Novato, CA, USA. He has also received research grants, participated in advisory boards, and received speakers' honoraria and travel support from BioMarin.

Paul Harmatz has provided consulting services to BioMarin Pharmaceutical Inc, Novato, CA, USA. He has also received research grants, participated in advisory boards, and received speakers' honoraria and travel support from BioMarin.

Manfred Schwarz received travel grants and speakers' fee from BioMarin Europe Ltd.

Michael Beck provided consulting services to BioMarin Pharmaceutical Inc, Novato, CA, USA. He also received research grants, participated in advisory boards, and received speakers' honoraria and travel support from BioMarin.

Christina Lampe received travel grants, speakers' fee, participated in advisory boards, provided consulting services and received article processing charge from BioMarin Europe Ltd and Shire.

Funding

The authors are grateful to Ismar Healthcare NV for their writing assistance, which was funded by BioMarin Europe Ltd. The authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

Ethical Approval

Ethical approval was not required for this retrospective study.

Patient Consent Statement

All patients included in the study gave informed consent.

References

Ain MC, Chaichana KL, Schkrohowsky JG (2006) Retrospective study of cervical arthrodesis in patients with various types of skeletal dysplasia. Spine 31:E169–E174

Ashraf J, Crockard HA, Ransford AO, Stevens JM (1991) Transoral decompression and posterior stabilisation in Morquio's disease. Arch Dis Child 66:1318–1321

Boor R, Goebel B, Taylor MJ (1998) Subcortical somatosensory evoked potentials after median nerve stimulation in children. Eur J Paediatr Neurol 2:137–143

Boor R, Miebach E, Brühl K, Beck M (2000) Abnormal somatosensory evoked potentials indicate compressive cervical myelopathy in mucopolysaccharidoses. Neuropediatrics 31:122–127

Castro S, Ayres-Basto M, Rodrigues E, Campos MM, Guimarães J, Leão-Teles E (2008) Vertebro-medular imaging findings in mucopolysaccharidosis types II and IV. J Inherit Metab Dis 31(1):109 (abs. 431-P)

Hughes DG, Chadderton RD, Cowie RA, Wraith JE, Jenkins JPR (1997) MRI of the brain and craniocervical junction in Morquio's disease. Neuroradiology 39:381–385

Kopits SE, Perovic MN, McKusick V, Robinson RA, Bailey JA III (1972) Congenital atlantoaxial dislocations in various forms of dwarfism. J Bone Joint Surg Am 54-A:1349–1350

Lachman R, Martin KW, Castro S, Basto MA, Adams A, Teles EL (2010) Radiologic and neuroradiologic findings in the mucopolysaccharidoses. J Pediatr Rehabil Med 3:109–118

Lipson SJ (1977) Dysplasia of the odontoid process in Morquio's syndrome causing quadriparesis. J Bone Joint Surg Am 59:340–344

McLaughlin AM, Farooq M, Donnelly MB, Foley K (2010) Anaesthetic considerations of adults with Morquio's syndrome - a case report. BMC Anesthesiol 10:2

Montaño AM, Tomatsu S, Gottesman GS, Smith M, Orii T (2007) International Morquio A Registry: clinical manifestation and natural course of Morquio A disease. J Inherit Metab Dis 30:165–174

Muenzer J (2011) Overview of the mucopolysaccharidoses. Rheumatology (Oxford) 50(Suppl 5):v4–v12

Northover H, Cowie RA, Wraith JE (1996) Mucopolysaccharidosis type IVA (Morquio syndrome): a clinical review. J Inherit Metab Dis 19:357–365

Ransford AO, Crockard HA, Stevens JM, Modaghegh S (1996) Occipito-atlanto-axial fusion in Morquio-Brailsford syndrome. A ten-year experience. J Bone Joint Surg Br 78:307–313

Roach JW, Duncan D, Wenger DR, Maravilla A, Maravilla K (1984) Atlanto-axial instability and spinal cord compression in children–diagnosis by computerized tomography. J Bone Joint Surg Am 66:708–714

Shukla D, Arvind S, Devi BI (2011) Myelopathy in a dwarf: a case of Morquio's syndrome without odontoid hypoplasia. Neurol India 59:126–127

Stevens JM, Kendall BE, Crockard HA, Ransford A (1991) The odontoid process in Morquio-Brailsford's disease. The effects of occipitocervical fusion. J Bone Joint Surg Br 73:851–858

Stöhr M, Dichgans J, Büttner U, Hess CW (2005) Evozierte Potenziale: SEP – VEP – AEP –EKP – MEP. Springer 4. Auflage, Berlin

Takeda E, Hashimoto T, Tayama M et al (1991) Diagnosis of atlantoaxial subluxation in Morquio's syndrome and spondyloepiphyseal dysplasia congenita. Acta Paediatr Jpn 33:633–638

Tomatsu S, Montaño AM, Oikawa H et al (2011) Mucopolysaccharidosis type IVA (Morquio A disease): clinical review and current treatment. Curr Pharm Biotechnol 12:931–945

Walker RWM, Darowski M, Morris P, Wraith JE (1994) Anaesthesia and mucopolysaccharidoses. A review of airway problems in children. Anaesthesia 49:1078–1084

White KK, Steinman S, Mubarak SJ (2009) Cervical stenosis and spastic quadriparesis in Morquio disease (MPS IV). A case report with twenty-six-year follow-up. J Bone Joint Surg Am 91:438–442

JIMD Reports
DOI 10.1007/8904_2013_224

RESEARCH REPORT

Outcome of Perinatal Hypophosphatasia in Manitoba Mennonites: A Retrospective Cohort Analysis

Edward C.W. Leung · Aizeddin A. Mhanni · Martin Reed · Michael P. Whyte · Hal Landy · Cheryl R. Greenberg

Received: 12 December 2012 / Revised: 12 March 2013 / Accepted: 13 March 2013 / Published online: 12 April 2013

Abstract Hypophosphatasia (HPP) is the metabolic bone disease caused by loss-of-function mutation within the gene that encodes the "tissue nonspecific" isoenzyme of alkaline phosphatase (TNSALP). Perinatal HPP is usually fatal due to respiratory insufficiency, and infantile HPP often has a similar outcome although no formal study into the natural history of these severe forms of HPP has been undertaken. We reviewed our 80-year (1927–2007) cohort of 15 Canadian patients with perinatal HPP. All had Mennonite heritage. Family linkage studies indicated that nine were homozygous for a TNSALP disease allele, likely Gly334Asp. Three patients had parents who were carriers for the Gly334Asp allele by mutation analysis. One patient was confirmed by mutation analysis to be homozygous for the *TNSALP* Gly334Asp mutation. One patient who had only one Mennonite parent was a genetic compound for the Gly334Asp mutation and the Val382Ile mutation. This patient's sibling was also affected. All 15 patients had profound skeletal hypomineralization, severe rickets, and respiratory insufficiency. All died by 9 months of age, usually soon after birth, from pulmonary failure.

Communicated by: Daniela Karall

E.C.W. Leung (✉) · A.A. Mhanni · M. Reed · C.R. Greenberg
Manitoba Institute of Child Health and Department of Pediatrics and Child Health, University of Manitoba, AE308 – 820 Sherbrook St, R3A 1R9, Winnipeg Manitoba, Canada
e-mail: eleung@hsc.mb.ca

M.P. Whyte
Center for Metabolic Bone Disease and Molecular Research, Shriners Hospital for Children and Division of Bone and Mineral Diseases, Washington University School of Medicine, St. Louis, MO, United States

H. Landy
Alexion Pharmaceuticals, Chesire, CT, United States

Introduction

The first report of hypophosphatasia (HPP) in the English-language medical literature has been attributed by some to Dr. Bruce Chown of the University of Manitoba, Winnipeg. In 1936, Chown described two sisters of Welsh descent with what he called "renal rickets" (Chown 1936; Fraser 1957). The two infants had hypercalcemia and died at age 3 and 6 months, respectively, but Chown was unable to further define the biochemical nature of the suspected underlying inborn error of metabolism. Similar patients were subsequently identified with the identical clinical and radiographic phenotype and the biochemical hallmark was eventually proven to be decreased activity of the liver/bone/kidney "tissue nonspecific" isoenzyme of alkaline phosphatase (TNSALP) (Whyte 1994). HPP severity is generally inversely related to the age at onset (Whyte 1994). Now, HPP is classified when there is skeletal disease as "perinatal," "infantile," "childhood," or "adult" based on age at presentation (Whyte 2013). Both autosomal recessive and autosomal dominant forms of the disease are described. All but odontohypophosphatasia manifest defective skeletal mineralization (Fraser 1957) and involve loss-of-function mutation of the TNSALP gene (Whyte 1994) which was characterized in 1988 (Weiss et al. 1988). To date, more than 260 mutations have been recorded in the TNSALP Gene Mutations Database (http://www.sesep.uvsq.fr/03_hypo_mutations.php).

In Canada, the prevalence in the general population of the most severe forms of HPP is estimated to be ~1:100,000 (Fraser 1957). In France, the prevalence for the severe forms is ~1:300,000 (Fraser 1957; Mornet et al. 2011). In perinatal HPP, there is profound hypomineralization of the skeleton, frequently with foreshortened and deformed limbs. Perinatal HPP has been reported in various case reports to be almost invariably fatal (Whyte 1994; Shohat

et al. 1991). Death results from respiratory insufficiency due to pulmonary hypoplasia and biomechanical compromise due to rachitic disease of the chest. Radiographic examination reveals skeletal hypomineralization which in some cases may appear as almost total absence of bony structures (Fraser 1957; Cole 2008). Patients with infantile HPP may appear normal at birth, but then present within the first 6 months of life with failure to thrive and progressive respiratory failure. Seizures may occur and are typically due to pyridoxine deficiency in the central nervous system (Greenberg et al. 1990). When such seizures occur, the prognosis is grim: In a recent review of the literature, no patients with HPP and pyridoxine-responsive seizures survived beyond 18 months of age (Baumgartner-Sigl et al. 2007). The outcome of infantile HPP is less certain. The natural history of the perinatal and infantile forms of HPP has not been systematically reviewed or published. Recent reports of spontaneous postnatal improvement of skeletal deformities identified antenatally in some babies with "benign prenatal" HPP (Moore et al. 1999; Pauli et al. 1999; Wenkert et al. 2011) and also in 2012, the potential of enzyme replacement treatment for the most severe forms of HPP (Whyte et al. 2012) prompted review of our experience with this disease in Manitoba, Canada. We had previously reported that autosomal recessive HPP is especially prevalent in the Manitoba Mennonite population, and the molecular basis for the perinatal/early infantile form in the population is a founder mutation Gly334Asp in the TNSALP gene. In 1993, the carrier frequency for this mutation was estimated to be 1/25 in the Manitoba Mennonite population predicting the frequency of homozygous affected babies to be 1/2,500 births in this community, significantly higher than the general population (Greenberg et al. 1993).

Herein, we present our 80-year (1927–2007) retrospective review of 19 Manitoba patients with perinatal HPP.

Methods

We conducted a retrospective study of the 80-year experience with patients with perinatal or infantile HPP in the province of Manitoba, Canada (1927–2007). All patients with a diagnosis of HPP by 5 years of age were reviewed. Nineteen patients were identified from our Genetics clinic database and from previously published cases (Macpherson, Kroeker and Houston 1972, McGuire et al. 1987; Chodirker et al. 1990).

Inclusion criteria required onset of signs and/or symptoms of HPP prior to 6 months of age. The diagnosis of HPP was made by the presence of radiographic evidence of HPP-related rickets and one or more of the following:

1. Two TNSALP alleles with previously reported HPP-causing mutation.
2. Both parents are carriers of at least one documented TNSALP allele with a previously reported HPP-causing mutation.
3. Confirmed or suspected homozygosity in the TNSALP gene based on the original family linkage study that contributed to the mapping of TNSALP gene to the Rh locus on chromosome 1p36.1 (Chodirker et al. 1987).

Survival information and other medical, surgical, and disease history data relevant to HPP of all patients was obtained from the medical records at primary, secondary, and tertiary care facilities, whenever necessary and feasible. We began TNSALP mutation analysis in 2000 in our center. Additional information previously reported concerning these patients in the medical/scientific literature could be included. This study was approved by the University of Manitoba Health Research Ethics Board.

Results

Diagnosis

Of the 19 patients whom we identified to be clinically diagnosed with perinatal or infantile HPP, four did not fulfill our study inclusion criteria because their medical records, including radiographic evidence of skeletal disease, were no longer available.

For the 15 patients who fulfilled our inclusion criteria, the parents of 13 patients were both Mennonites. Two patients were affected siblings from the same family where only one parent was Mennonite. HPP was confirmed with by *TNSALP* mutation analysis in two different families each with one affected patient. In one of these two families, the patient was shown to be homozygous for the Gly334Asp mutation. In the second family, the patient was a genetic compound for the founder Mennonite Gly334Asp mutation and a Val382Ile mutation which has previously been reported (Goseki-Sone et al. 1998). A sibling of this patient was similarly affected and their parents were shown to be carriers of either the Gly334Asp mutation or the Val382Ile mutation. The remaining 12 patients did not have a molecular diagnosis, but 3 sets of parents (all Mennonite) were shown to be carriers of the *TNSALP* Gly334Asp mutation and the other 9 sets of parents were shown to be carriers of a *TNSALP* mutation through linkage analysis that contributed to the mapping of HPP to the Rh locus chromosome 1p36.1 and subsequently to TNSALP gene identification (Greenberg et al. 1993; Chodirker et al. 1987, 1990).

Nine of the 15 patients had radiologic signs of HPP in utero, although reports were available for only 7 patients. Most abnormalities were detected using fetal ultrasound. The most common prenatal ultrasonographic findings were shortened limbs and decreased bone mineralization (Table 1).

Table 1 Summary of signs and symptoms of perinatal hypophosphatasia clinical cases

Point number	In utero signs?	Age at onset of HPP symptoms	Muscular hypotonia at presentation	Respiratory distress at presentation	Respiratory support	Seizures	First radiologic valuation	Age at death (days)
1	No	2 weeks	Yes	No	Unknown	Yes, Day 1	Date N/A, metaphyseal flaring	36
2	Yes; severe micromelia and undermineralized skull	At birth	No	Yes	No	No	N/A	30 min
3	Yes; fetal U/S, 29 wk GA, severe micromelia, undermineralized skull	At birth	No	Yes	No	No	N/A	10
4	Yes; fetal U/S 36 wk GA, micromelia, undermineralized skull, femoral fracture	At birth	No	Yes	Yes; at birth, intubated and ventilated	No	Day 1, osteopenia	1
5	Yes; fetal U/S, 25 wk GA, micromelia with bowing, bilateral forearm fractures	At birth	No	No	No	Yes, Day 36	Day 3, osteopenia; bilateral femoral bowing; left radial, left ulnar, and bilateral humeral fractures	263
6	Yes; fetal U/S, 21 wk GA, micromelia with bowing, demineralized skull	At birth	No	No	No	No	Prenatal X-ray (10 days before birth), osteopenia, thin gracile ribs, deformities of all long bones	3 h
7	Yes; fetal ultrasound, GA N/A, micromelia, demineralized skull	At birth	No	n.a.	n.a.	No	Day 1, report N/A	Stillborn
8	No	At birth	No	No	No	Yes, Day 1	Day 41, report N/A	281
9	Yes; report N/A	At birth	No	Yes	Yes; at Birth, intubated and ventilated	No	Day 2, osteopenia, marked metaphyseal irregularity of all tubular bones	1
10	No	At birth	Yes	Yes	Yes; at birth, supplemental oxygen	Yes, Day 1	Day 2, metaphyseal flaring	125
11	No	At birth	No	Yes	Yes; at birth, intubated and ventilated	No	Date N/A, metaphyseal flaring, rachitic chest	5
12	Yes; fetal X-ray, GA N/A, undermineralized bones	At birth	No	Yes	Yes; at birth, supplemental oxygen	No	Day 2, osteopenia, metaphyseal flaring	<1 h
13	Yes; fetal U/S, GA N/A, report N/A	At birth	No	n.a.	n.a.	No	Date N/A, absence of some bones	Stillborn
14	No	At birth	No	Yes	Yes; at birth, intubated and ventilated	No	Day 1, osteopenia, no ossification of forearm bones, deficient upper thoracic and cervical vertebrae	<1 day
15	No	At birth	No	No	Unknown	No	Date N/A, osteopenia, recent fracture	11

U/S ultrasound, *wk* weeks, *GA* gestational age, *N/A* not available, *n.a.* not applicable

Birth History and Congenital Anomalies

Two patients were born prematurely at 30 and 33 weeks while 2 others were born early at 36 weeks gestation. The remaining 11 patients were born at term. Birth weights were available for 6 patients and ranged from 2.3 kg (<5 percentile) to 3.3 kg (25–50 percentile). Length and head circumference measurements were available for three patients at birth. Length ranged from 40 cm (<5 percentile) to 51 cm (50–75 percentile). Head circumference ranged from 32.5 cm (5–10 percentile) to 33.5 cm (10–25 percentile).

At birth, 12 of the 15 patients had sufficient documentation for evaluation of skeletal anomalies. Ten patients had obvious skeletal anomalies while two did not. Of the ten with documented abnormalities, nine had severe shortening/bowing of limbs at birth, six had skull deformities, and two had rachitic chest deformities.

Disease Presentation and Progression

Two patients were stillborn, one at 30 weeks, another at term. Eleven patients presented with HPP at birth whereas the remaining patient presented at 2 weeks of life, consistent with perinatal HPP. Initial symptoms included respiratory distress (9 patients); abnormal skull shape (4 patients); rachitic chest deformity, hypotonia, fractures (2 patients each); and failure to thrive and seizures (1 patient each).

Six patients required respiratory support at birth (two initially with supplemental oxygen and four required intubation and ventilation). All four ventilated patients eventually succumbed to respiratory failure within the first 5 days of life.

Four patients developed seizures. One patient was treated with phenobarbital alone and another patient was treated with phenobarbital, phenytoin, and pyridoxine but there was insufficient documentation to determine whether either patient responded to his/her treatment. The remaining two patients responded to pyridoxine therapy. For all four patients, there was no further documentation of medically intractable seizures. However, all four patients succumbed to respiratory failure.

Radiographic Evaluation

Twelve patients had radiographic examinations shortly after birth although another patient had a prenatal X-ray 10 days before birth. Unfortunately, two patients' postnatal films and reports were not available for review. Common documented radiographic abnormalities included hypomineralization/osteopenia (7 patients), recent fractures (4 patients), metaphyseal flaring (4 patients), and apparent absence of some bones (2 patients). There was no improvement radiographically in any of the 6 patients who survived up to 281 days (Fig. 1).

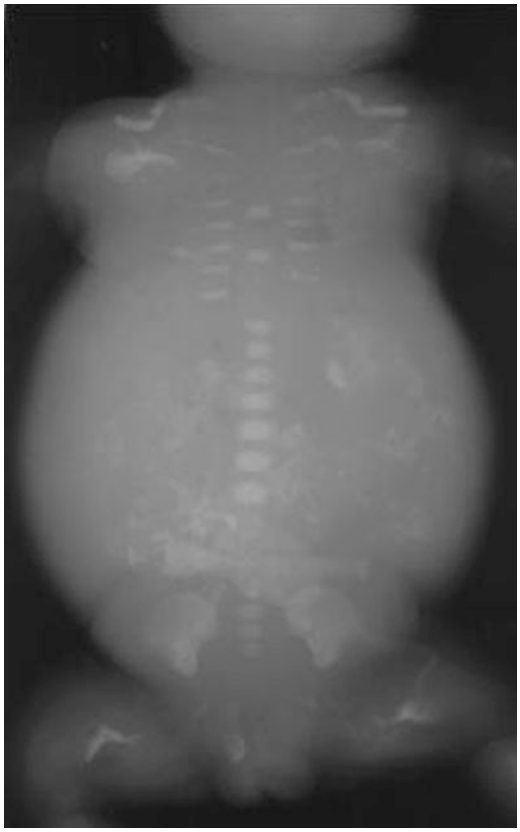

Fig. 1 Skeletal radiograph of Patient 11: Rachitic chest, metaphyseal flaring, and significant hypomineralization of bones to a point of near absence and absence of skeletal mineralization

Survival Data

For the 15 patients who fulfilled our study inclusion criteria for severe HPP, 9 (including the stillbirth) did not survive beyond 1 day of life (4 died despite respiratory support). The remaining patients died at days 4, 11, 36, 125, 263, 281 of life, respectively (Fig. 2). The cause of death was not available for five patients, one of whom died in utero shortly before birth. One patient died after a series of apneic, breath-holding spells, whereas the remaining nine patients died from respiratory complications.

Discussion

Our cohort of perinatal HPP patients in Manitoba, Canada, had major morbidity apparent in the neonatal period. All died within first 9 months of life. All were confirmed by mutation analysis to have autosomal recessive HPP or were presumed to have autosomal recessive HPP because both

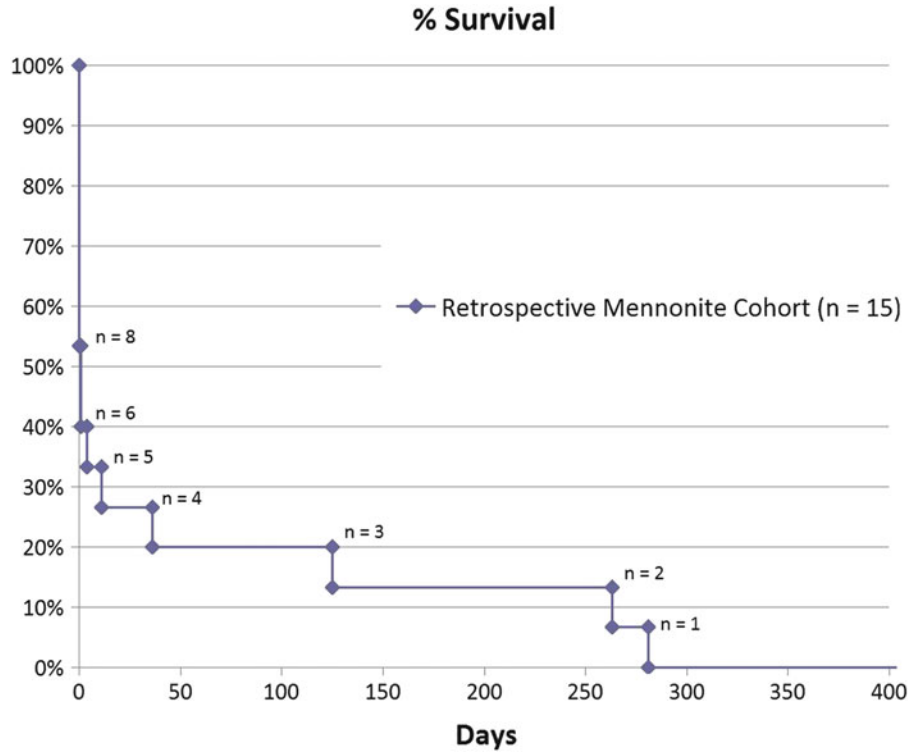

Fig. 2 Kaplan-Meier survival data for Manitoba perinatal hypophosphatasia cohort

parents were confirmed or presumed to be *TNSALP* Gly334Asp mutation carriers through linkage analysis. Hence, in our Mennonite population, the Gly334Asp mutation is a founder mutation, and appears to predict a lethal phenotype when homozygous or when compound heterozygous with the Val382Ile mutation.

For four patients who developed seizures, regardless of their anticonvulsant therapy regimen, their survival was longer than 1 month, unlike the rest of our cohort. It is unclear whether this is a coincidence due to the small number of patients or whether the development of seizures is a prognostic factor for longer survival.

In our cohort of patients who had prenatal ultrasonographic or prenatal radiographic evaluation, all seven reports described either shortened limbs or decreased bone mineralization. None appeared to have the long bone features of fetuses of spontaneous improvement postnatally who were later diagnosed with benign prenatal HPP (Wenkert et al. 2011) and our patients all followed a lethal course.

Most attempts to treat severe HPP have been unsuccessful. Two unrelated patients with worsening infantile HPP received marrow cell transplantations and survived a likely lethal outcome (Cahill et al. 2007; Whyte et al. 2003). Now, enzyme replacement therapy for severe HPP using human recombinant bone-targeted alkaline phosphatase provides promise for improved outcome (Whyte et al. 2012).

Acknowledgments The authors are grateful to the families who participated in this study and to Sharon Allentuck for administrative assistance.

Competing Interest

Dr. Edward C.W. Leung received a research stipend and research grant support from Alexion Pharmaceuticals. Dr. Cheryl Greenberg received research grant support from Alexion Pharmaceuticals. Dr. Michael P. Whyte received consulting fees and research grant support from Alexion Pharmaceuticals.

Synopsis

Perinatal hypophosphatasia in Manitoba Mennonites has been uniformly fatal.

References

Baumgartner-Sigl S, Haberlandt E, Mumm S et al (2007) Pyridoxine-responsive seizures as the first symptom of infantile hypophosphatasia caused by two novel missense mutations (c.677T>C, p.M226T; c.1112C>T, p.T371I) of the tissue-nonspecific alkaline phosphatase gene. Bone 40:1655–1661

Cahill RA, Wenkert D, Perlman SA et al (2007) Infantile hypophosphatasia: transplantation therapy trial using bone fragments and cultured osteoblasts. J Clin Endocrinol Metab 92:2923–2930

Chodirker BN, Evans JA, Lewis M et al (1987) Infantile hypophosphatasia–linkage with the RH locus. Genomics 1:280–282

Chodirker BN, Evans JA, Seargeant LE, Cheang MS, Greenberg CR (1990) Hyperphosphatemia in infantile hypophosphatasia: implications for carrier diagnosis and screening. Am J Hum Genet 46:280–285

Chown B (1936) Renal rickets and dwarfism: A pituitary disease. Br J Surg 23:552–556

Cole DE (2008) Hypophosphatasia update: recent advances in diagnosis and treatment. Clin Genet 73:232–235

Fraser D (1957) Hypophosphatasia. Am J Med 22:730–746

Goseki-Sone M, Orimo H, Iimura T et al (1998) Hypophosphatasia: identification of five novel missense mutations (G507A, G705A, A748G, T1155C, G1320A) in the tissue-nonspecific alkaline phosphatase gene among Japanese patients. Hum Mutat Suppl 1:S263–7

Greenberg CR, Evans JA, McKendry-Smith S, Redekopp S, Haworth JC, Mulivor R, Chodirker BN (1990) Infantile hypophosphatasia: localization within chromosome region 1p36.1-34 and prenatal diagnosis using linked DNA markers. Am J Hum Genet 46:286–292

Greenberg CR, Taylor CL, Haworth JC, Seargeant LE, Philipps S, Triggs-Raine B, Chodirker BN (1993) A homoallelic Gly317 → Asp mutation in ALPL causes the perinatal (lethal) form of hypophosphatasia in Canadian mennonites. Genomics 17:215–217

Macpherson RI, Kroeker M, Houston CS (1972) Hypophosphatasia. J Can Assoc Radiol 23:16–26

McGuire J, Manning F, Lange I, Lyons E, deSa DJ (1987) Antenatal diagnosis of skeletal dysplasia using ultrasound. Birth Defects Orig Artic Ser 23:367–384

Moore CA, Curry CJ, Henthorn PS et al (1999) Mild autosomal dominant hypophosphatasia: in utero presentation in two families. Am J Med Genet 86:410–415

Mornet E, Yvard A, Taillandier A, Fauvert D, Simon-Bouy B (2011) A molecular-based estimation of the prevalence of hypophosphatasia in the European population. Ann Hum Genet 75: 439–445

Pauli RM, Modaff P, Sipes SL, Whyte MP (1999) Mild hypophosphatasia mimicking severe osteogenesis imperfecta in utero: bent but not broken. Am J Med Genet 86:434–438

Shohat M, Rimoin DL, Gruber HE, Lachman RS (1991) Perinatal lethal hypophosphatasia; clinical, radiologic and morphologic findings. Pediatr Radiol 21:421–427

Weiss MJ, Cole DE, Ray K, Whyte MP, Lafferty MA, Mulivor RA, Harris H (1988) A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia. Proc Natl Acad Sci U S A 85:7666–7669

Wenkert D, McAlister WH, Coburn SP et al (2011) Hypophosphatasia: nonlethal disease despite skeletal presentation in utero (17 new cases and literature review). J Bone Miner Res 26:2389–2398

Whyte MP (1994) Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr Rev 15:439–461

Whyte MP, Greenberg CR, Salman NJ et al (2012) Enzyme-replacement therapy in life-threatening hypophosphatasia. N Engl J Med 366:904–913

Whyte MP, Kurtzberg J, McAlister WH et al (2003) Marrow cell transplantation for infantile hypophosphatasia. J Bone Miner Res 18:624–636

Whyte MP (2013) Hypophosphatasia. In: Thakker RV, Whyte MP, Eisman J, Igarashi T (eds) Genetics of bone biology and skeletal disease. Elsevier (Academic Press), San Diego, pp 327–360

JIMD Reports
DOI 10.1007/8904_2013_225

RESEARCH REPORT

Novel Deletion Mutation Identified in a Patient with Late-Onset Combined Methylmalonic Acidemia and Homocystinuria, cblC Type

Paul Hoff Backe · Mari Ytre-Arne · Åsmund Kjendseth Røhr · Else Brodtkorb · Brian Fowler · Helge Rootwelt · Magnar Bjørås · Lars Mørkrid

Received: 22 December 2012 / Revised: 12 March 2013 / Accepted: 14 March 2013 / Published online: 12 April 2013

Abstract Combined methylmalonic aciduria and homocystinuria, cblC type (MMACHC), is the most common inborn error of cellular vitamin B_{12} metabolism and is caused by mutations in the *MMACHC* gene. This metabolic disease results in impaired intracellular synthesis of adenosylcobalamin and methylcobalamin, coenzymes for the methylmalonyl-CoA mutase and methionine synthase enzymes, respectively. The inability to produce normal levels of these two coenzymes leads to increased concentrations of methylmalonic acid and homocysteine in plasma and urine, together with normal or decreased concentration of methionine in plasma. Here, we report a novel homozygous deletion mutation (NM_015506.2:c.392_394del) resulting in an in-frame deletion of amino acid Gln131 and late-onset disease in a 23-year-old male. The patient presented with sensory and motoric disabilities, urine and fecal incontinence, and light cognitive impairment. There was an excessive urinary excretion of methylmalonic acid and greatly elevated plasma homocysteine. The clinical symptoms and the laboratory abnormalities responded partly to treatment with hydroxycobalamin, folinic acid, methionine, and betaine. Studies on patient fibroblasts together with spectroscopic activity assays on recombinant MMACHC protein reveal that Gln131 is crucial in order to maintain enzyme activity. Furthermore, structural analyses show that Gln131 is one of only two residues making hydrogen bonds to the tail of cobalamin. Circular dichroism spectroscopy indicates that the 3D structure of the deletion mutant is folded but perturbed compared to the wild-type protein.

Communicated by: Matthias Baumgartner

Competing interests: None declared

P.H. Backe · M. Ytre-Arne · M. Bjørås
Department of Microbiology, Oslo University Hospital and University of Oslo, 4950, 0424 Oslo, Nydalen, Norway

P.H. Backe (✉) · M. Ytre-Arne · E. Brodtkorb · H. Rootwelt · M. Bjørås · L. Mørkrid
Department of Medical Biochemistry, Oslo University Hospital and University of Oslo, 4950, 0424 Oslo, Nydalen, Norway
e-mail: Paul.Hoff.Backe@rr-research.no

Å.K. Røhr
Department of Biosciences, University of Oslo, Oslo, Norway

B. Fowler
Division of Metabolism, Children's Research Center (CRC), University Children's Hospital, Zürich, Switzerland

Introduction

Combined methylmalonic acidemia and homocystinuria, cblC type, is an autosomal recessive inborn error of intracellular cobalamin metabolism that can cause a wide variety of symptoms including developmental, hematologic, neurological, metabolic, ophthalmologic, and dermatologic abnormalities (Martinelli et al. 2011). The clinical picture is divided into two main forms according to the debut age of symptoms: The early onset form starts usually within the first year of life with more serious and general organ manifestations, while the late-onset form is milder and tends to mainly involve the neurological system. The molecular basis for this distinction is poorly understood. The MMACHC disorder, which is the most common inborn error of cobalamin metabolism, is caused by mutations in the *MMACHC* gene and results in impaired intracellular synthesis of adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), coenzymes for the methylmalonyl-CoA mutase and methionine synthase enzymes, respectively. Reduced activity of these two enzymes leads to increased

concentrations of methylmalonic acid (MMA) and homocysteine in plasma and urine, together with normal or decreased concentration of methionine in plasma (Thiele and Van Raamsdonk 2006). The gene *MMACHC* was identified in 2006 and is located on chromosome 1p34.1 (Lerner-Ellis et al. 2006). The gene encodes a protein of 282 amino acids with a predicted molecular weight of 31.7 kDa. Although the exact function of the protein is currently unknown, it appears to play an important role in the synthesis of cobalamin intermediates. For instance, it has been shown that MMACHC can catalyze a reductive decyanation reaction of cyanocobalamin (CNCbl) to yield cob(II)alamin (Kim et al. 2008) and a dealkylation reaction of AdoCbl or MeCbl using glutathione as an electron donor (Hannibal et al. 2009; Kim et al. 2009). Furthermore, MMACHC has been proposed to interact with combined methylmalonic aciduria and homocystinuria cblD type (MMADHC), the downstream protein in the cobalamin pathway (Deme et al. 2012; Plesa et al. 2011). Recently the three-dimensional structure of human MMACHC was determined (Froese et al. 2012; Koutmos et al. 2011). This provides a structural framework to understand the effects of *MMACHC* mutations and gives new biochemical insight into the catalytic functions of MMACHC.

Here, we have used three-dimensional structure analyses in combination with fibroblast studies and spectroscopic MMACHC activity assay to verify the predicted enzyme impairment of a novel *MMACHC* deletion mutation from a patient suffering from this inherited cobalamin disorder. This study was performed after receiving written informed consent from the patient involved and in accordance with the guidelines of the institutional ethics committee.

Materials and Methods

Initial Clinical Picture

The 23-year-old patient was adopted from an East-Asian country at the age of 8 months. Nothing is known about his parents or relatives. Apart from some possible slightly impaired intellectual disability, he had an unremarkable medical history until January 2009 when he felt tired and worn out and complained of headache. It was interpreted as a flu-like respiratory tract infection. Four weeks later, he was admitted in the local hospital after an acute onset of atactic gait, numbness, and partial paralysis in the lower limbs. Within two days, he developed a fulminant picture of cerebellar edema, verified by CT and MR imaging, and was transferred to another hospital where he was treated with large doses of steroids and transient occipital cranioectomy for pressure relief. A new MR examination demonstrated an extended high intensity lesion in the spinal cord at levels Th9–Th11 and C4–C6. The cerebrospinal fluid contained traces of blood, leukocytes 33 per mm^3, and total protein 0.71 g/L. CSF isoelectric focusing yielded negative results. On the basis of the clinical picture and a long series of negative serum analyses, he was suspected to have an acute demyelinating encephalomyelitis (ADEM). Recovery was nearly complete when he, in November 2009, was vaccinated against the swine flu (epidemic influenza A H1N1). Two weeks later, he again experienced the same acute symptoms as in February, except for the cerebellar edema. However, this time the peripheral neurological disabilities progressed until he, in January 2010, ended up in a wheelchair with partial paresis both in upper and lower extremities. He became incontinent to urine and partially lost the control of the defecation. Neurological examination revealed atrophy, partial loss of peripheral sensibility in both upper and lower extremities, and total loss of deep sensibility in lower limbs. There were weak tendon reflexes, however the Achilles tendon reflexes were clonic, and there was a positive bilateral Babinski sign. Neurography revealed incipient sensomotoric polyneuropathy that was not present at the first episode. From the subsequent laboratory investigations, an intracellular defect of cobalamin handling was suspected (see Results and Discussion).

Fibroblast Studies

Methionine and serine formation was measured as described by Fowler et al. (1997). [^{57}Co]Cobalamin incorporation and coenzyme synthesis was performed as previously described (Suormala et al. 2004). Propionate incorporation in intact fibroblasts was studied according to the method of Willard et al. (1976).

Cloning and Site-Directed Mutagenesis of *MMACHC*

cDNAs encoding full length and a truncated version (residues 1–235) of *MMACHC* were synthesized with codons optimized for expression in *Escherichia coli* (*E. coli*) (GenScript). The cDNAs were subcloned into the EcoRI and HindIII sites of the pETM-11 vector (EMBL collection), which includes an N-terminal His_6-tag and a TEV (tobacco etch virus) protease cleavage site in front of the inserted gene.

The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce the patient's deletion mutation in the pETM-11-MMACHC construct. The oligonucleotide primers used were designed using the manufacturer's protocol: 5′-GCTTACTACTACC*GACAAGATGTGG-3′, 5′-CCACATCTTGTC*GGTAGTAGTAAGC-3′ (* indicates the position of the AAC trinucleotide deletion; ΔGln131). Mutant constructs were verified by DNA sequencing.

Expression and Purification of MMACHC

MMACHC constructs were transformed into *E. coli* BL21 (DE3) RIL Codon Plus cells (Stratagene) for overexpression. Cultures were grown in LB medium. Protein expression was induced when the cell density reached an OD_{600} of ~ 0.80 by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Induced cells were grown for 18 h at 18 °C prior to harvesting by centrifugation. Cell pellets were resuspended in 300–500 mM NaCl, 100 mM Hepes pH 7.0, 5 % Glycerol, and 10 mM imidazole; the cells were lysed by sonication. Cellular debris was removed by centrifugation, and the supernatant was applied to Ni-NTA resin equilibrated in lysis buffer and eluted using 300 mM imidazole. The purified protein was dialyzed against 100 mM HEPES pH 7.0, 150 mM NaCl, and 5 % Glycerol; and frozen before further use.

DNA Extraction and Sequencing

Genomic DNA was extracted from EDTA-blood using QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). Exons 1, 2, 3, and 4 with neighboring intronic and 5′ and 3′ untranslated regions (UTRs) were amplified using AmpliTaq Gold polymerase and Gold buffer (Applied Biosystems; manufactured by Roche, Branchburg, New Jersey, USA), in a MJ Research PTC 200 Thermal Cycler (Watertown, Massachusetts). Sequencing was carried out using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and ExoSAP-IT (USB, Cleveland, Ohio, USA) and analyzed on a 3730 DNA Analyzer (Applied Biosystems). The primers used for amplification and sequencing were from Lerner-Ellis et al. (2006). In addition, we used a second set of primers (5′-CGGACAAGGTCATAACTCCC-3′ (sense) and 5′-TCCTTTTCTTGGCAAACCC-3′ (antisense)) spanning exon 3 to eliminate the possibility of allele dropout caused by allele-specific PCR had there been a polymorphism under one of the original PCR primers.

Characterization of Cobalamin Intermediates Using UV-Vis Spectroscopy

Reaction intermediates in the MMACHC catalyzed cleavage of cyanide from cyanocobalamin were followed using UV-Vis spectroscopy. The activity of all MMACHC variants were monitored in the 250–800 nm range using a HP8454 spectrophotometer in the presence of cyanocobalamin and the reductant sodium dithionite. In all experiments, the protein concentration was maintained at about 20 μM in the working buffer consisting of 0.1 M HEPES pH 7.0, 0.5 M NaCl, and 5 % glycerol. Typically, a 0.95 mL protein sample was transferred to a screw-capped septum-sealed cuvette that went through several cycles of evacuation and purified argon gas flushing using a vacuum manifold. UV-Vis spectra were recorded before and after adding 10 μL of a 1 mM anaerobic aqueous cyanocobalamin stock solution to the sealed cuvette using an airtight Hamilton syringe. To initiate the cleavage reaction, 25–50 μL working buffer containing 10 mM sodium dithionite was added to the cuvette. While the products of dithionite oxidation do not absorb light in the 250–800 nm range, dithionite itself absorbs light at 315 nm. Thus we could ensure a reducing environment in the sealed cuvette by adding sodium dithionite until the 315 nm peak had an absorption of 0.8–1. Experiments were carried out with wild-type MMACHC, the deletion mutant ΔGln131, and the truncated variant.

Circular Dichroism Measurements

Circular dichroism spectroscopy was carried out to examine the folds of the wild-type and mutant MMACHC proteins. Samples containing 10–20 μM protein in 10 mM phosphate buffer pH 8.0 and 150 mM NaCl were transferred to 1 mm quarts cuvettes, and spectra were recorded using a Jasco J-810 spectropolarimeter at a scanning speed of 50 nm/min. For each spectrum, buffer background was subtracted and the final spectrum of each sample was the average of five consecutive scans.

Results and Discussion

Ordinary Laboratory Investigations

Measurement (ref. intervals in parentheses) of plasma amino acids revealed a low methionine 6 μmol/L (14–39), prompting a more complete metabolic screening. We found B-Hb 13.2 g/dL (13.4–17.0); normal leukocyte and platelet counts; S-vit B12 559 pmol/L (160–710); S-folate 29 nmol/L (7.1–27); P-homocysteine 158–176 μmol/L (6–16); S-MMA 118 μmol/L (0.07–0.30); P-C3-acylcarnitine 11.2 μmol/L (0.18–0.80); S-free carnitine 7 μmol/L (28–50); S-total carnitine 20 μmol/L (29–59). The urinary organic acid profile (qualitative investigation by GC-MS after methylation) showed substantially increased MMA and somewhat increased methylcitric acid. Further analyses showed the following component/creatinine ratios: U-glycine 1205 μmol/mmol (40–500); U-cystathionine 19 μmol/mmol (<8.1); U-homocystine 31 μmol/mmol (<1.1); U-guanidinoacetate 159 μmol/mmol (8–50).

Studies on Fibroblast Cells from Patient

The formation of methionine and serine in patient fibroblasts and controls grown in normal medium is shown in

Table 1 Methionine and serine formation. The cells were grown in normal medium and with medium supplemented with different amounts of OHCbl for 3 days

		[^{14}C] Formate incorporated into amino acids, nmol/16 h/mg protein	
	OHCbl μg/L	Methionine	Serine
	0	0.20	0.09
Patient cells	10	1.23	0.28
	100	2.55	0.85
	1000	2.30	0.97
Controls (n = 18)			
Range	0	1.0–4.0	0.38–3.7
Mean		2.42	1.25

Table 1. The cells were grown in normal medium and with medium supplemented with different amounts of hydroxocobalamin (OHCbl) for 3 days (Table 1). The patient cells show clearly deficient methionine and serine formation when cells were grown in normal medium, whereas in medium supplemented with varying concentrations of OHCbl, there is a clear increase in the synthesis of methionine and serine. The values after addition of 1000 μg/OHCbl were 2.3 and 0.90 nmol/16 h/mg protein, respectively, i.e., full rescue of activity. Table 2 shows total uptake of [^{57}Co]-CNCbl and cobalamin coenzyme synthesis. In the patient fibroblasts, the total content of radioactive cobalamin is very low and there is virtually no production of either methyl or adenosyl forms of cobalamin coenzymes. Propionate incorporation in intact fibroblasts is given in Table 3. When cells were grown in normal medium for 3 days, there is a clearly deficient propionate fixation. However, there is a substantial increase in cells when the medium is supplemented with a high concentration of OHCbl.

Molecular Defect

Sequencing of genomic DNA identified a homozygous deletion of three base pairs in exon 3 of MMACHC; NM_015506.2:c.392_394delAAC (Fig. 1). No other DNA sequence variants were identified in the four exons or their neighboring intronic sequences (60–289 nucleotides), 483 nucleotides of the 5' untranslated region (UTR), or the first 174 nucleotides of the 3' UTR. Since both primer sets yielded identical results for the exon 3 deletion, allelic dropout caused by a sequence variant under a primer was ruled out. The mutation identified deletes the last two nucleotides from the triplet coding for Gln131 and the first nucleotide of the codon for Arg132. However, as the first base in these two codons is identical, the result at the protein level is a deletion of the first and conservation of the second amino acid, producing the in-frame deletion mutant ΔGln131. Gln131 is conserved and lies in a highly conserved region of MMACHC that partly forms the cobalamin-binding pocket.

Characterization of the Reductive Conversion of CNCbl by MMACHC Wild-Type and Deletion Mutant

For the wild-type protein, a decreased intensity of cyanocobalamin bands in the 500–600 nm region of the UV-Vis spectrum is observed when the reaction is initiated by adding the reductant sodium dithionite to the reaction mixture. This observation is expected when cyanide is cleaved from cyanocobalamin. However, the peak typical for the product cob(II)alamin at 414 nm does not appear during the reaction (Fig. 2a), complicating the exact identification of the enzymatic product. In the control experiment, where the MMACHC protein was excluded from the reaction mixture, no change in the cyanocobalamin UV-Vis spectrum was observed, indicating that the enzyme catalyzes a cyanide cleavage reaction in the presence of sodium dithionite (data not shown). The truncated form of MMACHC had catalytic activity similar to the wild-type enzyme (data not shown), whereas the deletion mutant was inactive (Fig. 2b).

Structural Analysis of the Deletion Mutation ΔGln131 in the MMACHC Protein

We have performed structural analyses of the deletion mutant ΔGln131 based on the recently determined crystal structure of MMACHC in complex with methylcobalamin (Koutmos et al. 2011). The structure showed that MMACHC is comprised of one N-terminal and one C-terminal module connected by a long linker of about nine residues. The N-terminal core module contains a four-stranded antiparallel β-sheet flanked by α-helices and a short antiparallel two-stranded β-sheet, a fold that is characteristic of the NADP oxidase/flavin reductase family. The C-terminal module consists of four α-helices, of which the most C-terminal of the four is unstructured when MMACHC is in complex with methylcobalamin that caps the core N-terminal module. The corrin ring of cobalamin binds in the large cavity that is formed at the interface of the C-terminal cap and the N-terminal core module. The cobalamin tail is positioned in a shallow and narrow cleft on the surface of MMACHC, which is formed between helix F and the two-stranded antiparallel β-sheet consisting of β-strand 1' and 2' (Fig. 3). Gln131 is located in the loop connecting the two β-strands, and is one of only two hydrogen bonds between the cobalamin tail and protein in

Table 2 [^{57}Co] Total uptake of [^{57}Co]-CNCbl and cobalamin coenzyme synthesis in fibroblasts

	Total uptake pg/mg prot	OHCbl % of total	CNCbl % of total	AdoCbl % of total	MeCbl % of total	Others % of total
Patient cells	9	18	79	0.0	3.0	0.6
Controls						
Range ($n = 23$)	40–156	2.5–2.4	6.9–22	14–28	40–76	
Mean	98	10	12	20	58	

Table 3 Propionate incorporation in intact fibroblasts

	[1-^{14}C] Propionate fixation		
	OHCbl µg/L	nmol/mg protein/ 16h	^{14}C/^{3}H Phe incorporation
Patient cells	0	1.06	0.050
	1,000	12.0	0.472
Controls ($n = 33$)			
Range	0	3.5–24.4	
Mean		10.19	
Range	1,000	4.33–28.9	
Mean		10.3	

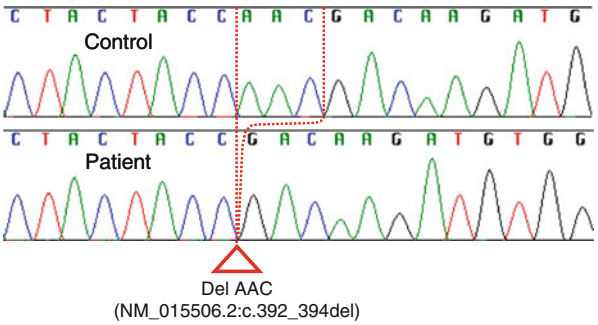

Fig. 1 Sequence chromatogram of genomic DNA from patient and control shows a deletion of three nucleotides in the patient DNA causing an in-frame deletion of Gln131

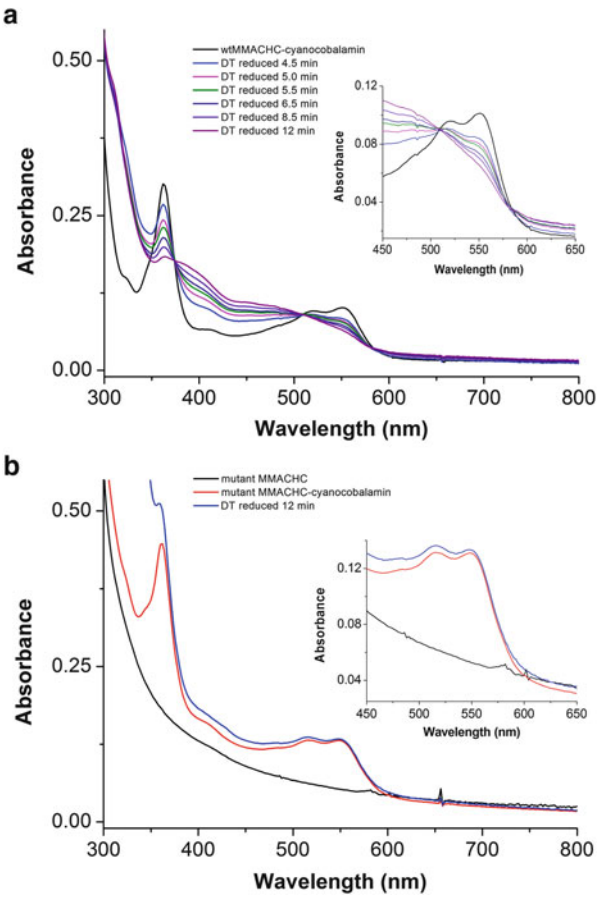

Fig. 2 Reductive conversion of CNCbl by wild-type or mutant MMACHC. (**a**) Conversion of CNCbl catalyzed by wt MMACHC in the presence of the reductant sodium dithionite. As CNCbl reacts, absorbance decreases at 550 nm. The inset shows a close up of the spectra around 550 nm. (**b**) No enzymatic activity can be observed for the deletion mutant ΔGln131

this shallow cleft (Fig. 3). The rest of the interactions are of a hydrophobic nature. It seems clear that Gln131 contributes to tethering the cobalamin tail to the protein and therefore is important for binding and positioning. Thus, a deletion of this residue will most likely disturb the complex formation between cobalamin and MMACHC. The overall folds of the wild-type protein and the deletion mutant were probed by CD-spectroscopy (Fig. 4). Both spectra exhibit features typical for proteins with mixed α-helical and β-sheet secondary structure; however, the spectral minimums are 10 nm apart. This indicates that the deletion mutant's three-dimensional structure is folded but perturbed compared to the wild-type protein.

Treatment, Laboratory Response, and Further Clinical Course

Once the diagnosis was established, the patient was substituted daily with 1 mg OHCbl i.m. and perorally 3 × 400 mg methionine, 4 mg calcium folinic acid, 2 × 6 g betaine (Cystadane®), and 3 × 3.75 mg carnitine (Biocarn®). There was an immediate decrease in the

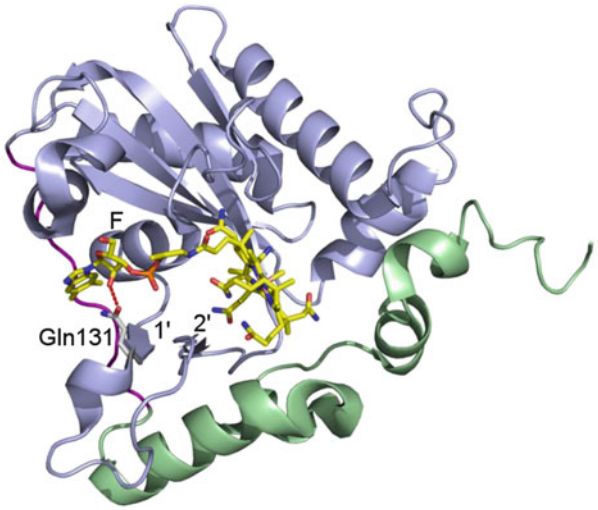

Fig. 3 Cartoon representation of the structure of MMACHC in complex with MeCbl. MMACHC is comprised of one N-terminal (*light blue*) and one C-terminal module (*light green*) connected by a long linker of about nine residues (*purple*). The corrin ring of cobalamin (*yellow*) binds in a large cavity that is formed at the interface of the C-terminal cap and the N-terminal core module. The cobalamin tail is positioned in a shallow and narrow cleft on the surface of MMACHC, which is formed between helix F and the two-stranded antiparallel β-sheet consisting of β-strand 1′ and 2′. Deletion of amino acid Gln131 abolishes a hydrogen bond between the cobalamin tail and the MMACHC protein (PDB id: 3SC0; (Koutmos et al. 2011)

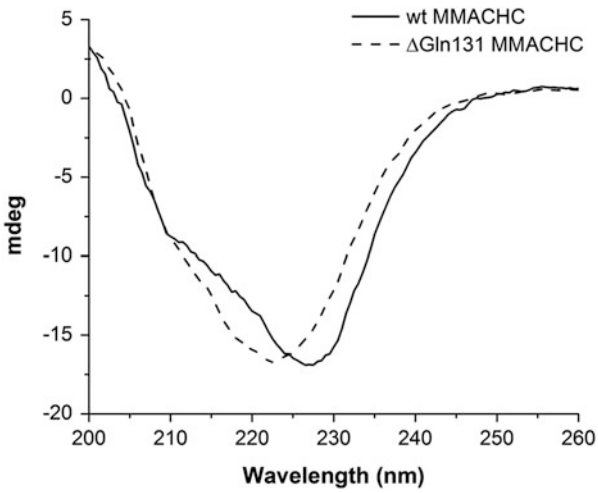

Fig. 4 Circular dichroism spectra of wt MMACHC (——) and ΔGln131 MMACHC (------) recorded in 10 mM phosphate pH 8.0, 150 mM NaCl. For each spectrum, buffer background was subtracted and the final spectrum of each sample was the average of five consecutive scans

disease markers, S-MMA: 32.0, 8.1, and 6.0 μmol/L; P-total homocysteine: 93, 43, and 44 μmol/L; P-methionine 29, n.a., and 25 μmol/L; C3-carnitine: 3.5, n.a., and 1.7 μmol/L 1 month, 1 year, and 2 years respectively, after start of treatment, (n.a. = not analyzed). He gradually regained control over the bladder and anal sphincters, and peripheral sensibility was somewhat improved. At present he normally prefers to use a wheelchair, but he is able to walk up to 100 m without support. The slow and gradual improvement in symptoms parallels the change in the disease markers and is quite consistent with what is observed in other patients with a late-onset cblC disorder. An analogous mode of onset and a similar clinical picture have been described in by (Wang et al. 2012).

Conclusion

We have identified a novel deletion mutation of Gln131 in a patient with late-onset methylmalonic acidemia and homocystinuria cblC type defect. Spectroscopic studies of recombinant mutant protein in addition to patient fibroblasts studies confirm functional deficiency of intracellular cobalamin metabolism. Furthermore, analysis of the recently determined crystal structure of MMACHC in complex with MeCbl shows that amino acid Gln131 is in close proximity to MeCbl and interacts with the vitamin through a hydrogen bond which seems essential for the activity of the protein. The mutation reported here, which deletes amino acid Gln131, also sheds light on the importance of a stable association of the coenzyme precursor in the binding pocket of MMACHC for conserved functional activity. Late-onset cblC disorder has a rare occurrence, and the mutation found in this particular patient has not been described elsewhere. It is therefore difficult to predict the onset mode solely from the genetic defect. Our experiments indicate that the mutated protein possibly retains some of its structural integrity and that some of its function can be restored by appropriate coenzyme access. Patients with late-onset cblC disorder can be remarkably normal until some triggering factors take place. These can be dietary changes, rapid growth, metabolic derangement, and fever (Martinelli et al. 2011; Tsai et al. 2007), but how they intervene is largely unknown. To which extent the initial virus infection and subsequent swine flu vaccination contributed to elicit the clinical symptoms in this particular patient with a late-onset manifestation of the disorder remains to be elucidated.

Acknowledgments This work was supported by grants from University of Oslo and the Norwegian Research Council of Norway and the South-East Health Authority of Norway. B.F. was supported by a grant from Swiss National Science Foundation (320000_122568 and 31003A_138521). P.H.B. also received grants from "Legatet til Henrik Homans Minde" and "Dr. Fürst medisinske laboratoriums fond til klinisk kjemisk og klinisk fysiologisk forskning".

Take-Home Message

A single amino acid deletion in MMACHC causes late-onset combined methylmalonic acidemia and homocystinuria, cblC type, and neurological damage.

References

Deme JC, Miousse IR, Plesa M, Kim JC, Hancock MA, Mah W, Rosenblatt DS, Coulton JW (2012) Structural features of recombinant MMADHC isoforms and their interactions with MMACHC, proteins of mammalian vitamin B(12) metabolism. Mol. Genet, Metab

Fowler B, Whitehouse C, Wenzel F, Wraith JE (1997) Methionine and serine formation in control and mutant human cultured fibroblasts: evidence for methyl trapping and characterization of remethylation defects. Pediatr Res 41:145–151

Froese DS, Krojer T, Wu X, Shrestha R, Kiyani W, von DF, Gravel RA, Oppermann U, Yue WW (2012) Structure of MMACHC reveals an arginine-rich pocket and a domain-swapped dimer for its B(12) processing function. Biochemistry 51:5083–5090

Hannibal L, Kim J, Brasch NE, Wang S, Rosenblatt DS, Banerjee R, Jacobsen DW (2009) Processing of alkylcobalamins in mammalian cells: a role for the MMACHC (cblC) gene product. Mol Genet Metab 97:260–266

Kim J, Gherasim C, Banerjee R (2008) Decyanation of vitamin B12 by a trafficking chaperone. Proc Natl Acad Sci U S A 105:14551–14554

Kim J, Hannibal L, Gherasim C, Jacobsen DW, Banerjee R (2009) A human vitamin B12 trafficking protein uses glutathione transferase activity for processing alkylcobalamins. J Biol Chem 284:33418–33424

Koutmos M, Gherasim C, Smith JL, Banerjee R (2011) Structural basis of multifunctionality in a vitamin B12-processing enzyme. J Biol Chem 286:29780–29787

Lerner-Ellis JP, Tirone JC, Pawelek PD, Dore C, Atkinson JL, Watkins D, Morel CF, Fujiwara TM, Moras E, Hosack AR, Dunbar GV, Antonicka H, Forgetta V, Dobson CM, Leclerc D, Gravel RA, Shoubridge EA, Coulton JW, Lepage P, Rommens JM, Morgan K, Rosenblatt DS (2006) Identification of the gene responsible for methylmalonic aciduria and homocystinuria, cblC type. Nat Genet 38:93–100

Martinelli D, Deodato F, onisi-Vici C (2011) Cobalamin C defect: natural history, pathophysiology, and treatment. J Inherit Metab Dis 34:127–135

Plesa M, Kim J, Paquette SG, Gagnon H, Ng-Thow-Hing C, Gibbs BF, Hancock MA, Rosenblatt DS, Coulton JW (2011) Interaction between MMACHC and MMADHC, two human proteins participating in intracellular vitamin B(1)(2) metabolism. Mol Genet Metab 102:139–148

Suormala T, Baumgartner MR, Coelho D, Zavadakova P, Kozich V, Koch HG, Berghauser M, Wraith JE, Burlina A, Sewell A, Herwig J, Fowler B (2004) The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. J Biol Chem 279:42742–42749

Thiele J, Van Raamsdonk JM (2006) Gene discovery in methylmalonic aciduria and homocystinuria. Clin Genet 69:402–403

Tsai AC, Morel CF, Scharer G, Yang M, Lerner-Ellis JP, Rosenblatt DS, Thomas JA (2007) Late-onset combined homocystinuria and methylmalonic aciduria (cblC) and neuropsychiatric disturbance. Am J Med Genet A 143A:2430–2434

Wang X, Sun W, Yang Y, Jia J, Li C (2012) A clinical and gene analysis of late-onset combined methylmalonic aciduria and homocystinuria, cblC type, in China. J Neurol Sci 318:155–159

Willard HF, Ambani LM, Hart AC, Mahoney MJ, Rosenberg LE (1976) Rapid prenatal and postnatal detection of inborn errors of propionate, methylmalonate, and cobalamin metabolism: a sensitive assay using cultured cells. Hum Genet 34:277–283

JIMD Reports
DOI 10.1007/8904_2013_226

RESEARCH REPORT

Substrate Reduction Therapy in Four Patients with Milder *CLN1* Mutations and Juvenile-Onset Batten Disease Using Cysteamine Bitartrate

M. Gavin · G.Y. Wen · J. Messing · S. Adelman · A. Logush · E.C. Jenkins · W.T. Brown · M. Velinov

Received: 05 January 2013 / Revised: 16 March 2013 / Accepted: 19 March 2013 / Published online: 16 April 2013

Abstract Homozygous mutations in the gene *CLN1* typically result in infantile-onset neuronal ceroid lipofuscinosis, a severe progressive neurological disorder with early death. The gene *CLN1* encodes the enzyme palmitoyl protein thioesterase (PPT1), which is involved in lysosomal degradation of S-fatty acylated proteins. Cysteamine bitartrate (Cystagon) has been shown to reduce the storage material in PPT1 deficient cells. We report the results of a 7-year, open label, nonrandomized trial using Cystagon in four individuals with juvenile-onset NCL resulting from milder *CLN1* mutations. The Cystagon doses were gradually increased with the goal of achieving 50 mg/kg bodyweight. The disease progression was monitored with parental questionnaires in four treated individuals and five untreated controls with the same *CLN1* mutations. Mononuclear leukocytes from the treated individuals were examined for submicroscopic lysosomal storage inclusions. Cystagon treatment resulted in decreased storage material in peripheral leukocytes of the treated individuals. No severe side effects were noted. An allergic rash occurred in one of the individuals that required a dose reduction. The treatment did not result in overall attenuation of the disease progression. Slower progression of the disease was observed in two of the individuals when they were analyzed separately. However, slower progression in these individuals was also observed prior to starting the treatment. This effect may have been due to the higher Cystagon dose achieved in this group, but it could also have been coincidental. The apparent lack of toxicity of Cystagon may warrant further Cystagon trials in infantile NCL, possibly in conjunction with other developing therapies.

Communicated by: Michael J Bennett, PhD
Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2013_226) contains supplementary material, which is available to authorized users.

M. Gavin · G.Y. Wen · J. Messing · S. Adelman · A. Logush · E.C. Jenkins · W.T. Brown · M. Velinov (✉)
NYS Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314

Introduction

The neuronal ceroid lipofuscinoses (NCL), also referred to as Batten disease, include at least 13 progressive genetic disorders, characterized with neurodegeneration and early death. A unifying feature of these devastating conditions is the lysosomal accumulation of autofluorescent lipopigment (Hobert and Dawson 2006). Homozygosity for inactivating mutations in the gene *CLN1* (encoding PPT1) typically results in the severe infantile form of NCL with an onset within the first 2 years of life, a fast downhill course, and early death (Mole et al. 2010). Milder *CLN1* mutations may present with later onset NCL at various ages (Das et al. 1998). PPT1 is a lysosomal enzyme that is abundant in the brain, and removes long chain fatty acids bound to cysteine residues of S-fatty acylated proteins (Hofmann et al. 1997). Cysteamine bitartrate (Cystagon), a lysosomotropic agent, has been shown in vitro to inhibit the formation of cysteine thioesters, the storage material in PPT1 deficient cells (Zhang et al. 2001). It was thus suggested that Cystagon might be used as a substrate reduction agent in infantile NCL due to *CLN1* mutations (Zhang et al. 2001). This effect on substrate accumulation was not replicated in later experiments (Lu and Hofmann 2006). Cystagon has been successfully used since the 1980s in the treatment of another progressive lysosomal storage condition, cystinosis.

Table 1 Treated and control individuals included in the Cystagon protocol

Patient ID	CLNI mutation	Age of first manifestations	Ages of treatment	Dose range
1-2	cA223C homozygote	8 years	16–24 years	30–50 mg/kg
1-6	gT125C homozygote	3 years	8–11 years	4.7–25 mg/kg
1-9	cC451T/cG749T compound heterozygote	5 years	13–18 years	17–38 mg/kg
1-10	cA223C homozygote	8 years	13–18 years	30–50 mg/kg
1-5	gT125C homozygote	6 years	Control	Control
1-7	cC451T/cG749T compound heterozygote	6 years	Control	Control
1-8	cC451T/cG749T compound heterozygote	6 years	Control	Control
1-11	cA223C homozygote	7 years	Control	Control
1-12	cA223C homozygote	6 years	Control	Control

The beneficial effect of Cystagon in the treatment of cystinosis is due to its ability to decrease/clear the abnormal lysosomal accumulation of cystine associated with this disorder (Goodyer 2011).

The lysosomal storage disorders, including NCL, are progressive conditions. Specific disease-modifying treatments in these conditions has been typically associated with better therapeutic outcomes when such treatment was initiated earlier in the course of the disease, and best prior to the onset of clinical manifestations. *CLN1* mutations are typically associated with infantile-onset NCL and a very fast progression that leads to early death between the ages of 2 and 9 years (Mole et al. 2010). Late-onset (atypical) forms of *CLN1* disease were previously described. These milder *CLN1* variants are associated with later disease onset and milder clinical course that more closely resembles juvenile NCL due to *CLN3* mutations (Das and Becerra 1998). Patients with such atypical forms of *CLN1* disease are better candidates for a pilot trial of specific disease treatment since they are often diagnosed at an earlier stage of the disorder, when stabilization/slowing of disease progression may be easier to achieve.

Patients and Methods

Patients Included in the Study

Four individuals, homozygous for atypical CLN1 mutations and with absent or very low PPT1 activity were included in this study. The treated individuals' mutations, age at initial presentation, and age of inclusion in the study are summarized in Table 1. The clinical manifestations in all individuals studied started after the age of 3 years and on initial evaluations were suggestive of the juvenile type of NCL. The two treated individuals, who had siblings previously diagnosed with NCL, had clinical manifestations of the disease reported at an earlier age than their siblings. This may be due to an increased parental awareness of the early signs and symptoms of the disease. Some of the data on one of the treated individuals were previously reported (Wen et al. 2012). The study was conducted under an IRB-approved protocol, and all participants were included after an informed consent was signed.

Nontreated Controls

Five control individuals, who had the same *CLN1* mutations as the treated individuals, were included in this study. Three of these individuals were older siblings of the treated individuals. Two were unrelated to the treated individuals. Mutations and age of onset of the control individuals are shown in Table 1.

Groups of Monitored Individuals

The treated subjects and the controls were divided into three observational groups consisting of individuals with identical mutations within each group. Three groups were established as follows:

- Group 1 included two treated and two control (untreated) individuals, who are homozygous for *CLN1* mutation c.223A>C.
- Group 2 included one treated individual and one untreated sibling, who are homozygous for *CLN1* intronic mutation g.125T>C.
- Group 3 included one treated and two untreated siblings, who are compound heterozygotes for the *CLN1* mutations c.749G>T/451C>T.

Treatment Regimen

Criteria for inclusion in the study were the ability to swallow and eat without the assistance of a gastric tube, and the lack of known allergies to Cystagon or penicillamine.

The initial evaluation consisted of general physical, neurological, and genetic evaluations.

The initial evaluation included CBC, SMA18, and electron microscopy study of peripheral white blood cells for inclusions. An EEG and brain MRI were also performed. After the initiation of the Cystagon treatment, the patients were evaluated at 3, 6, and 12 months during the first year, and then every 6 months. All evaluations, blood tests, brain MRIs, and EEGs were repeated every 6 months. The initial doses of Cystagon were 25–30 mg/kg that were gradually increased with a goal of 40–50mg/kg. In patients 1-6 and 1-9 (Table 1), this could not be achieved because of the discomfort associated with the unpleasant taste of Cystagon and/or because of the side effects of the medication (see Results). The age of the treated patients when the treatment was conducted is shown in Table 1.

Disease Severity Scores (DSS)

In order to monitor the disease progression and severity, disease severity scores (DSS) were established for every year of life of each treated and control individual. The previously established disease severity scale by Kohlschutter et al. (1988) was used with one modification: one additional scored parameter – psychiatric manifestations (behavior) was added. The additional parameter was also scored in scale 0–3 as were the other parameters. This scale is in use in the Batten Disease Registry in our Institute. The scoring for every year of life was done by the patients' parents. Simple, nonmedical language instructions for scoring were developed (see parental questionnaire in Supplement 1). Total DSS were obtained for every treated or control individual and for every year of life by adding the scores for all parameters.

Evaluation of Lysosomal Inclusions with Transmission Electron Microscopic (EM) Examination

Three of the treated individuals (patients 1-2, 1-6, and 1-9) had peripheral blood mononuclear leukocytes studied with electron microscopy (EM) for NCL-associated cytoplasmic inclusions at about 6 months intervals as previously described (Anderson et al. 2006).

Between 200 and 250 peripheral blood cells were studied on every specimen.

Results

Adverse Reactions

Minimal adverse reactions after the start of the Cystagon treatment were observed. One of the study participants (individual 1-9) had an allergic rash after starting the treatment. This resolved after the Cystagon dose was reduced. In addition, bad breath and lightening of the hair color were of concern during the Cystagon use in all participants. It is important to point out that maintaining the targeted dose of Cystagon required swallowing of large numbers of capsules every day. The parents were instructed to mix the content of the capsules with food. This may have created some degree of discomfort in the participants related to the unpleasant taste of Cystagon. An electroencephalogram (EEG) was performed prior to the initiation of Cystagon and every 6 months while on treatment. All EEGs revealed nonspecific abnormalities during wakefulness – an excess of diffuse irregular slow waves, rhythmic bilateral slow waves, or slowing of the posterior background. In individual 1-2, the posterior background was gradually lost as the disease progressed and epileptiform discharges (spikes and sharp waves) appeared later. In Individual 1-6, the waking background activity slowed over time and epileptiform discharges also emerged. In individual 1-9, frequent generalized and multifocal epileptiform discharges were seen at the outset both spontaneous and induced by photo stimulation and electrographic seizures were seen on two occasions. Patients 1-2, 1-6, and 1-9 all developed clinical seizures during the trial and were placed on anti-seizure medications.

An MRI was performed prior to the initiation of Cystagon and every 6 months while on treatment. Cerebellar atrophy was shown on all MRIs. Routine blood work including sodium, potassium, chloride, glucose levels, and hemoglobin, hematocrit, WBCs, RBCs, and platelets were performed prior to the initiation of Cystagon and every 6 months while on treatment and were all within normal limits with no significant changes from baseline.

Lysosomal Inclusions

The results of the EM studies and the Cystagon doses are shown on Fig. 1. The proportion of cells with inclusions and the Cystagon doses were plotted against the participants' age. A tendency towards decrease of the proportion of cells with storage inclusions correlating with the dose of Cystagon is apparent in all three participants that were monitored. A temporary increase of the storage inclusions in the peripheral cells of individual 1-2 was observed after a period when Cystagon administration was temporarily discontinued for about 6 months. Overall, these studies demonstrate that Cystagon use was associated with decrease in storage material in the peripheral blood cells of the treated individuals.

Questionnaire Results

Results of the Disease Severity Scoring of the treated patients and nontreated controls are shown on Fig. 2. The disease severity scores (DSS) were obtained from the completed disease questionnaire. The score of all six

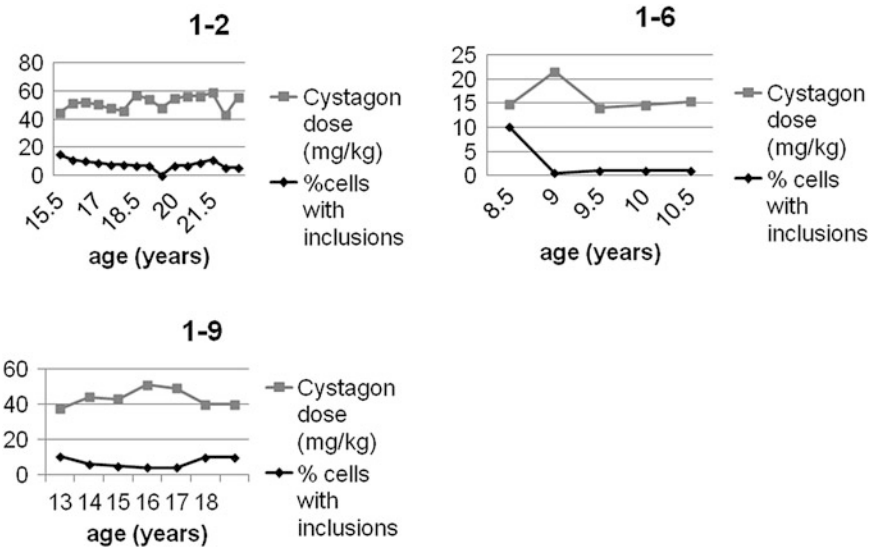

Fig. 1 Proportions of peripheral blood cells with NCL-specific inclusions identified on electron microscopy (EM) studies in three of the treated study participants are shown in relation to the Cystagon doses. Such EM studies were not done for participant 1-10. The ID number of each patient is shown on top of the graph. These studies demonstrate decrease in the NCL-specific inclusions with the Cystagon treatment

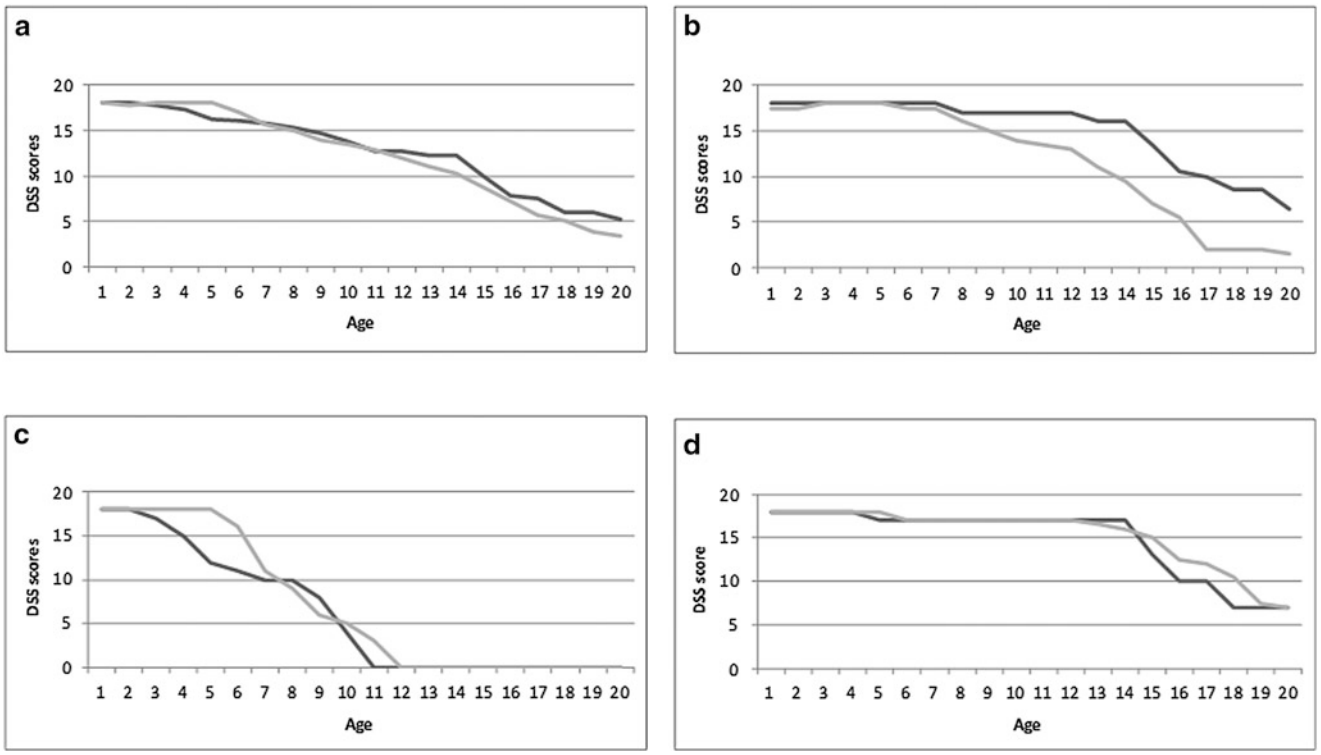

Fig. 2 *Disease severity scores (DSS) at different ages in the treated and control individuals.* Average disease severity scores (DSS) are shown on the Y-axis and patients' age in years are shown on the X-axis. The treated patients are indicated with *black lines* and the controls with *gray lines*. Lower scores show more advanced disease stage. (**a**) Results of all treated and control individuals combined. (**b**) Results in group 1. (**c**) Results in group 2. (**d**) Results in group 3. Only the results in group 1 show lower disease severity/ progression in the Cystagon treated individuals (see text for further explanation)

parameters were added (maximum score of 18) to obtain the DSS. The DSS plotted against the patients' age are shown for the participants in each of the three observational groups on Fig. 2. The combined scores of all study participants did not show any apparent difference in disease progression between treated and control individuals

(Fig. 2a). There was evidence of a slower disease progression in the two treated individuals in observation group 1 compared to nontreated controls (Fig. 2b). However, this slower disease progression was also observed in the treated individuals prior to the initiation of the treatment. In the other two observational groups, no treatment effect was noted as evidenced by the DSS. In addition, the parents of one of the patients from group 1 stated that they witnessed a decrease in the psychotic manifestations, auditory and visual hallucinations, and an improvement of the patient's gait and concentration paralleling the Cystagon administration. When the Cystagon was stopped for a brief period of time, the visual and auditory hallucinations returned. The parents also felt that the Cystagon administration improved the patient's overall functioning (Wen et al. 2012).

Limitations of the Study

Our study has a number of limitations. It involves a very small number of patients and it is not randomized. Also, the parents, who scored the patients' manifestations were not blinded regarding Cystagon administration. The scoring was done in a retrograde manner for the period prior the initiation of the study and some of the period of the study. All these limitations may have introduced a bias in the obtained results. The used DSS in its original development was validated only for patients between ages 3 and 20 (Kohlscutter et al. 1988). The scoring of our study participants was done from age 1. However, the Cystagon treatment was not started until after the third birthday in all treated patients.

Discussion

Various treatments for patients with *CLN1* mutations have been suggested, but no specific treatment was shown to be particularly successful to date. The cysteamine bitartrate (Cystagon) was shown to be able to metabolize the storage material in INCL. Study of phosphocysteamine, an agent with very similar structure and activity to Cystagon, was shown to prevent accumulation of storage material and to inhibit apoptosis in lymphoblasts of INCL patients (Zhang et al. 2001). However, later in vitro experiments with cysteamine showed inefficient cleavage of PTT substrates by this agent and by related aminothiols (Lu and Hofmann 2006). An ongoing trial using a combination of Cystagon and N-acetylcysteine in patients with typical *CLN1* mutations and infantile NCL, conducted in the National Institute of Child Health and Human Development, is listed on the NIH website "Clinicaltrials.gov", but per our knowledge no results of this trial were published to date. No other human trials using Cystagon in NCL patients were reported/listed to date.

Our study showed that the use of Cystagon was associated with a decrease in the storage material in individuals with *CLN1* mutations. Such decrease seemed to be reversible if the Cystagon intake was discontinued or reduced. Cystagon use overall did not show significant attenuation in the disease progression if all treated patients were analyzed together. In one of the groups studied, the disease progression in the treated patients was slower compared to controls with the same *CLN1* mutations (observational group 1). However, this slower progression was seen prior to the initiation of the Cystagon treatment and may not have been related to the Cystagon administration. Interestingly, in observational group 1 the highest Cystagon dose was achieved. High Cysteamine doses were needed for a significant therapeutic effect in cystinosis (Gahl et al. 1987). Cysteamine was recently suggested as a possible treatment for Huntington disease. A new neuroprotection mechanism of Cysteamine was demonstrated, related to its effect in increasing the level of brain-derived neurotrophic factor in a model of Huntington disease (Borrell-Pages et al. 2006). Therefore, beneficial effects of the Cystagon in NCL other than substrate reduction are also a possibility.

In conclusion, overall no significant clinical positive effect of Cystagon was seen in the current study involving four patients. It is not clear if the slower disease progression in the treated patients in observational group 1 is partially due to Cystagon administration since this slower progression was observed prior to the initiation of the Cystagon treatment. It is possible that the small size of the study limited our ability to see small positive effect.

Acknowledgments This study is dedicated to the memory of Dr. Krystyna Wisniewski. The study was initiated and continued under her supervision until her death in 2008. Without her leadership, the study would not have been possible.

Synopsis

Cystagon treatment in patients with *CLN1* mutations.

References

Hobert JA, Dawson G (2006) Neuronal ceroid lipofuscinoses therapeutic strategies: past, present and future. BBA 1762:945–953

Mole SE and Williams RE (2010) Neuronal ceroid lipofuscinoses. In: Pagon RA, Bird TD, Dolan CR, Stephens K (eds) Gene reviews. [Internet] Seattle (WA), University of Washington, Seattle

Das A, Becerra CHR, Yi W, Lu JY, Siakotos AN, Wisniewski KE, Hofmann SL (1998) Molecular genetics of palmitoyl-protein thioesterase deficiency in the US. J Clin Invest 102:361–370

Hofmann SL, Lee LA, Lu YY, Vekruyse LA (1997) Palmytoyl protein thioesterase and the molecular pathogenesis of infantile neuronal ceroid lipofuscinosis. Neuropediatrics 28:27–30

Zhang Z, Butler JD, Levin SW, Wisniewski KE, Brooks SS, Mukherjee AB (2001) Lysosomal ceroid depletion by drugs: therapeutic implications for a hereditary neurodegenerative disease of childhood. Nat Med 7(4):478–484

Lu JY, Hofmann SL (2006) Inefficient cleavage of palmitoyl-protein thioesterase (PPT) substrates by aminothiols: Implications for treatment of infantile neuronal ceroid lipofuscinosis. J Inher Metab Dis 29:119–126

Goodyer P (2011) The history of cystinosis: lessons for clinical management. Int J Nephrol 2011, 929456. doi:10.4061/2011/929456

Wen GY, Wisniewski KE, Messing J, Nealy V, Gavin M, Velinov M, Jenkins EC, Brown WT (2012) Cystagon treatment for neuronal ceroid lipofuscinosis: an 8-year case study. J Clinic Case Reports 2:108. doi:10.4172/jccr.1000108

Kohlscutter A, Laabs R, Albani N (1988) Juvenile neuronal ceroid lipofuscinosis (JNCL): quantitative description of its clinical variability. Acta Paediatr Scand 77:867–872

Anderson GW, Smith VV, Brooke I, Malone M, Sebire NJ (2006) Diagnosis of neuronal ceroid lipofuscinosis (Batten disease) by electron microscopy in peripheral blood specimens. Ultrastruct Pathol 30:373–378

Gahl WA, Reed GF, Thoene JG, Schulman JD, Rizzo WB, Denman DW, Schlesselman JJ, Corden BJ, Schneider JA (1987) Cysteamine therapy for children with nephropathic cystinosis. N Engl J Med 316:971–977

Borrell-Pages M, Canals JM, Cordelieres FP, Parker JA, Pineda JR, Grange G, Bryson EA, Guillermier M, Hirsh E, Hantraye P, Cheetham ME, Neri C, Alberch J, Brouillet E, Saudou F, Humbert S (2006) Cystamine and Cysteamine increase brain levels of BDNF in Huntington disease via HSJ1b and transglutaminase. J Clin Invest 116:1410–1422

JIMD Reports
DOI 10.1007/8904_2013_227

CASE REPORT

A Clinically Severe Variant of β-Mannosidosis, Presenting with Neonatal Onset Epilepsy with Subsequent Evolution of Hydrocephalus

Broomfield A • Gunny R • Ali I • Vellodi A • Prabhakar P

Received: 24 September 2012 / Revised: 19 March 2013 / Accepted: 20 March 2013 / Published online: 16 April 2013

Abstract β-Mannosidosis results from a functional deficiency of the lysosomal enzyme, β-mannosidase. While being a well recognised, naturally occurring disease in both goats and cattle, it is an extremely rare disorder in humans with the first cases only being recorded in 1986. Until now the severity of the human disease has not mirrored that of its bovine or caprine counterparts, whose presentation is typically in the neonatal period with both altered skull morphology and seizures. Human β-mannosidosis has previously appeared to be a more indolent disease, with its only consistent phenotypic feature being developmental delay of varying severity. We report a patient, homozygous for a private mutation, who presented in the immediate perinatal period with seizures and who subsequently developed communicating hydrocephalus at 2 years of age.

These are two new phenotypic features for β-mannosidosis. The first being the neonatal onset of the seizures, for while seizures have been seen in 3 out of the previous 20 documented cases, they have never before manifested prior to 6 months of age. However, as in the previous documented cases, the seizures were difficult to control and were associated with severe developmental delay.

The second unique feature about this case was the development of communicating hydrocephalus. We discuss the possible mechanisms of its development.

In summary, β-mannosidosis must thus now be considered in the differential diagnosis of neonatal onset seizures, and the potential for the development of hydrocephalus should be monitored during subsequent clinical follow-up.

Communicated by: Verena Peters

Competing interests: None declared

Broomfield A (✉) · Vellodi A
Metabolic Medicine Unit, Great Ormond Street Hospital for Children with UCL Institute of Child Health, London, UK WC1N 3JH
e-mail: alexander.broomfield@gosh.nhs.uk

Gunny R
Department of Radiology, Great Ormond Street Hospital for Children with UCL Institute of Child Health, London, UK

Ali I
Paediatric Department, Barts Health NHS Trust, Newham University Hospital, Glen Road, Plaistow, London E13 8SL, UK

Prabhakar P
Department of Neurology, Great Ormond Street Hospital for Children with UCL Institute of Child Health, London, UK

Introduction

β-Mannosidosis in man is a rare panethnic lysosomal storage disorder which results from a deficiency in the function of β-mannosidase, an enzyme that regulates the removal of N-linked mannose moieties during oligosaccharide catabolism. Currently there is a lack of a common clinical phenotype (Bedilu et al. 2002) which, when coupled to the absence of many of the overt hallmarks of classical storage disorders, i.e. organomegaly, dysostosis multiplex, ophthalmological changes or lymphocytic vacuolation (Thomas 2001), makes its clinical recognition difficult. We report an infant who presented in the immediate perinatal period with recurrent seizures who subsequently developed hydrocephalus. This presentation reflects a severity of disease that is more in keeping with the naturally occurring caprine or bovine diseases, than that previously documented in man.

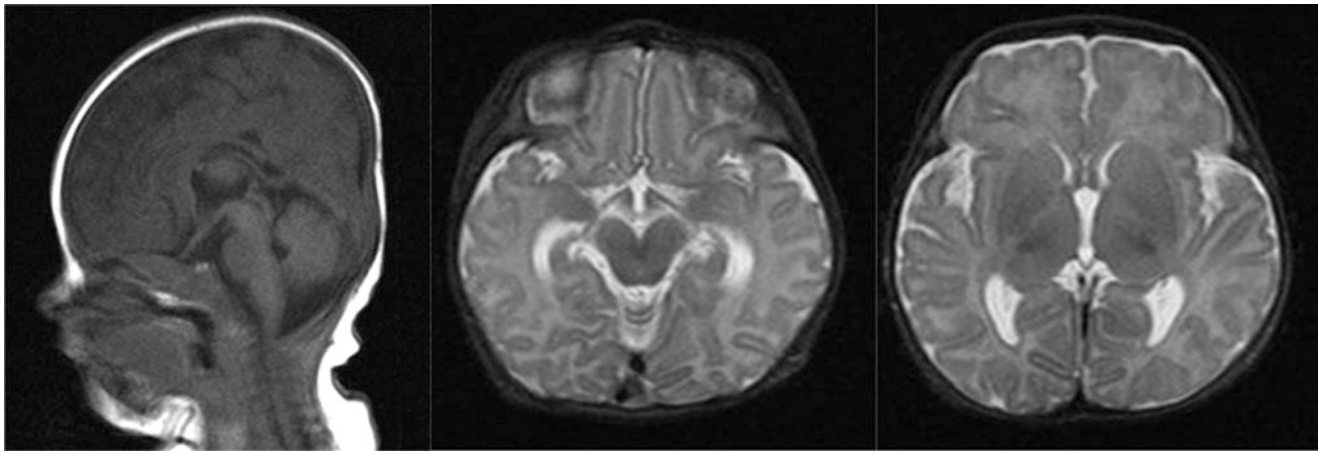

Fig. 1 Initial MRI performed at 4 weeks of age

Case Report

The patient, a girl, is the first child of consanguineous South Asian parents (the parents being 1st cousins). Three cousins have developmental delay and epilepsy of unknown aetiology. Antenatally there were no concerns, and she was born in good condition at term by vaginal delivery with forceps. However, at 24 h of age, she was noted to have abnormal clonic movements of her limbs and was poorly responsive. A full septic screen including lumbar puncture was normal, as was a cranial ultrasound and routine biochemistry. She was started on phenobarbitone and discharged home, pending further investigations. Prior to discharge, auditory brainstem response evaluation was performed which indicated she had impaired sensoneural hearing.

At 3 weeks of age, she developed recurrent generalised tonic-clonic seizures. Examination at that time revealed an increased truncal tone, brisk reflexes and a subtle facial dysmorphism with slight hypertelorism and flattening of the nasal bridge. Brain MRI at 4 weeks of age showed prominent ventricles and cerebrospinal fluid volume in the subarachnoid spaces, but no focal lesions were noted and myelination was age appropriate (see Fig. 1). The frequency of the seizures increased, despite the introduction of sodium valproate and a trial of pyridoxine, necessitating her transfer to the regional neurological centre for further assessment. A further panel of infective and metabolic investigations to investigate the cause of this perinatal onset of seizures was performed. This included both viral and bacterial plasma and CSF PCRs, urine organic acids, urine aminoadipic semialdehyde, urinary sulphite, urinary glycosaminoglycans, plasma electrolytes including calcium, paired plasma and CSF amino acids, ammonia, purine analysis, vacuolated lymphocytes, biotindase, very long chain fatty acids, CSF neurotransmitters and tetrahydrofolate and CSF/plasma glucose ratios. All of these investigations were unremarkable. The patient was also noted to have a degree of stridor at this time with laryngomalacia being confirmed on microlaryngobronchoscopy. Seizure control was established by the use of topiramate and phenytoin at 3 months of age.

At discharge at 3 months she had mild persistent stridor and pronounced gastroesophageal reflux, and required feeding by nasogastric tube. However, her seizures appeared to be well controlled. She made some progress over the next 2 months with a degree of resolution of her stridor and the development of some head control. However at 5 months of age, following further seizures, the diagnosis of β-mannosidosis was established by the demonstration of a leucocyte β-mannosidase activity of 0.2 nmol/h/mg of protein (normal range 100–187). This enzymic study was undertaken as urinary oligosaccharide analysis as a screening test for the glyocoproteinoses is not routinely available at our centre. Subsequent urine analysis has confirmed the presence of the typical disaccharide. Mutation analysis subsequently revealed homozygosity for c.293T>A, a private mutation in exon 3 of MANBA which encodes for a stop codon**.**

Her seizures again became more prominent at 8 months of age where despite the use of clobabazm, topiramate and midazolam she continued to have breakthrough seizures. She also developed diarrhoea requiring the use of a hypoallergenic infant formula (Neocate); loperamide was required for symptomatic relief. She remained severely developmentally delayed with signs of visual impairment.

At her current age of 23 months, she is unable to sit, has visual inattention and only babbling vocalisation. Her ears show retracted tympanic membranes suggestive of a mild ear effusion, with tympanometry confirming middle ear dysfunction.

Given her extremely poor clinical state, the slow increase in her head circumference (9th centile at birth, 75th at 9th months, to the 91st centile at 23 months of age) was not initially investigated. However at 22 months a further increase in her seizure activity, together with unequal upper limb tone, raised the possibility of raised intracranial pressure or focal pathology. Brain MRI was thus undertaken and demonstrated a communicating hydrocephalus. However shortly after the MRI, her clinical state stabilised without further intervention.

Now at 25 months of age, her head circumference is static on the 91st centile and her seizures are controlled by topiramate with additional midazolam when required. Although neurological stable, she remains severely neurological affected with generalised increased tone in all 4 limbs requiring baclofen. She also requires exclusively feeding by NG tube, due to the extent of her dysphagia. She continues to have only mild hepatosplenomegaly and minimal facial coarsening but has now also suffered a fracture of her left femur with bone mineral density scanning revealing a generalised decrease in bone mineral density.

Discussion

β-Mannosidosis is an extremely uncommon lysosomal storage disease with an estimated incidence of 0.1 per 100,000 (Poorthuis et al. 1999; Poupetova et al. 2010). It results from a deficiency in the activity of the lysosomal enzyme β-mannosidase which cleaves the unique β(1-4)-linked mannose sugar found in all N-linked oligosaccharides of glycoproteins (Thomas 2001). To the best of our knowledge, there have been 20 cases in 16 families reported in the medical literature, since it was first described in humans in 1986 by 2 independent groups (Cooper et al. 1986; Wenger et al. 1986). While the age of presentation ranges from a few weeks (Dorland et al. 1988) to adulthood (Gort et al. 2006), overall the disease has been thought to generally be less severe than that found naturally occurring in goats or cows (Bedilu et al. 2002), where frank dysmorphism and severe neonatal onset neurological disease is the norm (Jones et al. 1983; Abbitt et al. 1991). This has in part been attributed to the fact that β-mannosidosis deficient ruminants accumulate the trisaccharide Man(β1-4) GlcNAc (β1-4) GlcNAc to a greater extent than the disaccharide Man(β1-4) GlcNAc (Jones et al. 1992). However humans who, like other non-ruminants, have an additional lysosomal enzyme chitobiase (Zhu et al. 2006) accumulate the disaccharide (Cooper et al. 1988). This potential correlation between phenotype and predominating oligosaccharide is strengthened by the fact that the mouse model, where predominately disaccharides accumulate, also exhibits a mild phenotype with no obvious dysostosis (Zhu et al. 2006).

In a review of the literature, Bedilu found that the main phenotypic traits in man were developmental delay and hearing loss (Bedilu et al. 2002), although many of the more recent cases do not have the hearing impairment. Both of these features were present in our patient, with the hearing loss being suspected from an extremely early age. Interestingly, this review also indicated that there was little genotype/phenotype correlation with patients in that study surviving until middle age with null mutations. Thus, it cannot be inferred that the null mutation found in this patient was the major determinant of the severity of presentation.

Distinguishing whether the developmental delay was secondary to the seizures or the underlying mannosidosis is impossible, though it seems that once seizures are noted in β-mannosidosis, a loss of skills does ensue (Cooper et al. 1991; Cherian 2004). This lack of consistent clinical features means that there is increased reliance on screening tests diagnostically and would support the use of urinary oligosaccharides as part of initial investigations of potential metabolic disorders. The first unique feature of this case is the onset of seizures in the perinatal period. Previously the earliest manifestation of any neurological impairment in a patient with β-mannosidosis was the dysphagia seen at 8 weeks, in one of the Turkish siblings described by Dorland (Dorland et al. 1988). The earliest persistent seizure activity documented was in the Saudi siblings described by Cherian at 6 months of age, however in both of these patients the seizures were initially isolated and associated with temperatures, unlike our patient, and only became persistent later (Cherian 2004). The historical patient, who appears to be most similar to our patient, was the girl described by Cooper et al. (1991). She also presented with sudden onset of persistent generalised tonic-clonic seizures which were also difficult to control. However, the age of onset of the seizures was at 9 months of age and was preceded by a history of developmental delay. Including this case, seizures have occurred in 4 cases out of the 21 reported (Cooper et al. 1991; Cherian 2004) making seizures a relatively common presentation. As may be expected, seizures seem to be associated with a more severe phenotype, with all four cases presenting at less than 1 year of age and all of them having marked developmental delay. However, it is to be noted that EEG abnormalities are not restricted to those with clinical seizure activity (Wijburg et al. 1992).

The other unique feature in this case is the communicating hydrocephalus, a feature that has not been described before. While cerebral atrophy has been described (Labauge et al. 2009), there have been no documented cases of hydrocephalus. Given the scarcity and heterogeneity of the

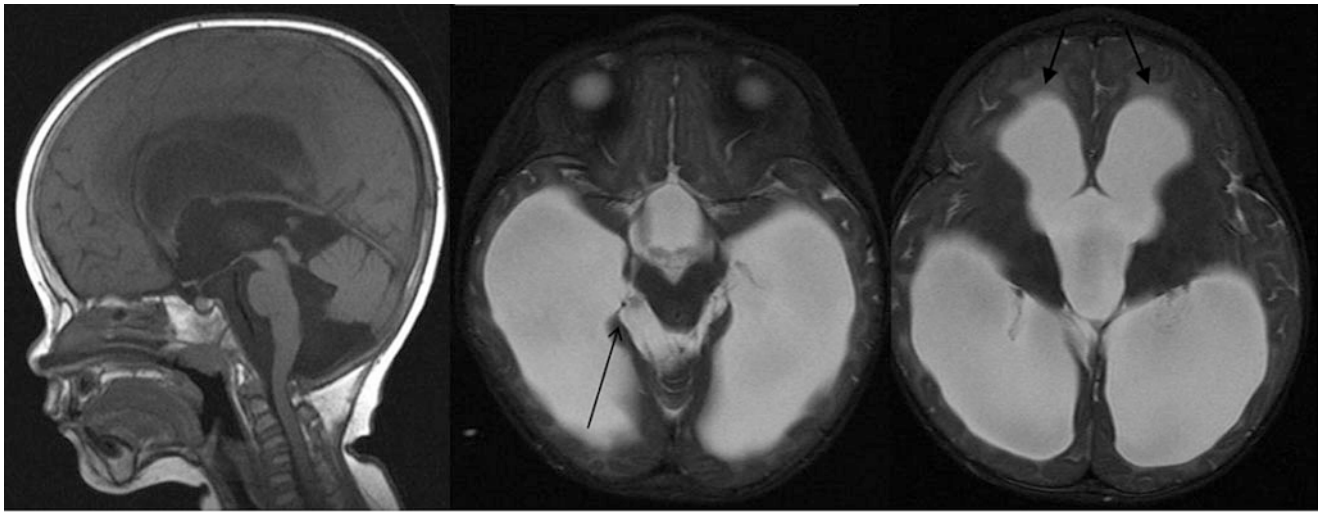

Fig. 2 Second brain MRI performed at 23 months. While there is some coexistent cerebral volume loss, the sagittal T1W and axial T2W images show macrocrania, dilatation of the lateral, third and fourth ventricles with transependymal oedema (*closed arrowheads*) and some bulging of the inferior recesses of the third ventricle in keeping with extraventricular hydrocephalus. The pons and medulla are displaced posteriorly, the inferior cerebellar vermis superiorly and there are new extra-axial cystic lesions within the ambient cisterns, larger on the right (*open arrowhead*) in keeping with loculated fluid within the arachnoid space

disease, it is hard to be certain that the hydrocephalus is directly attributable to the mannosidosis, but it is the most likely explanation. Indeed in storage disorders it is occasionally hard to differentiate between communicating hydrocephalus and cerebral atrophy (Manara et al. 2011) though the progressive macrocephaly would be indicative of the former.

The pathogenesis underlying the hydrocephalus, however, remains unclear. There have been a number of different mechanisms suggested for the development of hydrocephalus in storage disorders. This has been best described in the mucopolysaccharidoses (MPS) where a number of different theories have been put forward for their development. These include venous hypertension secondary to skull base abnormalities (Vedolin et al. 2007); however, there was no obvious crowding of the posterior fossa structures or bony craniovertebral junction anomaly in our patient. Other potential mechanisms include the deposition of storage material in the leptomeninges (Matheus et al. 2004) with a subsequent decrease in CSF absorption. The loculation of fluid in the extra-axial cystic lesions suggests the trapping of fluid in the arachnoid space (see Fig. 2). This feature of arachnoid loculation, including the development of hydrocephalus, is a recognised feature of MPS and has been attributed to the deposition of glycosaminoglycans in the subarachnoid space. In this case, there was no enlargement of the perivascular spaces, which is an additional feature, described in MPS. The possibility of alternative pathological mechanisms cannot be discounted, however, since both the type and degree of substrate deposition differ between disorders giving rise to the potential triggering of different pathological cascades.

In summary, this case would indicate that β-mannosidosis although phenotypically heterogeneous in presentation is not necessarily a milder disease in humans than in other animals and is a potential cause of neonatal seizures. Children with β-mannosidosis should also be closely observed as they do have the potential to develop hydrocephalus.

References

Abbitt B, Jones MZ, Kasari TR, Storts RW, Templeton JW, Holland PS, Castenson PE (1991) Beta-mannosidosis in twelve Salers calves. J Am Vet Med Assoc 198:109–113

Bedilu R, Nummy KA, Cooper A, Wevers R, Smeitink J, Kleijer WJ, Friderici KH (2002) Variable clinical presentation of lysosomal beta-mannosidosis in patients with null mutations. Mol Genet Metab 77:282–290

Cherian MP (2004) Beta-mannosidase deficiency in two mentally retarded girls with intractable seizures. Ann Saudi Med 24:393–395

Cooper A, Sardharwalla IB, Roberts MM (1986) Human beta-mannosidase deficiency. N Engl J Med 315:1231

Cooper A, Hatton C, Thornley M, Sardharwalla IB (1988) Human β-mannosidase deficiency: biochemical findings in plasma, fibroblasts, white cells and urine. J Inher Metab Dis 1:17–29

Cooper A, Wraith JE, Savage WJ, Thornley M, Noronha MJ (1991) Beta-mannosidase deficiency in a female infant with epileptic encephalopathy. J Inherit Metab Dis 14:18–22

Dorland L, Duran M, Hoefnagels FE et al (1988) Beta-mannosidosis in two brothers with hearing loss. J Inherit Metab Dis 11(Suppl 2): 255–258

Gort L, Duque J, Fabeiro JM, Zulaica A, Coll MJ, ChabÄs A (2006) Molecular analysis in two beta-mannosidosis patients: description of a new adult case. Mol Genet Metab 89:398–400

Jones MZ, Cunningham JG, Dade AW et al (1983) Caprine beta-mannosidosis: clinical and pathological features. J Neuropathol Exp Neurol 42:268–285

Jones MZ, Rathke EJ, Gage DA, Costello CE, Murakami K, Ohta M, Matsuura F (1992) Oligosaccharides accumulated in the bovine beta-mannosidosis kidney. J Inherit Metab Dis 15:57–67

Labauge P, Renard D, Castelnovo G, Sabourdy F, de Champfleur N, Levade T (2009) Beta-mannosidosis: a new cause of spinocerebellar ataxia. Clin Neurol Neurosurg 111:109–110

Manara R, Priante E, Grimaldi M et al (2011) Brain and spine MRI features of Hunter disease: frequency, natural evolution and response to therapy. J Inherit Metab Dis 34:763–780

Matheus MG, Castillo M, Smith JK, Armao D, Towle D, Muenzer J (2004) Brain MRI findings in patients with mucopolysaccharidosis types I and II and mild clinical presentation. Neuroradiology 46:666–672

Poorthuis BJ, Wevers RA, Kleijer WJ et al (1999) The frequency of lysosomal storage diseases in The Netherlands. Hum Genet 105:151–156

Poupetova H, Ledvinova J, Berna L, DvorÄkovÄ L, Kozich V, Elleder M (2010) The birth prevalence of lysosomal storage disorders in the Czech Republic: comparison with data in different populations. J Inherit Metab Dis 33:387–396

Thomas GH (2001) Disorders of glycoprotein degradation: a-mannosidosis, b-mannosidosis, fucosidosis, and sialidosis. In: Scriver CR, Beudet AL, Valle D et al (eds) The Metabolic and molcular basis of inherited disease, 8th edn. McGraw-Hill, New York, pp 3507–3535

Vedolin L, Schwartz IV, Komlos M et al (2007) Brain MRI in mucopolysaccharidosis: effect of aging and correlation with biochemical findings. Neurology 69:917–924

Wenger DA, Sujansky E, Fennessey PV, Thompson JN (1986) Human beta-mannosidase deficiency. N Engl J Med 315: 1201–1205

Wijburg H, de Jong J, Wevers R, Bakkeren J, Bakkeren J, Trijbels F, Sengers R (1992) Beta-mannosidosis and ethanolaminuria in a female patient. Eur J Pediatr 151:311

Zhu M, Lovell KL, Patterson JS, Saunders TL, Hughes ED, Friderici KH (2006) Beta-mannosidosis mice: a model for the human lysosomal storage disease. Hum Mol Genet 15: 493–500

JIMD Reports
DOI 10.1007/8904_2013_228

RESEARCH REPORT

A Novel Exonic Splicing Mutation in the *TAZ* (*G4.5*) Gene in a Case with Atypical Barth Syndrome

Yuxin Fan • Jon Steller • Iris L. Gonzalez • Wim Kulik • Michelle Fox • Richard Chang • Brandy A. Westerfield • Anjan S. Batra • Raymond Yu Jeang Wang • Natalie M. Gallant • Liana S. Pena • Hu Wang • Taosheng Huang • Sunita Bhuta • Daniel J. Penny • Edward R. McCabe • Virginia E. Kimonis

Received: 25 January 2012 / Revised: 19 March 2013 / Accepted: 22 March 2013 / Published online: 19 April 2013

Abstract Objective: Barth syndrome is an X-linked recessive disorder characterized by dilated cardiomyopathy, neutropenia, 3-methylglutaconic aciduria, abnormal mitochondria, variably expressed skeletal myopathy, and growth delay. The disorder is caused by mutations in the tafazzin (*TAZ/G4.5*) gene located on Xq28. We report a novel exonic splicing mutation in the *TAZ* gene in a patient with atypical Barth syndrome.

Patient & Methods: The 4-month-old proband presented with respiratory distress, neutropenia, and dilated cardiomyopathy with reduced ejection fraction of 10%. No 3-methylglutaconic aciduria was detected on repeated urine organic acid analyses. Family history indicated that his maternal uncle died of endocardial fibroelastosis and dilated cardiomyopathy at 26 months. TAZ DNA sequencing, mRNA analysis, and cardiolipin analysis were performed.

Communicated by: Eva Morava, MD PhD

Competing interests: None declared

Y. Fan and J. Steller are considered joint first authors

Y. Fan · B.A. Westerfield · L.S. Pena · H. Wang · D.J. Penny
John Welsh Cardiovascular Diagnostic Laboratory, Section of Cardiology, Department of Pediatrics, Texas Children's Hospital, Baylor College of Medicine, Houston, TX, USA

R. Chang · R.Y.J. Wang
Division of Metabolic Disorders, Children's Hospital of Orange County, Orange County, CA, USA

J. Steller · R. Chang · A.S. Batra · R.Y.J. Wang · T. Huang · V.E. Kimonis
Children's Hospital of Orange County and Department of Pediatrics, University of California Irvine, Orange, CA, USA

M. Fox · N.M. Gallant · E.R. McCabe
Department of Pediatrics, University of California, Los Angeles, CA, USA

I.L. Gonzalez (✉)
Nemours Biomedical Research Department, Alfred I. duPont Hospital for Children, Wilmington, DE, USA
e-mail: irislgonz@gmail.com

S. Bhuta
Department of Pathology, University of California, Los Angeles, CA, USA

W. Kulik
Department of Clinical Chemistry, University of Amsterdam, Laboratory Genetic Metabolic Diseases, Amsterdam, The Netherlands

E.R. McCabe
Linda Crnic Institute for Down Syndrome, University of Colorado School of Medicine, Aurora, CO, USA

V.E. Kimonis (✉)
UCI Division of Genetics and Metabolism, Dept. of Pediatrics, Univ. of California-Irvine Med. Center, Mail: 101, The City Drive South, ZC4482, Orange, CA 92868, USA
e-mail: vkimonis@uci.edu

Results: A novel nucleotide substitution c.553A>G in exon 7 of the *TAZ* gene was identified in the proband, predicting an amino acid substitution p.Met185Val. However, this mutation created a new splice donor signal within exon 7 causing mis-splicing of the message, producing two messages that only differ in the presence/absence of exon 5; these retain intron 6 and have only 11 bases of exon 7. Cardiolipin analysis confirmed the loss of tafazzin activity. The proband's mother, maternal aunt, and grandmother carry the same mutation.

Conclusions: The identification of a *TAZ* gene mutation, mRNA analysis, and monolysocardiolipin/cardiolipin ratio determination were important for the diagnosis and genetic counseling in this family with atypical Barth syndrome that was not found to be associated with 3-methylglutaconic aciduria.

Abbreviations

3-MGCA	3-methylglutaconic acid
BNP	Brain natriuretic peptide
BTHS	Barth Syndrome
hs-CRP	High sensitivity C-reactive protein
MLCL/CL	Monolysocardiolipin/cardiolipin
TAZ	Tafazzin

Introduction

Barth syndrome (OMIM 302060, BTHS), first described in 1983 by Barth and colleagues, is manifested clinically by an array of characteristics varying in severity and presentation including dilated cardiomyopathy, skeletal muscle weakness, growth delay with delayed puberty, and neutropenia (Barth et al. 1983; Kelley et al. 1991). Other features include abnormal mitochondria associated with the reduced concentration and altered composition of cardiolipin, hypocholesterolemia, and elevated urinary levels of 3-methylglutaconate (3-MGCA), 3-methylglutarate, or 2-ethylhydracrylate (Kelley et al. 1991; Schlame and Ren 2006). Considerable variability in the age of onset and progression of the disease is observed, and while mortality is highest during the first 4 years, the survival curve for BTHS patients has improved thanks to discovery of the gene (Bione et al. 1996) and to earlier detection and treatments. While previous reports (Barth et al. 2004) cited lifespan peaking at puberty, a current update cites much reduced mortality leading to extended lifespan (Clarke et al. 2013).

Barth syndrome is associated with mutations in the *TAZ* (Tafazzin) gene located on chromosome Xq28; this gene produces four main transcripts: full length, delta5 (lacking exon 5), delta7 (lacking exon 7), and delta5/7 (lacking both exons 5 and 7) (Gonzalez 2005). We report a case of BTHS caused by an exonic splice mutation in exon 7 of the *TAZ* (or *G4.5*) gene; this case lacks the characteristic 3-methylglutaconic aciduria.

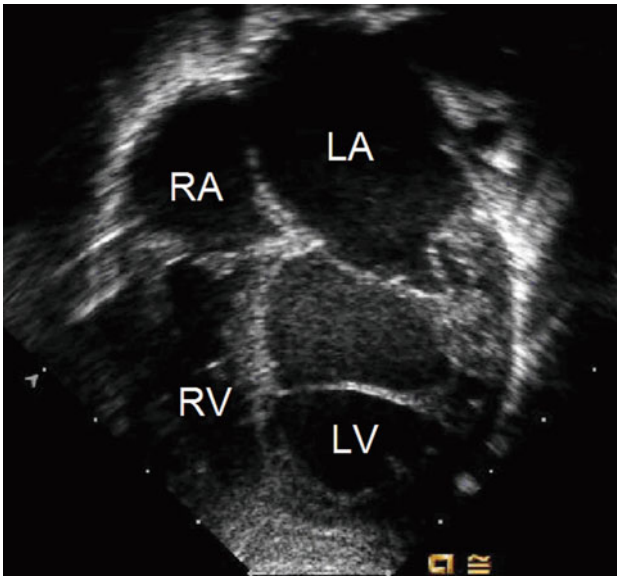

Fig. 1 An echocardiogram displaying severe left ventricular and atrial dilation. *LA* left atrium, *LV* left ventricle, *RA* right atrium, *RV* right ventricle

Patients and Methods

The proband of German/Irish descent was first brought to the emergency room at the age of 7 weeks with shortness of breath, tachypnea, tachycardia, and cyanosis. Upon physical examination, he had microcephaly (HC = 37 cm, < 3rd percentile) and short stature (Height = 57 cm, 3rd percentile; Weight = 3.89 kg, 10th–25th percentile) with no craniofacial dysmorphic features or other notable organomegaly. A chest x-ray revealed significant cardiomegaly, while an echocardiogram demonstrated a dilated left ventricle with an ejection fraction of only 10%. A second echocardiogram at 11 weeks confirmed the presence of severe systolic dysfunction of the dilated left ventricle. Additionally, there was moderate dilation of the left atrium, moderate insufficiency of the mitral valve, and mild tricuspid valve insufficiency at high velocity, which indicated a moderate degree of pulmonary hypertension. Further work-up disclosed normal liver function tests with normal levels of lactate, glucose, and electrolytes. Acetylcarnitine levels were found to be elevated (35.01 umol/L, normal: 1.62–16.06) and plasma amino acids, namely, Ala, Val, Leu, Ile, Lys, and Trp, were slightly below the lower limit of normal; however, these were due to levocarnitine supplementation and reduced protein intake. Multiple urine organic acid analyses did not demonstrate any abnormalities, even with special attention paid to assess for

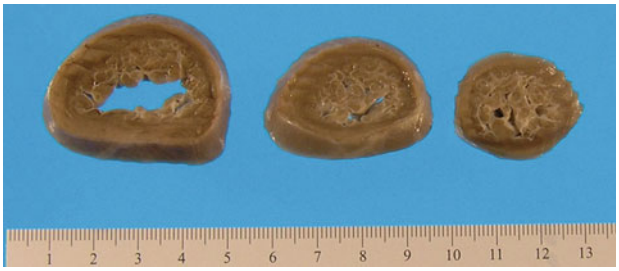

Fig. 2 Cross sections from the 64.0 g explanted heart exhibit prominent trabeculations involving greater than 50% of the left ventricular wall thickness. Also note thickened fibroelastotic endocardium

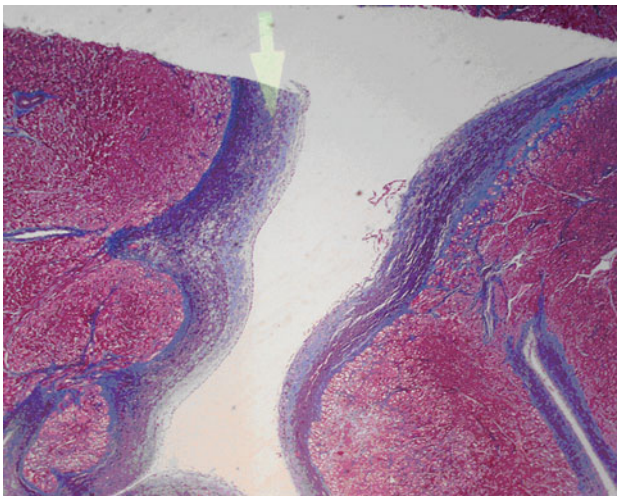

Fig. 3 Hematoxylin- and eosin-stained section of left ventricle shows hypertrophic cardiac myocytes (x10)

3-methylglutaconic, 3-methylglutaric, and 2-ethylhydracrylic aciduria. A complete blood cell count displayed a low white blood cell count (3.3 K/uL, normal 4.9–17.8) and mild neutropenia (15.4%, normal: 17–65% of WBC; absolute neutrophil count: 508), while normal levels of CPK and MB CPK were obtained. Additionally, B-type natriuretic peptide was greater than 5,000 (normal: <100 pg/mL), and Troponin I and high sensitivity CRP were found to be very high.

Over the next 5 months, our proband's condition worsened until he received a heart transplant from a 17-month-old donor. An echo performed a week prior to his surgery showed severe dilation of the left atrium and ventricle with global severe depression of left ventricular function and wall motion, severe mitral valve regurgitation, and an ejection fraction of less than 20% (Fig. 1), while an echo the day of his surgery additionally revealed a normal aorta, a dilated mitral valve annulus, regurgitation of both the mitral and tricuspid valves, and an ejection fraction of <10%. His final pre-transplant chest x-ray reported

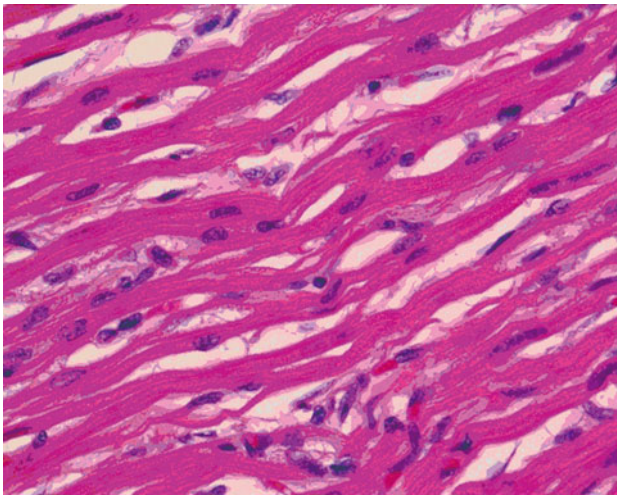

Fig. 4 This photomicrograph shows significantly thickened endocardium with duplication of elastic fibers (*red*) and collagen fibers (*blue*) highlighted with Trichrome-EVG stain (Elastic Van Gieson Stain). (x10). In general, the normal endocardium has fewer than five layers of elastic fibers

persistent cardiomegaly with a known dilated left atrium and mild pulmonary edema (data not shown).

He successfully underwent cardiac transplantation, and at 2 months post-transplant, his echocardiogram showed normal left ventricular size with increased wall thickness, normal wall motion and systolic function, normal right ventricular size and systolic function, an ejection fraction of 64%, and abnormal septal motion consistent with a post-cardiac transplant heart. His chest x-ray showed stable cardiomediastinal structures, right parahilar subsegmental atelectasis, and normal lung aeration. His laboratory investigations (also at 2 months post-transplant) showed a brain natriuretic peptide (BNP) of 43 (normal: <100 pg/mL); a high sensitivity C-reactive protein (hs-CRP) of 0.3 (low risk: <1.0 mg/L); a completely normal electrolyte panel, an HA1c of 4.7 (normal: 4.4–5.9); a normal kidney panel with slightly elevated alkaline phosphatase of 158 (normal: 31–103 U/L); and a normal complete blood count with exceptions, including a neutrophil percent of 11.6% (normal: 40.1–75.9%) and an absolute neutrophil count of 0.5 (normal: 1.3–7.0 $\times$ 10^3/uL). For his neutropenia, following sampling of his bone marrow, he was started on GCSF treatments of 20 mcg SC 3x/week; 5 mcg/kg/dose. Post-transplant urine organic acid analysis at a second trustworthy lab again failed to demonstrate any 3-methylglutaconic, 3-methylglutaric, or 2-ethylhydracrylic aciduria.

The explanted heart weighed 64 g. There was non-compaction of the left ventricle with secondary endocardial fibroelastosis confirmed by trichrome stain and normal glycogen content confirmed with PAS stain with and without diastase (Figs. 2, 3, and 4). Electron micrographs

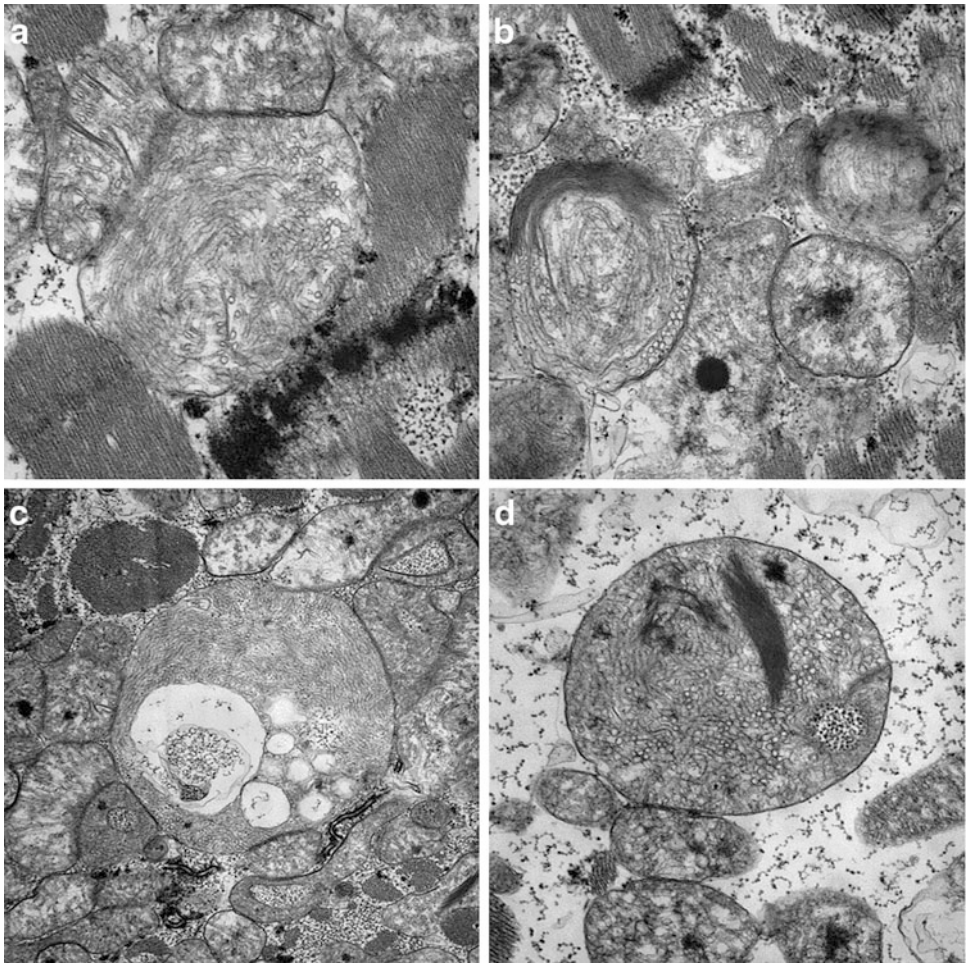

Fig. 5 (**a**) Noticeable onion skinning of the mitochondrial cristae, fusion of intracristae inner mitochondrial membrane preventing transport, and marked variation in size of mitochondria. (**b**) Note many inclusions of glycogen in intramitochondrial vacuoles replacing sarcomeres. (**c, d**) Notice disorganized stacks of cristae, intramitochondrial glycogen, and glycogen surrounding mitochondria

revealed marked myofibrillar disarray; widening of Z-bands; and fragmented, disorganized, intercalated disks. Loss of myofibrils with perinuclear pools of glycogen particles were noted, and his mitochondria showed marked variation in size, shape, and cristae. Additionally, many greatly enlarged mitochondria (giant forms) displayed stacks of closely packed cristae or had tubular or concentric cristae, and intramitochondrial glycogen was frequently noted (Fig. 5a–d).

Upon review of the family history, the proband has no siblings, and other notable phenotypes include a maternal uncle who displayed similar symptoms to the proband before his death at 2 years old. Examination of the uncle's past medical records revealed an initial admission for respiratory distress, fever, and diaphoresis at 5 months of age. A chest x-ray and echocardiogram displayed cardiomegaly with increased pulmonary vascular markings and poor function of the left ventricle resulting in a diagnosis of endocardial fibroelastosis. All growth measurements were noted below the 5th percentile at that time. Subsequent admissions to the hospital over the next year and a half revealed an EKG displaying tall R waves and inverted T waves, gastroenteritis, mild generalized hypotonia, jerking movements of the head, severe failure to thrive, pneumonia and eventual cardiac arrest, coma and severe encephalopathy before his death. Genetic testing was not available at the time of his death, and it is uncertain if urine organic acid analysis was performed. Our proband also had a second maternal aunt who was found to have a very unusual right hypoplastic heart complex and received a prostaglandin E1 infusion. Cardiac findings included tricuspid stenosis, pulmonary valve atresia, a small hypertrophied right ventricle, marked left ventricular hypertrophy, and an atrial septal defect. A Gore-Tex shunt from the right subclavian to the right pulmonary artery was created and she was discharged on post-operation day 7. At 2½ months of age, she was readmitted with cyanotic heart disease and expired despite massive resuscitative efforts. Unfortunately, no

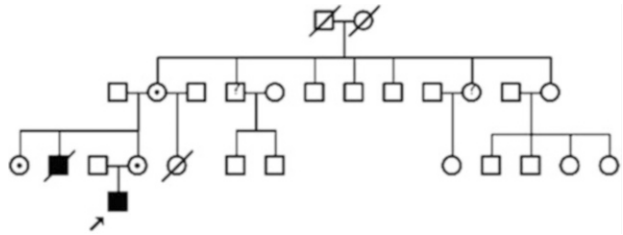

Fig. 6 A pedigree of our family with atypical Barth syndrome. *Filled symbols* indicate clinically affected individuals; *open symbols* indicate unaffected individuals; a *slash through a circle* or *square* indicates a deceased individual; symbol with *"?"* indicates an individual who did not have clinical evaluation and genetic testing; *squares* indicate males; *circles* indicate females. The proband patient is marked with an *arrow*

karyotype analysis appears to have been done and we were not able to conduct any further studies on her due to unavailability of saved frozen tissue.

Results

Mutation analysis for the tafazzin gene (OMIM 300394, *TAZ*) in our proband revealed a novel adenine to guanine transition mutation at position c.553 in exon 7, resulting in a predicted p.Met185Val amino acid substitution. Family studies showed that the proband's mother, maternal aunt, and grandmother carry the same mutation (Figs. 6, 7). Subsequent bioinformatic analyses by both PolyPhen-2 and PANTHER algorithms (http://genetics.bwh.harvard.edu/pph/; http://www.pantherdb.org/tools/csnpScoreForm.jsp) predicted that this sequence alteration would be deleterious. The substitution c.553A>G found in the proband was not observed in 115 X chromosomes from 81 ethnically matched control subjects (47 males and 34 females); it is also known that methionine is evolutionarily conserved at position 185 in all vertebrate classes and many invertebrate classes as well. However, SplicePort analysis (http://spliceport.cs.umd.edu; Dogan et al. 2007) predicted that c.553A>G generates a strong splice donor signal within exon 7 which would result in a truncated protein; this was supported by another splice site prediction tool (http://www.fruitfly.org/seq_tools/splice.html). To determine whether this mutation leads to amino acid substitution or to mis-splicing, we obtained RNA from the patient's lymphoblast cell line. RT-PCR with labeled primers showed only two abnormally sized *TAZ* mRNA fragments instead of the four known splice variants. The RT-PCR fragments were subjected to capillary electrophoresis separation to obtain precise fragment sizing and were also sequenced. These analyses showed two main mRNAs differing in the presence/absence of exon 5, both of which retain intron 6 and use a new splice donor site within exon 7 after only 11 bases of the exon. Thus, c.553A>G results in r.[541+1_542-1ins;r.553_583del]; p.Lys182Glnfs*4 (Fig. 8).

Biochemical testing for monolysocardiolipin/cardiolipin (Kulik et al. 2008; Houtkooper et al. 2009) was performed in order to confirm the loss of tafazzin activity. The MLCL/CL results are shown in Fig. 9, yielding an m/z 582.4 to m/z 723.5 ratio of 91, consistent with BTHS.

Discussion

The c.553A>G exonic splice mutation is being reported for the first time and is considered to be a pathogenic mutation for the following reasons: (a) abnormal splicing of the *TAZ* message, (b) abnormal MLCL/CL ratio, (c) absence of this change in 115 control chromosomes, and (d) family history of an early male infant death.

If this single base pair change resulted in p.Met185Val, it would only affect splice variants that contain exon 7 (full length and delta 5). Instead, this mutation generated a strong donor splice signal within exon 7 causing retention of intron 6 and partial deletion of exon 7 (r.[541+1_542-1ins; r.553_583del]), resulting in p.Lys182Glnfs*4. BTHS-causing mutations have been found in all *TAZ* exons, and known mutations include small deletions and insertions, termination mutations, and splice signal mutations. The Human Tafazzin (*TAZ*) Gene Mutation and Variation Database lists only one other exonic splice mutation (http://www.barthsyndrome.org/).

To date, no clear genotype-phenotype correlation has emerged (Bione et al. 1996; D'Adamo et al. 1997; Johnston et al. 1997; Sakamoto et al. 2001; Chen et al. 2002; Roberts et al. 2012). In fact, to the authors' best knowledge, there is also no clear genotype-phenotype correlation between the six reported cases of Barth syndrome not displaying 3-methylglutaconic aciduria (Table 1). There is significant variation in the severity of presentation between the patients as noted in the table, with varying degrees of neutropenia – heart defects and outcome with three patients passing before the age of 5 months and three patients still alive at publication. However, it is noted that BTHS not displaying elevated 3-MGCA may be more common than reported, in that there is considerable variability in laboratory methods used to detect 3-MGCA. There is also noteworthy variability seen in two very similar cases reported by Brady et al. in 2006 and Sakamoto et al. in 2002. Brady presented a proband with an Arg94His missense mutation in exon 3 with dilated cardiomyopathy, neutropenia, and no organic aciduria leading to death within 12 days, while Sakamoto reported an Arg94Ser missense mutation in exon 3 in a functioning 17-year-old boy with cardiac manifestations, organic aciduria, and a normal neutrophil count. However,

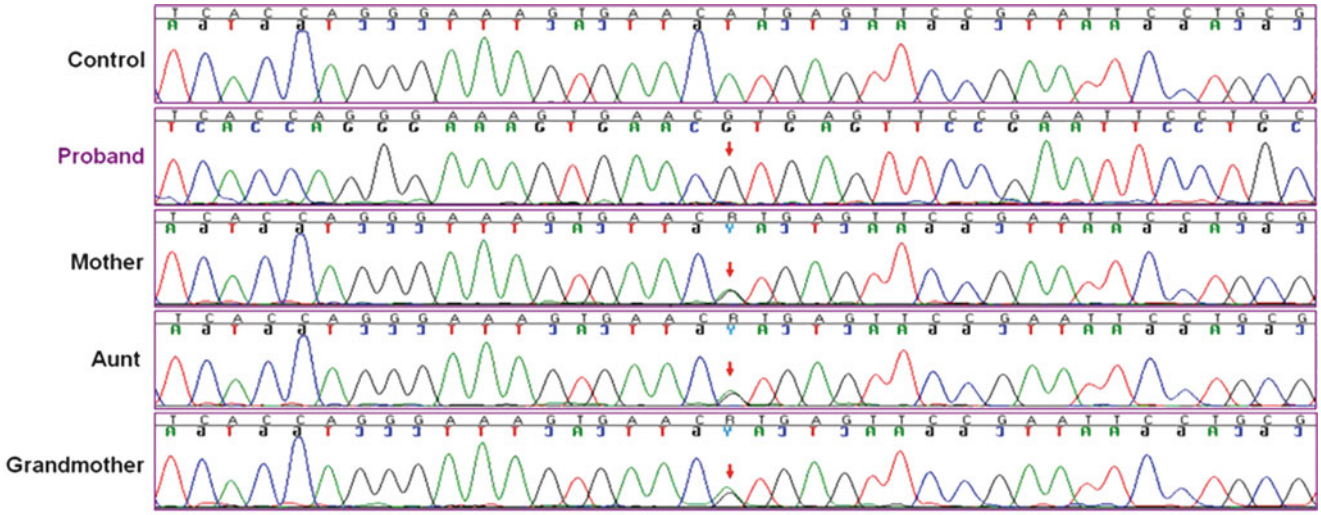

Fig. 7 TAZ sequencing of the family members revealed that the proband's mother, maternal aunt, and grandmother all carry the c.553A>G substitution

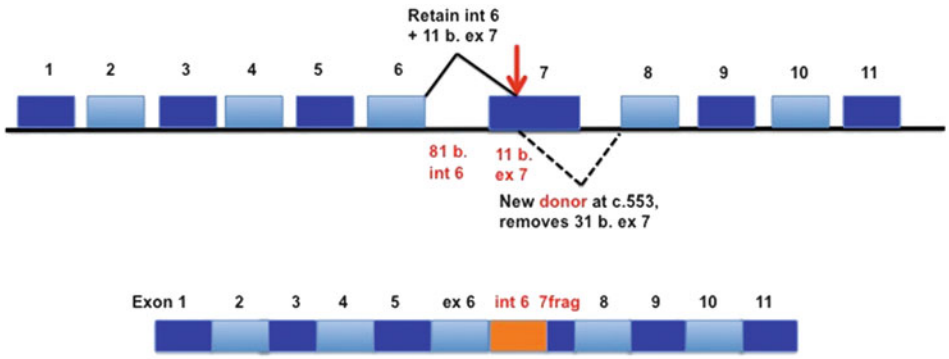

Fig. 8 (*Top*) TAZ gene diagram showing mutation location and its effect on splicing. (*Bottom*) Processed mRNA including complete intron 6 retention and only 11 bases of exon 7

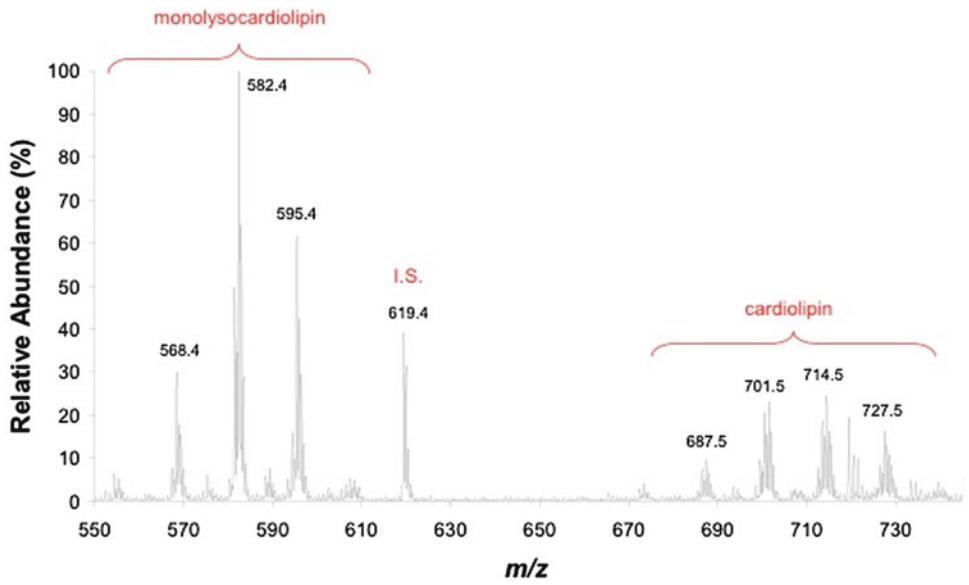

Fig. 9 MLCL and CL analysis performed on patient lymphoblast cell line shows the monolysocardiolipin and cardiolipin profiles with their characteristic doubly charged ions. The ratio of monolysocardiolipin/cardiolipin species is strongly increased as compared to normal controls (see Houtkooper et al. 2009). *m/z* mass/charge

Table 1 Clinical presentation among 6 reported cases of BTHS without 3-methylglutaconic aciduria. Values for 3-methylglutaconic acid levels were only available for the first two cases. A minus sign indicates that no elevation of 3MGCA was observed; normal range is 2-12 mmol/mol creatinine. The mutation of the 6th patient, initially described by Gedeon et al. 1995, was identified by D'Adamo et al. 1997.

Mutation	Dilated cardiomyopathy	Neutropenia	3-methylglutaconic aciduria	Age at report or death (D)	Source
c.553A>G, p.Lys182Glnfs*4	+	+	*6 mmol/mol creatinine*	A (3 y/o)	Current report
c.589G>A, p.Gly197Arg	+	+ (transient)	*<10 mmol/mol creatinine*	D (4.5 m/o)	Schmidt et al. 2004
c.589G>A, p.Gly197Arg	+	+	–	A (4.5 y/o)	Bleyl et al. 1997
c.281G>A, p.Arg94His	+	+	–	D (0.5 m/o)	Brady et al. 2006
c.605_608del, p.Glu202Valfs*15	–	+	–	A (11 y/o)	Marziliano et al. 2007
Mapped to Xq28c.634delC, p.Leu212Cysfs*6	+	–	–	D (5 m/o)	Gedeon et al. 1995 D'Adamo et al. 1997

it is possible that Brady's proband may have deceased before 3-methylglutaconic acids could be detected, or that the difference is due to the different amino acid substitutions in their cases.

While Bione had predicted that tafazzins may function as membrane anchors or soluble cytoplasmic proteins (Bione et al. 1996), more recent studies have demonstrated that they are phospholipid acyltransferases involved in the acyl-specific remodeling of cardiolipin (Vreken et al. 2000; Valianpour et al. 2002; Schlame et al. 2002). Tetralinoleoyl cardiolipin is a phospholipid found in the inner membrane of mitochondria that is essential for mitochondrial membrane structure and correct insertion and function of electron transport components; it has been shown that incorrect remodeling due to mutated tafazzin may compromise the assembly and stability of the electron transport chain (Schlame et al. 2000). Thus, the resultant mitochondrial dysfunction is an underlying mechanism of the skeletal and cardiac myopathy seen in Barth syndrome. This is supported by *Drosophila* studies where *Taz* mutants showed an 80% reduction in cardiolipin, abnormal mitochondria, and weakness in their indirect flight muscles (Xu et al. 2006).

However, the complex array of symptoms seen in patients with BTHS is much more sophisticated than a breakdown in energy metabolism, and continued research is imperative in determining the function of multiple tafazzin proteins and in elucidating the pathogenesis of BTHS. Further, the variability in presentation and severity of the syndrome not only suggest that multiple factors during multiple time periods may be influencing the expression of the phenotype. For example, *TAZ* expression has been found to decline with age, suggesting that tafazzins may be more essential during fetal and early postnatal periods (Marziliano et al. 2007; Malhotra et al. 2009). Intrafamilial phenotypic variability and the variability between families that carry the same mutation further suggest that there are modifier genes that modulate severity (Malhotra et al. 2009; Steward et al. 2010).

Conclusion

We have presented a novel substitution mutation in the *TAZ* gene that is associated with an atypical presentation of BTHS lacking one of the cardinal signs: 3-methylglutaconic aciduria. There have only been five other patients reported in the literature possibly because (a) it truly is a rare variant of Barth syndrome or (b) its actual prevalence is presented falsely low either because of variability in lab methods, or because molecular testing for mutations in the *TAZ* gene or biochemical testing of urine organic acids are not considered by health providers. These six cases highlight the current lack of genotype-phenotype correlation. Tafazzin gene mutations are now recognized (Barth et al. 2004; Steward et al. 2010; Roberts et al. 2012) as being implicated in a spectrum of cardiac presentations that include endocardial fibroelastosis, isolated non-compaction of left ventricular myocardium, hypertrophic cardiomyopathy, ventricular arrhythmia, which are also part of syndromes caused by various other genes. The variability in clinical presentation and severity complicate early recognition and may lead to underdiagnosis (Cantlay et al. 1999; Barth et al. 1999; Spencer et al. 2006; Steward et al. 2010) delaying appropriate treatment for BTHS patients. We propose that every male child with dilated cardiomyopathy should be tested for Barth syndrome.

Synopsis

We present a novel exonic splicing mutation in the *TAZ* gene in a patient with an atypical presentation of Barth syndrome lacking one of the cardinal signs: 3-methylglutaconic aciduria.

Conflicts of Interest

There are no conflicts of interest.

References

Barth PG, Scholte HR, Berden JA et al (1983) An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leucocytes. J Neurol Sci 62(1–3):327–355

Barth PG, Wanders RJ, Vreken P (1999) X-linked cardioskeletal myopathy and neutropenia (Barth syndrome)-MIM 302060. J Pediatr 135(3):273–276

Barth PG, Valianpour F, Bowen VM et al (2004) X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): an update. Am J Med Genet A 126A(4):349–354

Bione S, D'Adamo P, Maestrini E et al (1996) A novel X-linked gene, *G4.5*. is responsible for Barth syndrome. Nat Genet 12(4):385–389

Bleyl SB, Mumford BR, Thompson V et al (1997) Neonatal, lethal noncompaction of the left ventricular myocardium is allelic with Barth syndrome. Am J Hum Genet 61(4):868–872

Brady AN, Shehata BM, Fernhoff PM (2006) X-linked fetal cardiomyopathy caused by a novel mutation in the *TAZ* gene. Prenat Diagn 26(5):462–465

Cantlay AM, Shokrollahi K, Allen JT et al (1999) Genetic analysis of the *G4.5* gene in families with suspected Barth syndrome. J Pediatr 135(3):311–315

Chen R, Tsuji T, Ichida F et al (2002) Mutation analysis of the *G4.5* gene in patients with isolated left ventricular noncompaction. Mol Genet Metab 77(4):319–325

Clarke SL, Bowron A, Gonzalez IL et al (2013) Barth syndrome. Orphanet J Rare Dis 8:23

D'Adamo P, Fassone L, Gedeon A et al (1997) The X-linked gene *G4.5* is responsible for different infantile dilated cardiomyopathies. Am J Hum Genet 61(4):862–867

Dogan RI, Getoor L, Wilbur WJ et al (2007) SplicePort–an interactive splice-site analysis tool. Nucleic Acids Res 35(Web Server issue):W285–W291, Epub 2007 Jun 18

Gedeon AK, Wilson MJ, Colley AC et al (1995) X linked fatal infantile cardiomyopathy maps to Xq28 and is possibly allelic to Barth syndrome. J Med Genet 32:383–388

Gonzalez IL (2005) Barth syndrome: *TAZ* gene mutations, mRNAs, and evolution. Am J Med Genet A 134(4):409–414

Houtkooper RH, Rodenburg RJ, Thiels C et al (2009) Cardiolipin and monolysocardiolipin analysis in fibroblasts, lymphocytes, and tissues using high-performance liquid chromatography-mass spectrometry as a diagnostic test for Barth syndrome. Anal Biochem 387(2):230–237

Johnston J, Kelley RI, Feigenbaum A et al (1997) Mutation characterization and genotype-phenotype correlation in Barth syndrome. Am J Hum Genet 61(5):1053–1058

Kelley RI, Cheatham JP, Clark BJ et al (1991) X-linked dilated cardiomyopathy with neutropenia, growth retardation, and 3-methylglutaconic aciduria. J Pediatr 119(5):738–747

Kulik W, van Lenthe H, Stet FS et al (2008) Bloodspot assay using HPLC-tandem mass spectrometry for detection of Barth syndrome. Clin Chem 54(2):371–378

Malhotra A, Edelman-Novemsky I, Xu Y et al (2009) Role of calcium-independent phospholipase A2 in the pathogenesis of Barth Syndrome. Proc Natl Acad Sci U S A 106(7): 2337–2341

Marziliano N, Mannarino S, Nespoli L et al (2007) Barth syndrome associated with compound hemizygosity and heterozygosity of the *TAZ* and *LDB3* genes. Am J Med Genet A 143A(9): 907–915

Roberts AE, Nixon C, Steward CG et al (2012) The Barth Syndrome Registry: distinguishing disease characteristics and growth data from a longitudinal study. Am J Med Genet Part A 158A:2726–2732

Sakamoto O, Ohura T, Katsushima Y et al (2001) A novel intronic mutation of the *TAZ* (*G4.5*) gene in a patient with Barth syndrome: creation of a 5' splice donor site with variant GC consensus and elongation of the upstream exon. Hum Genet 109(5):559–563

Sakamoto O, Kitoh T, Ohura T et al (2002) Novel missense mutation (R94S) in the *TAZ* (*G4.5*) gene in a Japanese patient with Barth syndrome. J Hum Genet 47(5):229–231

Schlame M, Rua D, Greenberg ML (2000) The biosynthesis and functional role of cardiolipin. Prog Lipid Res 39(3):257–288

Schlame M, Towbin JA, Heerdt PM et al (2002) Deficiency of tetralinoleoyl-cardiolipin in Barth syndrome. Ann Neurol 51(5): 634–637

Schlame M, Ren M (2006) Barth syndrome, a human disorder of cardiolipin metabolism. FEBS Lett 580(23):5450–5455

Schmidt MR, Birkebaek N, Gonzalez I et al (2004) Barth syndrome without 3-methylglutaconic aciduria. Acta Paediatr 93(3): 419–421

Spencer CT, Bryant RM, Day J et al (2006) Cardiac and clinical phenotype in Barth syndrome. Pediatrics 118(2):e337–e346

Steward CG, Newbury-Ecob RA, Hastings R et al (2010) Barth Syndrome: an X-linked cause of fetal cardiomyopathy and stillbirth. Prenal Diagn 30(10):970–976

Valianpour F, Wanders RJ, Overmars H et al (2002) Cardiolipin deficiency in X-linked cardioskeletal myopathy and neutropenia (Barth syndrome, MIM 302060): a study in cultured skin fibroblasts. J Pediatr 141(5):729–733

Vreken P, Valianpour F, Nijtmans LG et al (2000) Defective remodeling of cardiolipin and phosphatidylglycerol in Barth syndrome. Biochem Biophys Res Commun 279(2):378–382

Xu Y, Condell M, Plesken H et al (2006) A *Drosophila* model of Barth syndrome. Proc Natl Acad Sci U S A 103(31): 11584–11588

JIMD Reports
DOI 10.1007/8904_2013_229

RESEARCH REPORT

Selective Screening for Lysosomal Storage Diseases with Dried Blood Spots Collected on Filter Paper in 4,700 High-Risk Colombian Subjects

Alfredo Uribe • Roberto Giugliani

Received: 14 December 2012 / Revised: 18 March 2013 / Accepted: 27 March 2013 / Published online: 23 April 2013

Abstract Lysosomal storage disorders (LSDs) are a very heterogeneous group of hereditary disorders. The diagnostic process usually involves complex sampling, processing, testing, and validation procedures, performed by specialized laboratories only, which causes great limitations in reaching a diagnosis for patients affected by these diseases.

There are few studies about LSDs in Colombia. The diagnostic limitations often make medical practitioners disregard the possibility of these disorders while diagnosing their patients. The current study documents the results of a 7-year screening in high-risk patients, aimed to detect LSDs using dried blood spots (DBS) collected on filter paper, with a micromethodology that facilitates diagnosis even with a large number of samples.

The activities of α-galactosidase A, α glucosidase, α-L-iduronidase, arylsulfatase B, β-galactosidase, β-glucosidase, total hexosaminidase, iduronate sulfatase, and chitotriosidase were analyzed in high-risk patients for lysosomal disease. The catalytic activity was evaluated with fluorometric micromethods using artificial substrates marked with 4-methylumbelliferone.

The reference values for a control population were established for the enzymes listed above, and 242 patients were found to have an enzyme deficiency, guiding to the following diagnoses: Fabry disease ($n = 31$), Pompe disease ($n = 16$), Hurler Syndrome ($n = 15$), Maroteaux-Lamy Syndrome ($n = 34$), GM1 Gangliosidosis ($n = 10$), Morquio B ($n = 1$), Gaucher disease ($n = 101$), Sandhoff disease ($n = 1$), Mucolipidosis ($n = 2$), and Hunter Syndrome ($n = 31$). In conclusion, this protocol provides a comprehensive diagnostic approach which could be carried out in Colombia and made it available to medical services spread around the country, enabling the identification of a large number of patients affected by LSDs, which could potentially benefit from the therapeutic tools already available for many of these diseases.

Introduction

Lysosomal storage disorders (LSDs) include approximately 50 different diseases with a combined incidence of 1:1,500 to 1:7,000 births (Wraith 2002; Staretz-Chacham et al. 2009). Each of these diseases occurs due to a deficiency in the enzymes, activating proteins or transport proteins involved in the catabolism of macromolecules that takes place in the lysosomes, causing substrate storage and progressive cell damage (Gieselmann 1995). The majority of these genetic anomalies is inherited in an autosomal recessive manner, with the exceptions of Fabry, Hunter, and Danon diseases, which are linked to the X chromosome (Staretz-Chacham et al. 2009).

The clinical findings surrounding these pathologies demonstrate multisystemic and progressive difficulties with

Communicated by: Frits Wijburg

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2013_229) contains supplementary material, which is available to authorized users.

A. Uribe (✉)
Centro de Investigaciones en Bioquimica (CIBI), Departamento de Ciencias Biologicas, Universidad de Los Andes, Carrera 1 No. 18A – 12 of. M-302, Bogota, Colombia
e-mail: jeuribe@uniandes.edu.co

R. Giugliani
Department of Genetics, Federal University of Rio Grande do Sul (UFRGS), Medical Genetics Service, Hospital de Clinicas de Porto Alegre (HCPA) and INAGEMP, Porto Alegre, Brazil

a broad spectrum of manifestations, including skeletal abnormalities, visceromegalies, and, often, central nervous system dysfunction, ranging from behavior problems to profound mental retardation (Vellodi 2005; Scriver et al. 2001). The phenotypic expression of these diseases is so diverse and heterogeneous that their definitive identification and diagnosis must be made through specialized clinical and laboratory studies (Wenger et al. 2002)

The laboratory diagnostic approach, directed at high-risk populations, first uses protocols typically based on the detection of metabolites that are excreted in the urine after accumulating in tissues as a result of enzymatic deficiencies. Lysosomal storage disorders usually involve an accumulation of three groups of compounds: sphingolipids, mucopolysaccharides, and oligosaccharides, molecules that may be detected in affected individuals but do not offer specific diagnostic clues (Futerman et al. 2004; Filocamo and Morrone 2011). Next, these preliminary tests were followed by specific enzymatic studies – which involved plasma, leukocytes, or cultured fibroblasts samples – aimed to provide definitive diagnosis (Civallero et al. 2006).

In Colombia, and in the majority of Latin American countries, laboratory diagnosis of these metabolic disorders is not a routine practice in health services, due to the complexity of the process and also because these disorders have a very low incidence and are not among the health priorities. According to the European Union, a rare disease is classified as having a prevalence of fewer than five cases for every 10,000 inhabitants (Stolk et al. 2006). Since a complex infrastructure is required to perform the diagnostic tests for LSDs, the protocols for screening and diagnosis are primarily developed by few specialized centers. This situation becomes a limitation for the investigation of a large number of patients, either because they do not have direct access to these centers or because sending liquid samples to these laboratories (plasma, whole blood, or urine) requires strict storage conditions and short transportation times. These circumstances can either cause underdiagnosis or late diagnosis, leading to a delay in starting the treatment and, in some cases, to the death of affected individuals without the benefit of a diagnosis.

Recently, a new methodology to facilitate patient access to this type of screening has been developed in Latin America and implemented worldwide. It consists in the enzymatic analysis of dried blood spots (DBS) collected on filter paper (Fig. 1) (Chamoles et al. 2001a, 2001b; Niizawa et al. 2005; Civallero et al. 2006; Müller et al. 2010). This methodology facilitates the packing and shipment of samples because it does not require strict refrigeration and eases the collection of samples for analysis at reference testing centers, allowing the study of high-risk populations without regard to their geographical location. Given that in the recent years a number of treatments are available to treat some of these diseases, diagnosis becomes relevant. This point is especially important in the case of enzyme replacement therapy for disorders such as Fabry, Gaucher, Pompe, and Mucopolysaccharidosis type I, II, and VI for which an early diagnosis could dramatically improve the outcome of the disease (Brady 2006).

The protocols for LSDs screening are usually made with a punch of 3.2 mm diameter (1/8 in.). This is considered the "universal punch" and is the most widely used because its blood volume (3.2 ul) has been reported with the greater exactitude (Reuser et al. 2011). In Colombia, there are no government screening programs that include these kinds of diseases, so all the initiatives to study them are from private universities, which leads to the development of sustainable projects based on donations and self-reliance. In this context, we propose the use of a 1.2 mm punch with the aim of diminishing the sample, materials, and reagents quantities used and in that way, reduce costs even in a 40 %, making the project viable and sustainable in time.

The results presented here correspond to seven years of selective screening for LSDs in Colombia. This study involves the enzymatic evaluation of DBS samples from 4,700 patients, with ages ranging from 4 months to 73 years. This study also involved the analysis of control samples to obtain reference values for α-galactosidase A, α-glucosidase, α-L-iduronidase, arylsulfatase B, β-galactosidase, β-glucosidase, total hexosaminidase, iduronate sulfatase, and chitotriosidase using modified protocols for a punch of filter paper with a diameter of 1.2 mm. These enzymatic analyses of DBS resulted in the identification of 242 individuals affected by LSDs, an unprecedented accounting in Colombia. The techniques reported here have facilitated the diagnostic approach and rapid incorporation of affected individuals into available treatment programs, which helped to change the clinical trajectory of these pathologies and to improve the quality of life for these patients and their families.

Materials and Methods

Sample Collection for the Controls and Patients in the Study

A total of 4,700 blood samples were collected on filter paper for analysis. These samples were taken from patients, aged between 4 months and 73 years, who had clinical findings related to disorders affecting glycosaminoglycan and sphingolipid metabolism. The samples were subjected to different enzymatic analyses according to clinical criteria and informed consent was obtained. Blood samples were

Table 1 Analytical conditions for the lysosomal enzymes under study

Enzyme	Reaction buffer	Substrate (Concentration in reaction buffer)	Incubation time/T°	Stop buffer
Arylsulfatase B	40 µl of 0.5 M acetate buffer 0.5 M, pH 6.0 with 10 mM barium acetate	4-Methylumbelliferyl Sulfate 5.5 mM	24 h/37°C	150 µl 0.17 M glycine: carbonate, pH 10
α-Galactosidase A	20 µl of 0.15 M citrate phosphate buffer, pH 4.4 with 10 µl of 0.25 M N-acetil-D-galactosamine	4-Methylumbelliferyl α-D-Galactoside 5 mM	20 h/37°C	150 µl 0.17 M glycine: carbonate, pH 10
α-I-Iduronidase	20 µl of 0.2 M sodium formiate buffer, pH 2.8 with 10 µl of 3.1 mM D-saccharic acid 1,4-lactone monohydrate	4-Methylumbelliferyl α-L-Iduronide 2 mM	20 h/37°C	200 µl 0.17 M glycine: carbonate, pH 10
α-Glucosidase (AαG)[a]	Total AαG determination: 40 µl of 0.04 M acetate buffer, pH 3.8. : Residual AαG: 40 µl of 0.04 M acetate buffer, pH 3.8 with 40 µl of 40 µM acarbose. Neutral isoforms: 40 µl of 0.04 M acetate buffer, pH 7.0	4-Methylumbelliferyl α-D-Glucopyranoside 2.8 mM	24 h/37°C	200 µl 0.17 M glycine: carbonate, pH 10
β-Galactosidase	40 µl of 0.1 M citrate phosphate buffer, pH 4.4 with 20 µl of 0.15 M NaCl	4-Methylumbelliferyl β-D-Galactoside 0.8 mM	3 h/37°C	200 µl 0.17 M glycine: carbonate, pH 10
β-Glucosidase	60 µl of 0.3 M citrate phosphate buffer, pH 5.0 with sodium 1% taurodeoxycholate and conduritol-β-epoxide 0.5 mM[b]	4-Methylumbelliferyl β-D-Glucoside 20 mM	24 h/37°C	150 µl 0.17 M glycine: carbonate, pH 10
Total Hexosaminidase	60 µl of 0.01 M citrate phosphate buffer, pH 4.4	4-Methylumbelliferyl 2-Acetamide-2 deoxy-β-D-Glucopyranoside 3 mM	1 h/ 37 °C	120 µl 0.17 M glycine: carbonate, pH 10
Iduronate Sulfatase	Pre-Incubation: Place each 1.2 mm punch in 25 µl of BSA 0.2 % Incubation 1: Add 16 µl of 0.1 M of acetate buffer, pH 5.0 with lead acetate 10 mM and the substrate to 1.25 mM. Incubation 2: Add 25 µl of 0.2 M of citrate phosphate buffer, pH 4.5 with 0.02 % sodium azide and auxiliary enzyme	Incubation 1: 4-Methylumbelliferyl α-Iduronate-2-Sulfate 1.25 mM Incubation 2: Enzyme α-I-Iduronidase 3.4 mg/ml in distilled water	Pre-incubation: 20 min/room T° Incubation 1: 24 h/37 °C Incubation 2: 24 h/37 °C	200 µl 0.17 M glycine: carbonate, pH 10 (Added at the end of Incubation 2)
Chitotriosidase	30 µl of 0.2 M acetate buffer, pH 5.5 with 10 mM barium acetate	4-Methylumbelliferyl-β-D-N,N′,N″ Triacetylchitotrioside 0.25 mM	20 min/37°C	150 µl 0.17 M glycine: carbonate, pH 10

[a] For each reaction mixture, the experimental assay requires a 1.2 mm punch of filter paper. An evaluation of AαG in the presence of the inhibiting agent acarbose permits the elimination of maltase glucoamylase activity, that can degrade the substrate, but it is not related with Pompe disease. Evaluation at pH 7.0 determines the activity of neutral isoforms (Glucosidase II and α-Glucosidase C). The quotient obtained from dividing this value over activity in the presence of acarbose offers a discrimination criterion for individuals affected by Pompe disease and healthy controls

[b] See Olivova et al. 2008

obtained via intravenous or capillary puncture, depending on the age of the person being evaluated and were collected directly on grade 903 filter paper (Schleicher and Schuell, Whatman®) provided by GE Healthcare Life Sciences (Piscataway, NJ, USA). The specimens were kept at room temperature for 8–12 h to assure complete drying and then stored at 4 °C in self-sealing plastic bags to prevent deterioration from humidity. The samples were received from different regions in Colombia, did not undergo refrigeration, and had transport times ranging from 2 to 7 days.

To establish reference values for each enzyme tested in this study, a total of 2,520 healthy subjects were analyzed during 7 years of study (approximately 360 subjects per year, 30 per month), following the same sampling procedure applied to patients. Control subjects are distributed in two groups: the first one consisted of 250 completely healthy newborns (10%), who were <36 days old (range

between 4–36 days). These samples came as part of the extended newborn screening program offered by our laboratory.

The second group consisted of 2,270 control subjects, from 3 months to 88 years, distributed as follows: 1,463 samples were from control subjects (58%) >12 years old. These samples were taken from students and employees of Universidad de los Andes, and their relatives (all of them healthy subjects). Another 605 samples (24%) were from subjects ranging from 1.5 to 11.5 years old, who were in routine growth and nutritional control in pediatric services. Finally, 202 samples (8%) corresponded to subjects clinically defined who were between 3 months and 1.3 years old. These subjects did not show clinical findings related with inborn errors of metabolism.

Enzyme Activity Assay

DBS analysis employed substrates marked with 4-methylumbelliferone (4-MU) provided by Sigma (St. Louis, MO, USA), with the exception of 4-methylumbelliferyl-α-L-iduronide (enzyme: α-L-iduronidase), which was provided by Toronto Research Chemicals (Toronto, Canada), and 4-methylumbelliferyl-iduronate 2-sulfate (enzyme: iduronate sulfatase), which was provided by Moscerdam Substrates (Rotterdam, the Netherlands). All solutions were prepared using reagents with a high level of purity.

Analytical assays on DBS for β-galactosidase, total hexosaminidase, α-galactosidase A, α-L-iduronidase, β-glucosidase, and chitotriosidase were based on the protocols proposed by Chamoles et al. (2001a, 2001b, 2002) and Civallero et al. (2006). The analysis of α-glucosidase was performed according to a standardized methodology described by Kallwass et al. (2007) and Li et al. (2004). The analysis of arylsulfatase B was performed using a standardized methodology based on the analytical principle of the leukocyte analysis proposed by Shapira et al. (1989) and Civallero et al. (2006). The analysis of iduronate sulfatase was performed using an adaptation of the methods of Voznyi et al. (2001) and Civallero et al. (2006).

The aforementioned methodologies were modified to implement the use of 1.2 mm punches (~ 0.52 µl of blood) and the corresponding volumes of substrate and buffers (Table 1). To determine the approximate blood volume in the 1.2 mm punch, we calculate and average quantity according to what Reuser et al. (2011), Daitx et al. (2012), and Rodrigues et al. (2009) reported before (see Supplementary Fig. 1).

A volume of substrate, equal to that used in the samples, was included to test unspecific substrate degradation. This volume was incubated with the samples in a separate Eppendorf tube for each protocol. At the end of the incubation, the stop buffer (glycine:carbonate buffer 0.17 M, pH 10) was added to samples and blanks. Then, the incubated substrate was added to the blank wells to evaluate unspecific substrate degradation in the reaction buffer. The difference between sample and blank fluorescence values allowed differentiating the real enzymatic activity from the unspecific degradation.

Assays were carried out by combining the punches of filter paper and the reaction solutions in black, 96-well polypropylene microplates. Evaporation was prevented using aluminum foil thermo-sealing sheets provided by Corning (Lowell, USA). The elution (5 min at room temperature) and incubation processes were performed with the orbital agitation of samples (200 rpm for elution and 120 rpm for incubation), using a Titramax 1000 plate vibrator and agitator and a Unimax 1010 incubator/agitator from the Heidolph group (Schwabach, Germany). A Spectramax M2 (Molecular Devices Corp.) was used as a fluorescence reader (excitation 360 nm, emission 460 nm), and the results were compared with a 10 points 4-MU calibration curve. Enzymatic activity was expressed as nanomoles of hydrolyzed substrate per milliliter of blood per hour (nmol/ml/h).

As quality control measures for the procedure, every referred patient was evaluated for betagalactosidase and total hexosaminidase as control enzymes, besides the requested enzyme (Supplementary Fig. 2). To avoid enzyme activity loss due to sample storage, the maximum processing time between sample taking and processing was 30 days. All samples were processed by triplicate to establish an intra assay coefficient of variance and in each plate, besides the group of patients, a control sample and a previously detected patient were included to see the feasibility of the assay. It is important to note that these control samples were not the same in each plate but they were evaluated several times to determine inter-assay variation. Additionally, since 2010 we are involved in an external quality control that includes sample exchange between research groups from Argentina, Brazil, and Mexico (Burin et al. 2011).

Deficient activity values were found in the screening assays for the DBS for the different enzymes under study and were confirmed in leukocyte assays performed in accordance with the protocols designed by Shapira et al. (1989), Voznyi et al. (2001), and Kallwas et al. (2007) (see Supplementary Table). Protein quantities were determined according to Lowry et al. (1951) and BCA (ThermoScientific) assays.

Statistical Analysis

An inter- and intra-assay coefficient of variance (CV) was determined as the average value taken from all the enzymes analyzed. Descriptive statistics were performed using the

IBM SPSS Statistic 19 software provided by SPSS Inc. (Special Package for the Social Sciences, Chicago, USA). A nonparametric Shapiro-Wilk test was used to evaluate the distribution of control groups and a Mann-Whitney test was used to determine the difference between the populations under study. The cutoff between the enzymatic activities of controls and patients was established using ROC (Receiver Operating Characteristics) curves with the same program.

Results and Discussion

Enzymatic analysis of the control population samples on filter paper allowed establishing reference values for the Colombian population for nine enzymes that are directly related to hereditary disorders involving lysosomal metabolism. Samples from a total of 4,700 patients, whose clinical results were indicative of an LSD, were received during the study period (2005–2011) from all over the nation.

This high-risk screening led to the detection of 242 patients (5.2 %) affected by different LSDs. We found 242 with enzyme deficiencies, leading to the following diagnoses: Fabry disease ($n = 31$), Pompe disease ($n = 16$), Hurler Syndrome ($n = 15$), Maroteaux-Lamy Syndrome ($n = 34$), GM1 Gangliosidosis ($n = 10$), Morquio B ($n = 1$), Gaucher disease ($n = 101$), Sandhoff disease ($n = 1$), Mucolipidosis ($n = 2$), and Hunter Syndrome ($n = 31$). The enzymatic activities obtained showed significant differences ($p < 0.001$) in the catalytic agents measured with respect to the control group (Table 2).

Internal quality controls provided evidence that the analytical techniques used here are reproducible. To this end, samples were evaluated in triplicate and the time between sampling and processing was interior to 30 days. The assays demonstrated an average intra-assay coefficient of variance of 7.7 % for all the enzymes analyzed and an inter-assay variability coefficient of 11.9 % ($n = 30$). The highest values found for any the enzymes analyzed in this study were detected in the β-glucosidase assay, where the intra- and inter-assay coefficients showed values of 15 % and 14.7 %.

When working with DBS samples, the suggested storage temperature for the filter paper is − 20 °C. However, in accordance to our experience and previous studies (Gasparotto et al. 2009; de Castilhos et al. 2011; De Jesus et al. 2009), the decrease in enzymatic activity from DBS samples stored at 4 °C in 60 days is less than 6 %, which means that there is no significant difference in the sample storage at 4 °C or − 20 °C.

Upon following up on the condition of the patients remitted for β-glucosidase, all cases showed evidence of various levels of leukopenia, which can affect sample quality and has been reported to be a variant that might affect the analytical processes (Civallero et al. 2006; Jiménez et al. 2011) (Table 2). In the present study, DBS analyses of β-glucosidase detected 103 deficient patients and the leukocyte assay confirmed only 101 Gaucher patients, finding two false positives and suggesting a possible interference due to low leukocyte counts. The other enzymes analyzed did not have this problem, and showed a 100% correlation between DBS and leukocyte samples.

In regard to the interferences reported in the analysis of β-glucosidase, both the studies in DBS and the leukocytes included the use of conduritol-β-epoxide (CBE, 0.5 mM), which has been described as a potent inhibitor of β-glucosidase, and allowed us to discriminate the activity of other isoenzymes that could degrade the fluorogenic substrate (Olivova et al. 2008). Additionally, it is important to highlight that the described DBS protocols are not made for diagnostic purposes, they are designed to orient the diagnosis, but definitely this must be confirmed with the measurement of enzymatic activities in cellular extracts.

The different methodologies led to a significant differentiation of enzymatic activities between control subjects and patients with an enzymatic deficiency. However, the evaluation of α-glucosidase levels from whole blood samples presented a challenge, given that three enzyme forms unrelated to the disease showed catalytic activity on 4-MU-α-D-glucopyranoside. This finding was demonstrated in the present study, where total catalytic activity was not different between the healthy controls and the patients ($p < 0.176$). A modified technique was used based on the one reported by Kallwass et al. (2007) and Li et al. (2004), which permitted the discrimination of the activity of the different isoforms using acarbose as an inhibitor. This method achieved significant differences between the controls and the individuals affected by Pompe disease (see Table 3).

Chitotriosidase analyses, which were originally intended to assist in the diagnosis of patients suspected to suffer from Gaucher disease, also allowed for the evaluation of other LSDs in many cases ($n = 74$). Patients with Niemann-Pick disease, Mucopolysaccharidosis, and Gangliosidosis, among others, showed variable levels of this biomarker (Table 2). While its detection was nonspecific, an increase in the levels of this biomarker could be of great use in the detection of these disorders. In relation to Gaucher patients, Table 2 shows two different groups classified according to their chitotriosidase activities (elevated and non elevated with respect to the reference values), indicating that, although useful, chitotriosidase cannot always be used as a tool for a diagnostic approach. In the present study, we found that 14.9 % of patients with Gaucher disease did not show a marked increase in this biomarker. Moreover, in a previous study that evaluated Colombian healthy subjects and patients with LSDs, 2.6 % of control subjects showed no activity for this enzyme (data not shown, article in preparation).

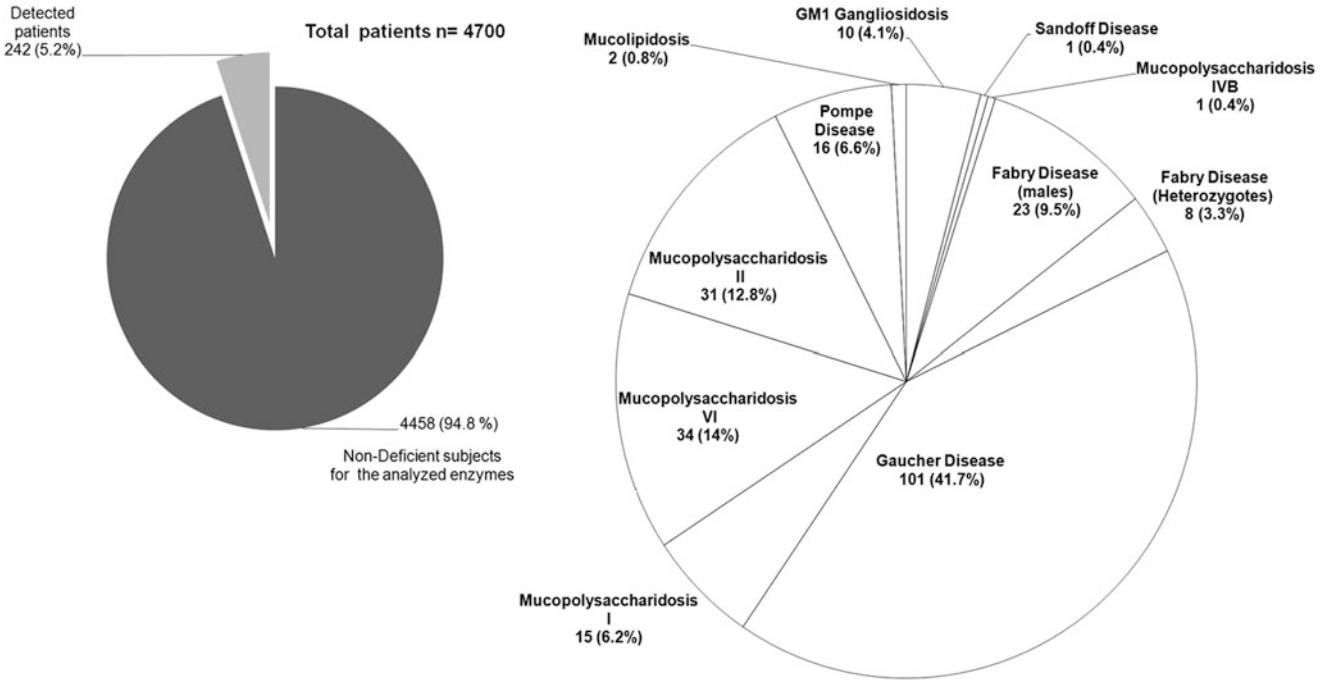

Fig. 1 Final results of the application of enzymatic studies for dried blood collected on filter paper

The present study also allowed the detection of one patient with Morquio IVB. In this case, the patient was referred because he showed dysmorphic features and short stature without mental commitment. The obtained results for arylsulfatase B, α-L-iduronidase, and iduronate sulfatase showed normal values. However, this patient showed a marked decrease in the enzymatic activity of one of the control enzymes (β-galactosidase). In order to support diagnosis, we made a quantification of glycosaminoglycans in urine samples, obtaining a value 1.5 times above the reference value. The electroforetic pattern fund in this sample showed excretion of keratan sulfate, which, in correlation with clinical symptoms, allowed the diagnosis of a mucopolysaccharidosis type IVB (Morquio B). We also find a patient with Sandhoff disease, whose deficiency was confirmed measuring total hexosaminidase activity and the percentage of hexosaminidase A in leukocytes (see Supplementary Table).

Another finding of great relevance was observed while evaluating β-galactosidase and total hexosaminidase in all samples as a quality control for blood extraction, drying, and sample transport. The total hexosaminidase analysis exhibited variable levels of increased enzymatic activity ($n = 32$, from 1.2 to 6.5 times above the reference value) with significant differences ($p < 0.001$) compared to the control groups. In conducting a follow-up with previously confirmed cases, increases in activity could be related to patients with Mucopolysaccharidosis, Gangliosidosis, Gaucher disease, Pompe disease, and Mucolipidosis. This result might suggest that for this enzymatic quantification, beyond the ability to detect Sandhoff disease, a nonspecific elevation might be a useful indicator of a lysosomal disease. This aspect is currently under investigation and could be of great assistance for the high-risk screening of these disorders (Table 2).

When comparing DBS and leukocyte samples, we could not demonstrate a quantitative exact correlation between both samples because of the sample's nature. While the activity in DBS is calculated over an approximate blood volume in the punch, the leukocyte activity is normalized to the protein quantity measured in the sample, making the latter a more accurate measure. Nevertheless, qualitatively the correlation is very good.

We could not perform molecular studies for all the deficiencies found due to the costs that these would imply. However, in association with another research group, we were able to develop a molecular protocol for Pompe disease, and 11 volunteer patients were tested for a complete study (Niño et al. 2012). At this time, patients with Fabry disease and Hunter syndrome are under mutation analyses.

Some articles have described interferences due to fluorescence quenching caused by hemoglobin (Oermardien et al. 2011). Our protocol uses a 1.2 mm punch, which dilutes the quantity of hemoglobin in the reaction, considerably diminishing this kind of interference. These modifications also allow to make the sample processing easier, since there is no need to make a previous elution (the punch is directly placed in the 96-well plate) and does not need addition of other reagents or posterior centrifugation to get better lectures, as

Table 2 Results of enzymatic activity on control[a] and patient DBS

Enzyme	n	Activity (nmol/ml/h) Average DS Range	CV_1	CV_2	P[b]	Cutoff[c]
		Arylsulfatase B				
Mucopolysaccharidosis type VI (age range: 1.5–17 years)	34	0.0–2.06 M: 1.2 DS:0.67	6.4	11.47	<0.0001	2.7
Controls (<36 days)	21	4.0–31.5 M: 10.1 DS: 5.9				
Controls (3 months–59 years)	625	2.9–43.2 M: 9.2 DS: 5.6				
		α-Galactosidase A				
Fabry disease – men (age range: 10–50.1 years)	23	0.0 –0.3 M: 0.08 DS: 0.09	8.2	10.7	<0.0001	1.15
Fabry disease[d] - heterozygotes (age range: 28–67 years)	8	0.21–3.34 M: 1.0 DS: 1.0				
Controls (<36 days)	80	2.5–21.9 M: 11.3 DS: 5.2				
Controls (3 months–88 years)	2337	2.0–21.8 M: 7.4 DS: 3.5				
		α-Iduronidase				
Mucopolysaccharidosis type I (age range: 8 months–27.7 years)	15	0.0–0.65 M: 0.25 DS: 0.23	5.2	11.3	<0.0001	1.1
Normal control (<36 days)	48	3.0–19.6 M: 9.9 DS: 3.6				
Normal controls (3 months–59 years)	1585	1.5–20.1 M: 9.5 DS: 3.8				
		β-Galactosidase				
Gangliosidosis GM 1 (age range: 7 months–4 years)	10	0.1–2.3 M: 1.4 DS: 0.7	5.3	13.4	<0.01	11.4
Mucopolysaccharidosis type IVB (age: 4 years)	1	2.1				
Controls (3 months–88 years)	2354	19–99 M: 47 DS: 16				
Controls (<36 days)	166	28–164 M: 71 DS: 26				
		β-Glucosidase				
Gaucher disease (age range: 4 months–72.8 years)	101	0.39–5.3 M:3.6 DS:1.14	15	14.7	<0.0001	5.6
Controls (6 months–63 years)	715	5.9–16.8 M:9.6 DS: 2.6				
		Total Hexosaminidase				
Sandoff disease (age: 1 years)	1	42.1	4.1	10.2	<0.0001	111.4
Normal controls (3 months–88 years)	1820	180.8–750.2 M: 396.3 DS:120.8				
Controls (<36 days)	114	284.3–884.1 M: 527.2 DS: 130.2				
Overexpression of total Hexosaminidase activity**	32	856.4–4882.8 M: 1538.3 DS: 913.1				
		Iduronate Sulfatase				
Mucopolysaccharidosis type II (age range: 1.1–35 years)	31	0.0 –1.86 M: 0.86 DS: 0.43	8.0	11.2	<0.0001	6.3
Controls (<36 days)	11	19.1–44.2 M:32.8 DS: 9.3				
Controls (3 months–59 years)	210	10.7–45.2 M: 24.8 DS: 7.3				

(continued)

Table 2 (continued)

Enzyme	n	Activity (nmol/ml/h) Average DS Range	CV_1	CV_2	P[b]	Cutoff[c]
		Chitotriosidase				
Patients affected by Gaucher disease and elevated chitotriosidase	86	120.9–3479.8 M:1108.9 DS: 789.9	9.4	12.4	<0.0001	96.7
Patients affected by Gaucher disease without elevated chitotriosidase*	15	0.0–80.8 M: 41.3 DS: 34.8				
Patients affected by other lysosomal disorders***	74	20.3 – 1649.6 M: 229.1 DS:231.5				
Controls (3 months–78 years)	518	2.3 –96.1 M:36.3 DS:23.2				

[a] According to the Shapiro-Wilk test, all control groups analyzed failed to show a normal distribution (Sig. <0.05 with 95% confidence)
[b] Cited values correspond to the results of the non-parametric Mann-Whitney test comparing activity values of individuals affected by enzyme deficiency and healthy controls
[c] Obtained through ROC (Receiver Operating Characteristics) analysis, based on a comparison between results for patients and control individuals (99 % confidence, 100 % sensitivity, and 100 % specificity). Both sensitivity and specificity values refer to the certainty offered by each cutoff for the enzyme analyzed. The ROC analysis was made with the complete dataset
[d] Results correspond to obligated heterozygotes (mothers of Fabry patients) who at the time of this writing are still not under clinical follow-up
*All referred patients with clinical findings suggesting Gaucher disease were evaluated for chitotriosidase. Eight cases showed increased Chitotriosidase activities (range: 105–1650 nmol/ml/h) with normal β–glucosidase activity in leukocyte samples; subsequent studies indicated Niemmann Pick disease (data not shown)
**The values shown do not establish a cutoff for the overexpression. Some patients with a confirmed diagnosis of LSD ($n = 32$) showed increases of 1.5 to 6.5 times above the reference value. (range: 180.8–750.2 nmol/ml/h). This study is in progress
***Patients with other LSDs and increased chitotriosidase: Gangliosidosis GM1 ($n = 3$), Fabry disease ($n = 3$), MPS I (n=2), MPS II ($n = 1$), MPS VI (n=1), MPS VII ($n = 1$)
CV_1: Intra assay variability coefficient. (%)
CV^2: Inter assay variability coeffcient. (%) ($n = 30$)

has been described by Oermardien et al. (2011). Additionally, a smaller punch allowed us to make retrospective studies, which is a very important fact given the difficulty of finding some of these patients after the first sampling. The use of 1.2 mm punches allows us to reanalyze them several times and orient to a diagnosis that can, in some cases, be different from the initial approach.

Given the little information available in the literature about the state of the art of these entities in our countries, it is difficult to implement early detection protocols for LSDs, leading to a late diagnosis and, in the worst situation, to the death of patients without a diagnosis. Given that physicians and health management entities are more interested in the studies when these are made in their own countries than when they take place outside, we think this study will generate more interest from the medical community toward lysosomal storage diseases.

Although working with 4-MU substrates is not new and several authors have reported similar assays before, in Colombia, Andean countries, and several Central American countries, there are no high-risk screening studies for lysosomal storage diseases that involve such a large number of patients with a representative number of affected subjects, over such a long period of time. In our country, the funding for research in these orphan entities and the investment from the government in these diseases is very low. With this study we want to present an alternative solution that would help achieve diagnosis for patients in Colombia and the neighboring countries.

Conclusions

The results of this study offer reference values for healthy controls and individuals affected by nine lysosomal enzymes, allowing for the diagnostic approach of eight lysosomal disorders.

To date in Colombia, Andean countries, and several Central American countries, there are no high-risk screening studies for lysosomal storage diseases that involve such a large number of patients with a representative number of affected subjects, over such a long period of time. In this regard, in the 10 years prior to the start of this project (1995–2004), our laboratory performed enzymatic evaluations on only 751 patients with clinical characteristics related to these disorders which resulted in 26 confirmed cases. This limitation was due to the aforementioned difficulties regarding the handling of liquid samples, which led us to believe that the implementation of dried blood spots collected on filter paper would increase the number of patients that could be tested in the upcoming years. Here we report our increased testing capacity that allows individuals with clinical results related to these disorders

Table 3 Results of DBS enzyme activity for α-glucosidase

	Healthy controls		
Age	<36 days	6 months–89 years	Affected by Pompe disease*
n	209	1,574	16
Range/Median/Standard deviation[a]			
α-Glucosidase Total Activity[1] (α-Glu)=A	R: 5.3–70.6 M:18 DS: 7.6	R: 3.7–57.6 M:18.2 DS:7.7	R: 4.8–64.2 M:20.1 DS:16.1
Inhibited α-Glucosidase[2] (α-GluINH)=B	R: 1.8–10.9 M:4.4 DS: 1.9	R: 1.8–10.7 M:4.9 DS:2.0	R: 0.3–4.6 M:1.5 DS:1.2
Neutral α-Glucosidase[3] (α-GluNeu)=C	R: 12.1–96.5 M:36.3 DS: 13.6	R: 9.8–147.3 M:38.6 DS:14.6	R: 24.2–191.6 M:57.0 DS:42.9
Ratio[4,5] α-GluNeu/α-GlulNH	R: 3.0–16.0 M:8.0 DS: 2.9	R: 1.4–16.0 M:9.5 DS:3.2	R: 19.0–114.2 M:46.6 DS:24.8
% Inhibition[5]	R: 40.4–86.0 M:71.0 DS:9.8	R: 25.8–85.0 M:74.7 DS:8.4	R: 87.2–95.5 M:92.4 DS:2.4

[1] A comparative statistical analysis of α-Glu between healthy controls and affected patients did not reveal significant differences (Mann-Whitney test $P<0.176$, Sig. 0.05)
[2] The significant differences (Mann-Whitney test $P<0.0001$, Sig. 0.05) among different evaluation of α-GlulNH is performed using acarbose as an inhibitor. Comparative analysis demonstrates control groups and patients affected with Pompe disease
[3] α-GluNeu corresponds to an isoform of α-Glucosidase evaluated at pH 7.0. Statistical analysis shows significant differences between healthy controls and affected patients (Mann-Whitney test $P<0.0001$, Sig. 0.05)
[4] Ratio neutral / inhibited = C/B, % inhibition = (B*100)/A
[5] Enzymatic evaluations for α-Glu, α-GlulNH and α-GluNeu allowed the calculation of the relation between enzymatic isoforms and the percentage of inhibition generated by the presence of acarbose. Values found for individuals affected by Pompe disease were significantly different from control groups analyzed (Mann-Whitney test $P<0.0001$, Sig. 0.05)
[a] Activity in nmol/ml/h
*Molecular studies has been made for some of these patients (see Niño et al. 2012 and Supplementary Table)

to be more easily studied regardless of their geographical location, and, in addition, permits the analysis of a high volume of samples.

The final results of this study offer a general view of LSDs in Colombia, disorders that were, until recently, considered an extreme rarity in the medical field. It is expected that these preliminary findings could stimulate state-sponsored health systems to systematically screen for these disorders and to support reference centers which could offer diagnostic testing, facilitating the detection of these disorders and enabling a timely treatment for many patients affected by LSDs.

Acknowledgments The authors would like to express thanks to Dr. Barbara Zimmermann at the University of los Andes, Bogota, Colombia; Dr. Maira Burin and the laboratory of Inborn Errors of Metabolism, Medical Genetics Service of Hospital de Clinicas de Porto Alegre, Federal University of Rio Grande do Sul (UFRGS), Brazil; and Dr. Nestor Chamoles (deceased) and his research group at the Neurochemistry Laboratory, Buenos Aires, Argentina, for their guidance and support in the development of this study.

The author also wishes to thank the Faculty of Sciences and Department of Biological Sciences at the University of los Andes, Genzyme of Colombia, and Biomarin of Colombia for the support they provided for this project.

Synopsis

In this report, of a 7-year screening study, we demonstrate the usefulness of dried blood spots on filter paper for the high-risk screening of lysosomal storage disorders.

References

Beratis N, Aaron A, Hirschhorn K (1973) Metachromatic leukodystrophy: detection in serum. J Pediatr 83(5):824–827

Brady RO (2006) Enzyme replacement for lysosomal diseases. Annu Rev Med 57:283–296

Burin M, D'Almeida V, Carrillo J, Kallwas H, Schenone A, Uribe A (2011) Latin American external quality control: preliminary experience in analysis lysosomal enzymes from blood collected in filter paper. Oral presentation. Rev Gastroenterol Peru 31 (Suppl 1):29

Chamoles NA, Blanco MB, Gaggioli D (2001a) Fabry disease: enzymatic diagnosis in dried blood spots on filter paper. Clin Chim Acta 308:195–196

Chamoles NA, Blanco MB, Gaggioli D, Casentini C (2001b) Hurler-like phenotype: enzymatic diagnosis in dried blood spots on filter paper. Clin Chem 47:2098–2102

Chamoles NA, Blanco MB, Gaggioli D, Casentini C (2002) Gaucher and Niemann-Pick diseases – Enzymatic diagnosis in dried blood

spots on filter paper: retrospective diagnoses in newborn-screening cards. Clin Chim Acta 317:191–197
Chamoles NA, Niizawa G, Blanco M, Gaggioli D, Casentini C (2004) Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper. Clin Chim Acta 347:97–102
Civallero G, Michelin K, de Mari J, Viapiana M, Burin M, Coelho JC et al (2006) Twelve different enzyme assays on dried-blood filter paper samples for detection of patients with selected inherited lysosomal storage diseases. Clin Chim Acta 372(1–2):98–102
Daitx VV, Mezzalira J, Goldim MP, Coelho JC (2012) Comparison between alpha-galactosidase A activity in blood samples collected on filter paper, leukocytes and plasma. Clin Biochem 45(15): 1233–1238
de Castilhos CD, Mezzalira J, Goldim MP, Coelho JC (2011) Influence of pre-analytical factors on α-galactosidase A, arylsulfatase B and α-glucosidase activities measured on dried blood spots on filter paper. Clin Biochem 44(10–11):922–926
De Jesus VR, Zhang XK, Keutzer J, Bodamer OA, Mühl A, Orsini JJ, Caggana M, Vogt RF, Hannon WH (2009) Development and evaluation of quality control dried blood spot materials in newborn screening for lysosomal storage disorders. Clin Chem 55 (1):158–164
Filocamo M, Morrone A (2011) Lysosomal storage disorders: molecular basis and laboratory testing. Hum Genomics 5(3):156–169
Futerman AH, van Meer G (2004) The cell biology of lysosomal storage disorders. Nat Rev Mol Cell Biol 5:554–565
Gasparotto N, Tomanin R, Frigo AC, Niizawa G, Pasquini E, Blanco M, Donati MA, Keutzer J, Zacchello F, Scarpa M (2009) Rapid diagnostic testing procedures for lysosomal storage disorders: alpha-glucosidase and beta-galactosidase assays on dried blood spots. Clin Chim Acta 402(1–2):38–41
Gieselmann V (1995) Lysosomal storage diseases. Biochim Biophys Acta 1270:103–136
Jiménez LM, Bobillo J, Caro A, Duran P (2011) β-galactosidase activity as an index of quality control of dried blood sample collected on paper. Revista Laboratorio Clínico 4(3):153–157
Kallwass H, Carr C, Gerrein J, Titlow M, Pomponio R, Bali D, Dai J, Kishnani P, Skrinar A, Corzo D, Keutzer J (2007) Rapid diagnosis of late-onset Pompe disease by fluorometric assay of alpha-glucosidase activities in dried blood spots. Mol Genet Metab 90(4):449–452
Li Y, Scott CR, Chamoles NA, Ghavami A, Pinto BM, Turecek F, Gelb MH (2004) Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. Clin Chem 50(10):1785–1796
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193 (1):265–275
Müller KB, Rodrigues MD, Pereira VG, Martins AM, D'Almeida V (2010) Reference values for lysosomal enzymes activities using dried blood spots samples - a Brazilian experience. Diagn Pathol 29(5):65
Niizawa G, Blanco MB, Levin C, Aranda C, Chamoles NA (2005) Retrospective diagnosis of glycogen storage disease type II by use of newborn-screening card. Clin Chim Acta 359: 205–206
Niño MY, Mateus HE, Fonseca DJ, Kroos MA, Ospina SY, Mejía JF, Uribe JA, Reuser AJ, Laissue P (2012) Identification and functional characterization of GAA mutations in Colombian patients affected by Pompe disease. JIMD Rep 7:39–48
Oemardien LF, Boer AM, Ruijter GJ, van der Ploeg AT, de Klerk JB, Reuser AJ, Verheijen FW (2011) Hemoglobin precipitation greatly improves 4-methylumbelliferone-based diagnostic assays for lysosomal storage diseases in dried blood spots. Mol Genet Metab 102(1):44–48
Olivova P, Cullen E, Titlow M, Kallwass H, Barranger J, Zhang K, Keutzer J (2008) An improved high-throughput dried blood spot screening method for Gaucher disease. Clin Chim Acta 398 (1–2):163–164
Reuser AJ, Verheijen FW, Bali D, van Diggelen OP, Germain DP, Hwu WL, Lukacs Z, Mühl A, Olivova P, Piraud M, Wuyts B, Zhang K, Keutzer J (2011) The use of dried blood spot samples in the diagnosis of lysosomal storage disorders–current status and perspectives. Mol Genet Metab 104(1–2):144–148
Rodrigues MD, de Oliveira AC, Müller KB, Martins AM, D'Almeida V (2009) Chitotriosidase determination in plasma and in dried blood spots: a comparison using two different substrates in a microplate assay. Clin Chim Acta 406(1–2):86–88
Scriver CR, Sly WS, Childs B, Beaudet AR (2001) The metabolic and molecular basis of inherited disease, 8th edn. McGraw-Hill, New York
Shapira E, Blitzer MG, Miller JB, Africk DK (1989) Biochemical genetics. A laboratory manual. Oxford University Press, New York, pp 17–36
Singh J, Tavella D, Ferrante ND (1975) Measurements of arylsulfatases A and B in human serum. J Pediatr 86(4):574–576
Staretz-Chacham O, Lang TC, LaMarca ME, Krasnewich D, Sidransky E (2009) Lysosomal storage disorders in the newborn. Pediatrics 123:1191–1207
Stolk P, Willeme JC, Leufkens GM (2006) Rare essentials: drugs for rare diseases as essential medicines. Bull World Health Organ 84(9):745–751
Vellodi A (2005) Lysosomal storage disorders. Br J Haematol 128:413–431
Voznyi YV, Keulemans J, Beyer EM, van Diggelen OP (2001) A fluorogenic assay for the diagnosis of Hunter disease (MPS II). J Inherit Metab Dis 24:675–80
Wenger DA, Coppola S, Liu SL (2002) Lysosomal storage disorders: diagnostic dilemmas and prospects for therapy. Genet Med 4(6): 412–419
Whiteman P (1973) The Quantitative measurement of Alcian Blue glycosaminoglycan complexes. Biochem J 131:343–350
Wraith JE (2002) Lysosomal disorders. Semin Neonatol 7:75–83

JIMD Reports
DOI 10.1007/8904_2013_230

ORIGINAL ARTICLE

Mitochondrial Infantile Liver Disease due to *TRMU* Gene Mutations: Three New Cases

Pauline Gaignard · Emmanuel Gonzales · Oanez Ackermann · Philippe Labrune · Isabelle Correia · Patrice Therond · Emmanuel Jacquemin · Abdelhamid Slama

Received: 21 February 2013 / Revised: 01 April 2013 / Accepted: 02 April 2013 / Published online: 27 April 2013

Abstract Combined respiratory chain defect is a common feature in mitochondrial liver disease during early infancy. Mitochondrial DNA depletions, induced by mutations of the nuclear genes *POLG*, *DGUOK*, and *MPV17*, are the major causes of these combined deficiencies. More recently, mutations in *TRMU* gene encoding the mitochondrial tRNA-specific 2-thiouridylase were found in infantile hepatopathy related to mitochondrial translation defect. It is characterized by a combined defect of respiratory chain complexes without mitochondrial DNA depletion.

We report here clinical, biochemical, and genetic findings from three unrelated children presenting with hepatopathy associated with hyperlactatemia and respiratory chain defect due to bi-allelic mutations in *TRMU* gene. Two patients recovered spontaneously in a few months, whereas the other one died of acute liver failure. Spontaneous remission is a rare feature in mitochondrial liver diseases, and early identification of *TRMU* mutations could impact on clinical management. Our results extend the small number of *TRMU* mutations reported in mitochondrial liver disorders and allowed accumulating data for genotype–phenotype correlation.

Communicated by: Shamima Rahman, PhD, BMBCh

Competing interests: None declared

P. Gaignard · I. Correia · P. Therond · A. Slama (✉)
AP-HP, Hôpital Bicêtre, Hôpitaux Universitaires Paris-Sud, Laboratoire de biochimie, 78 rue du Général Leclerc, Le Kremlin-Bicêtre Cedex 94275, France
e-mail: abdel.slama@bct.aphp.fr

P. Gaignard · E. Gonzales · O. Ackermann · I. Correia · P. Therond · E. Jacquemin · A. Slama
Centre de référence des maladies mitochondriales, Paris, France

P. Labrune
AP-HP, Hôpital Antoine-Béclère, Hôpitaux Universitaires Paris-Sud, Service de pédiatrie, Clamart, France

E. Gonzales · O. Ackermann · E. Jacquemin
AP-HP, Hôpital Bicêtre, Hôpitaux Universitaires Paris-Sud, Service d'hépatologie pédiatrique, Le Kremlin-Bicêtre, France

Introduction

Liver involvement is a frequent clinical presentation of neonatal mitochondrial cytopathies (García-Cazorla et al. 2005). In most cases, liver biochemical analysis showed a combined deficiency of mtDNA-dependent complexes (I, III, IV, and V) with a reduction of mtDNA copy numbers (depletion), due to mutations in nuclear genes encoding proteins implicated in mtDNA stability, mainly *POLG*, *DGUOK*, and *MPV17* genes (Naviaux and Nguyen 2004; Mandel et al. 2001; Spinazzola et al. 2006; Sarzi et al. 2007). In a few cases, activities of mtDNA-dependent complexes in liver are decreased, whereas mtDNA copy number is normal: a defect in mitochondrial protein synthesis machinery is then to consider. Mutations of three nuclear gene-encoding proteins involved in mitochondrial protein translation have been recently associated with liver disorders in the neonatal period: *GFM1*, *TUFM*, and *TRMU* (Coenen et al. 2004; Valente et al. 2007; Zeharia et al. 2009).

First, *TRMU* mutations in infantile mitochondrial hepatopathy were reported by Zeharia et al. in 2009 by homozygosity mapping in a cohort of 13 patients of predominately Yemenite Jewish origin (OMIM 610230) (Zeharia et al. 2009). Nuclear *TRMU* gene encodes the mitochondrial tRNA-specific 2-thiouridylase 1 (EC 2.1.1.61), responsible for the 2-thiolation of uridine at the first anticodon position of the mitochondrial tRNALys, tRNAGlu, and tRNAGln (Hagervall et al. 1987). This post-transcriptional modification of uridine in the wobble position contributes to precise and

efficient codon recognition (Umeda et al. 2005). Defect of TRMU protein leads to a reduced 2-thiolation and a decrease of mitochondrial tRNA level that may impair the translation of mtDNA-dependent complexes and cause combined RC defect in liver (Zeharia et al. 2009; Guan et al. 2006).

Since 2009, to the best of our knowledge, only two other cases of *TRMU* mutations associated with neonatal hepatopathy have been described (Schara et al. 2011; Uusimaa et al. 2011). In this study, we report clinical, biochemical, and genetic findings in three unrelated patients affected by cholestasis, lactic acidosis, recurrent hypoglycemia, and combined respiratory chain (RC) deficiency. All harbored pathogenic *TRMU* mutations, including two novel mutations.

Material and Methods

Patients

We studied three unrelated patients with combined hepatic mitochondrial RC defect. Clinically relevant results are summarized next.

Patient 1 (P1) was born at term after an uneventful pregnancy and delivery (Apgar score 9/10) with a height of 47 cm, a body weight of 2.830 kg, and a head circumference of 33 cm. Her mother was Caucasian and her father was Asian. At 3 months of age, she presented with faltering growth, vomiting, and poor feeding. At 6 months, clinical examination confirmed growth retardation: height 58 cm (−3 SD), weight 4.970 kg (−3 SD), head circumference 38 cm (−3 SD). Hepatomegaly (without splenomegaly) and jaundice were also noticed. Abdominal ultrasound disclosed hyperechogenic liver, without ascites. The child presented axial and peripheral hypotonia. Cerebral MRI (magnetic resonance imaging) revealed myelination delay, without abnormal lactate peak on spectroscopy examination.

Laboratory investigations showed elevated serum transaminase activities when compared to upper limit of normal (ULN) (AST 8ULN, ALT 4ULN), cholestasis (total bilirubin 84 μmol/L, N < 17 μmol/L, conjugated bilirubin 71 μmol/L, N < 10 μmol/L) with elevated gamma-glutamyltransferases (GGT 6ULN). Factor V was normal (85 %). CPK and alpha-fetoprotein were 55 UI/l (N < 240 UI/L) and 28780 UI/ml (N < 25 UI/mL), respectively. Metabolic analysis revealed recurrent hypoglycemia and hyperlactatemia (5.0 mmol/L, N < 1.8 mmol/L) with elevated lactate/pyruvate ratios (L/P 28, N = 7–15). Lactate concentration in cerebrospinal fluid (CSF) was also increased (3.3 mmol/L, N < 1.9 mmol/L) with a slightly elevated L/P ratio (17, N = 7–15).

Liver histology showed a micronodular cirrhosis, an important canalicular cholestasis, and some oncotic change in hepatocytes. Microvesicular steatosis was absent.

Within a few months, her condition worsened and she was considered for a liver transplantation. Unfortunately, she died from variceal bleeding due to portal hypertension at 8 months of age.

Patient 2 (P2) was born at term from healthy unrelated Caucasian parents after an uneventful pregnancy and delivery (Apgar score 10/10, height 50 cm, weight 3.455 kg, head circumference 35 cm). At 4 months of age, hepatomegaly (without splenomegaly) and jaundice were detected. Abdominal ultrasound revealed hyperechogenic enlarged liver, with diffuse steatosis. Clinical examination noted regular growth and normal axial and peripheral tone. Cerebral MRI showed normal myelination but abnormal lactate peak on spectroscopy. Routine biochemical analysis disclosed similar liver disorders as P1 (AST 17ULN, ALT 13ULN, total bilirubin 42 μmol/L, conjugated bilirubin 21 μmol/L, GGT 11ULN) without coagulopathy (factor V 85 %). CPK and alpha-fetoprotein were 42 UI/L and 100600 UI/mL, respectively. P2 presented recurrent hypoglycemia and hyperlactatemia (8.0–10.0 mmol/L) with elevated lactate/pyruvate ratios (L/P 42–53). In CSF, lactate concentration was also increased (3.9 mmol/L) with a slightly elevated L/P ratio (18). Liver histology showed portal and perisinusoidal fibrosis with microvesicular steatosis and oncocytic hepatocytes.

P2 is currently 2 years old. Tailored diet allows good tolerance to fasting. Physical examination shows persistent liver enlargement, and abdominal ultrasound discloses some liver nodules (<1 cm).

Patient 3 (P3) is the third child of consanguineous Egyptian parents. At 33 weeks of gestation, intrauterine growth retardation was detected and the delivery was triggered 1 week later because of hemorrhagic placenta previa (delivery 36 weeks + 1 day, Apgar score 2/7/10, height 46 cm, weight 1.765 kg, head circumference 31 cm).

At 2 days of age, P3 presented cholestatic jaundice with hepatomegaly, hepatocellular deficiency (prothrombin time 45 %), and hypoglycemia with favorable outcome.

At 4 months of age, clinical examination noted hepatomegaly with splenomegaly. Abdominal ultrasound revealed several small liver nodules. P3 also presented growth retardation (height 55 cm [−3 SD], weight 4.400 kg [−2.5 SD], head circumference 39 cm [−2 SD]) and slight axial hypotonia. Cerebral MRI was not performed.

Biochemical investigations disclosed raised aminotransferase serum activities (AST 5ULN, ALT 2ULN), elevated serum bilirubin concentration (total 84 μmol/L, conjugated 63 μmol/L) and alpha-fetoprotein concentration (208000 UI/mL) but normal prothrombin time and GGT. Lactate concentrations were increased in blood (4.0–6.0 mmol/L; L/P 30–35) and slightly elevated in CSF (2.0 mmol/L, L/P not determined).

Table 1 Mitochondrial enzymatic activities in liver biopsies. Abnormal results are indicated in bold

	Patient 1 (liver)	Patient 2 (liver)	Patient 3 (liver)	*Normal range*
Mitochondrial enzymatic activities (nmol.min^{-1}.mg^{-1} of proteins)				
Complex I	**7**	**7**	**7**	19–26
Complex II	188	311	256	168–277
Complex III	248	400	238	143–192
Complex IV	**193**	**157**	**123**	202–319
Complex V	229	597	325	74–167
Citrate synthase	153	315	233	63–131
Activities ratios				
Complex IV/complex I	**27.6**	**22.4**	**17.6**	6.0–10.0
Complex II/citrate synthase	1.2	1.0	1.1	0.8–2.4
Complex IV/citrate synthase	**1.3**	**0.5**	**0.5**	2.5–3.3

Liver biopsy showed patent signs of fibrosis, irregular cirrhosis with nodulation, severe cholestasis, and moderate macrovesicular steatosis. Some oncocytic and swollen hepatocytes were detected.

At 5 years of age, this patient is still alive. Blood tests, clinical liver and neurological examinations were normal, but abdominal ultrasound noted a persistent multinodular liver.

Biochemical Assays

Mitochondrial enzymatic activities were measured in liver biopsies from P1, P2, and P3, in muscle biopsy from P1, and in cultured fibroblasts from P1 and P2. Fibroblasts were cultured in HAM F10 medium with 10 % FCS. Enzymatic activities of RC complexes (I, II, III, IV, and V) and mitochondrial enzyme marker (citrate synthase) were performed according to Rustin et al. (1994).

Molecular Analysis

Mutations in mtDNA were screened by sequencing the whole mitochondrial genome, and mtDNA copy numbers were measured in liver by quantitative PCR based on the ratio of mtDNA to nuclear DNA (*MTND2*/*ATP5B*) (Chabi et al. 2003).

Exon and exon–intron junctions of *POLG*, *DGUOK*, *MPV17*, and *TRMU* genes were sequenced on genomic DNA (*TRMU* reference sequence NM_018006).

Putative mutations were validated by sequencing PCR products on both strands. The segregation of alleles was confirmed by parents' DNA analysis.

For the new point mutation (p.Glu217Lys), 100 alleles from healthy Caucasian subjects were sequenced on the exon 5. The tool PolyPhen-2 based on multiple-sequence alignment was used to assign a score from 0.00 (benign) to 1.00 (probably damaging) reflecting the impact of amino acid substitution (www.genetics.bwh.harvard.edu/pph2).

For P1, total RNA was extracted from cultured fibroblasts, and the *TRMU* cDNA was sequenced using standard procedure.

Written informed consent was obtained from parents of each patient.

Results

Enzymatic Activities

Results of the activities of RC complexes in liver are summarized in Table 1. Deficiencies of complexes I and IV were detected in liver biopsies of every patient. The activity ratio of complex IV to complex I was strongly increased, suggesting that the enzymatic defect was more pronounced for complex I than for complex IV.

Muscle biopsy from P1 was also carried out because of axial hypotonia. The activities of RC complexes were found markedly reduced: complex I 4 nmol.min^{-1}.mg^{-1} of proteins (normal range 16–52); complex II 12 nmol.min^{-1}.mg^{-1} of proteins (normal range 43–102); complex III 31 nmol.min^{-1}.mg^{-1} of proteins (normal range 125–418); complex IV 85 nmol.min^{-1}.mg^{-1} of proteins (normal range 125–520); complex V not determined. Normal citrate synthase activity (84 nmol.min^{-1}.mg^{-1} of proteins, normal range 69–240) indicated that the decrease of the activities of RC complexes was not caused by low mitochondrial content in the tissue.

In cultured fibroblasts from P1, the activities of complexes III and IV were decreased, whereas the activities of complexe II and citrate synthase were normal: complex II 18 nmol.min^{-1}.mg^{-1} of proteins (normal range 11–17); complex III 62 nmol.min^{-1}.mg^{-1} of proteins (normal range 98–180);

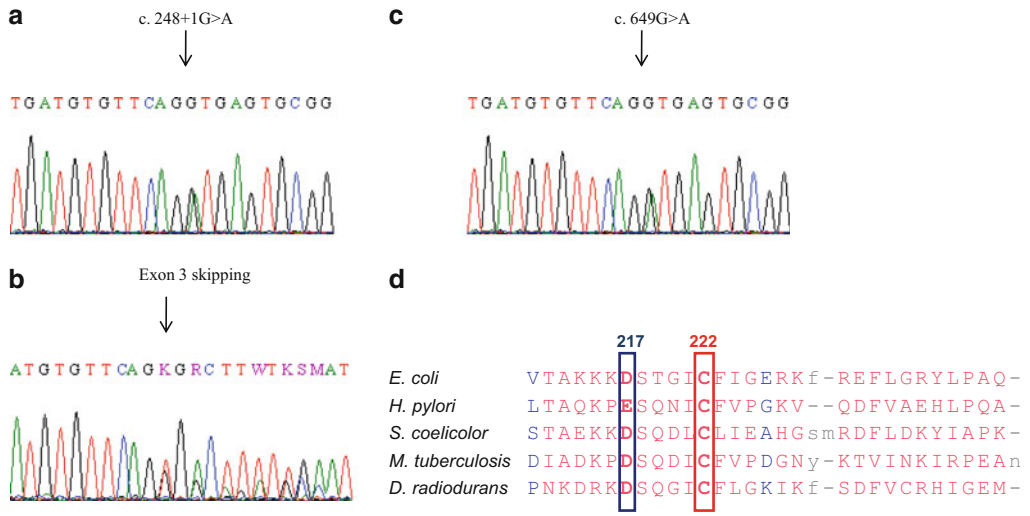

Fig. 1 Analysis of novel *TRMU* mutations. (**a**) Sequencing analysis of *TRMU* on P1 genomic DNA with the c. 248 + 1G>A heterozygous mutation. (**b**) Sequencing analysis of *TRMU* on P1 cDNA showing the exon 3 skipping. (**c**) Sequencing analysis of *TRMU* on P2 genomic DNA with the c. 649G>A heterozygous mutation (p.Glu217Lys). (**d**) Amino acid sequence of the conserved domain of the tRNA methyl-transferase family with the active Cys residue in position 222. Amino acids with carboxylic acid functional group (Glu or Asp) are conserved in position 217

complex IV 57 nmol.min^{-1}.mg^{-1} of proteins (normal range 72–143); complexes I and V not determined; citrate synthase 79 nmol.min^{-1}.mg^{-1} of proteins (normal range 32–72).

For P2, the activities of RC complexes were normal in cultured fibroblasts.

Genetic Analysis

A combined RC defect may result from defect of mtDNA maintenance, leading to mtDNA depletion, or alterations in mitochondrial proteins synthesis machinery. Liver mtDNA copy numbers were 40 %, 114 %, and 86 % of controls for P1, P2, and P3, respectively, excluding a mtDNA depletion. The sequencing of key nuclear genes involved in mtDNA instability in liver (*POLG*, *DGUOK*, and *MPV17*) did not show pathogenic mutations. Then, when sequence analysis of the entire mtDNA from liver failed to reveal mutations in either tRNA or rRNA genes, we considered a translation defect caused by mutations in a nuclear gene. *TRMU* gene emerged as a good candidate in the context of infantile hepatopathy. *TRMU* mutations were found in patients 1 and 2: c.835G>A/c.248 + 1G>A (P1); c.835G>A/c.649G>A (P2). Patient 3 was homozygous for the c.697C>T mutation. The c.835G>A (p.Val279Met) and c.697C>T (p.Leu233Phe) mutations have previously been described by Zeharia et al. (2009). We report here two new mutations: c.248 + 1G>A and c.649G>A (p Glu217Lys), both predicted to be highly damaging for protein function.

The new intronic c.248 + 1G>A mutation alters the natural 5' splice donor site of intron 2 (Fig. 1a). cDNA analysis on cultured fibroblasts from P1 showed that it leads to exon 3 skipping (Fig. 1b). The predicted resulting protein is likely to be inactive because it is truncated before the active site Cys222 that mediates uridine thiolation (p.Ser83ArgfsX18).

The missense c.649G>A mutation causes a p.Glu217Lys transition (Fig. 1c). A study of 100 control alleles excludes a common polymorphism. Evolutionary conservation of the Glu217 residue across orthologous genes is strong (conservation score Polyphen 0.999), indicating a structurally or functionally important role in the thiouridylase activity. Moreover, the Glu217 residue is situated in a conserved domain of the tRNA methyl-transferase family (pfam O3054), which includes TRMU protein, and is close to the Cys222 residue that mediates the thiolation of the tRNAs. As a consequence, the exchange between a carboxylic amino acid (Glu) and a basic amino acid (Lys) in position 217 is likely to be a disease-causing mutation (Fig. 1d).

Discussion

TRMU gene encodes a thiouridylase necessary to the maturation of mitochondrial tRNA and a correct translation (Umeda et al. 2005; Hagervall et al. 1987). The role of TRMU protein defect in mitochondrial diseases was first mentioned to modulate the phenotypic manifestation of the deafness-associated mitochondrial 12S rRNA mutation (m.1555A>G) (Guan et al. 2006; Yan et al. 2006). In 2009, Zeharia et al. first described *TRMU* mutations in mitochondrial hepatopathy characterized by infantile liver

injury with a decrease of mtDNA-encoded complexes in liver without mtDNA depletion (Zeharia et al. 2009). Only two other cases of *TRMU*-related hepatopathy have been described since (Kemp et al. 2011; Low et al. 2008; Schara et al. 2011; Uusimaa et al. 2011).

We report a series of three unrelated cases of infantile liver disease caused by *TRMU* mutations. All three children suffered from hepatomegaly with cytolysis and cholestasis in the first months of life. Two of them also presented growth retardation and abnormal tone. In all cases, hyperlactatemia with elevated lactate/pyruvate ratio suggested a RC defect.

The activities of RC complexes studies were undertaken in liver, muscle, and fibroblasts and our results clearly showed that liver biopsy is the most discriminating tissue to direct molecular investigations toward a RC combined defect. In all cases, strong decreases of complexes I and IV were noticed in the liver. The activities of complexes I and IV were diminished in all other cases of *TRMU*-related RC combined defects, even if other complexes may also be reduced (Zeharia et al. 2009; Low et al. 2008; Schara et al. 2011).

The activities of RC complexes could also be diminished in muscle biopsy, but not in all cases. Previous studies reported normal activities of complexes I, II, II + III, and IV (Zeharia et al. 2009) or deficiencies of complex IV (Zeharia et al. 2009), complexes I and IV (Schara et al. 2011; Low et al. 2008), or complexes I, II + III, and IV (Zeharia et al. 2009). In muscle biopsy from P1, mtDNA-encoded complexes I, III, and IV activities were decreased (V not determined), but, intriguingly, the nuclear-encoded complex II activity was also reduced. Decrease of complex II activity may reflect a secondary defect, since it has been suggested that TRMU protein deficiency could alter the addition of clusters Fe–S to complex II subunits (Sasarman et al. 2011).

Fibroblasts are the less informative tissue: a decrease of mtDNA-dependent complexes III and IV was noted in cultured fibroblasts from P1, whereas no abnormality was detected in fibroblasts from P2.

Following the identification of combined RC defect in liver, a depletion syndrome was first envisaged, but mtDNA depletion screening in liver and sequencing of the main genes involved in mtDNA maintenance failed to reveal anomalies. A translation defect was then investigated. As mitochondrial tRNA or rRNA mutations were excluded by whole sequencing of liver mtDNA, mutations in a nuclear gene were suspected. In addition to the *TRMU* gene, two genes have also been recently associated with early-onset liver disorders by translation defect: *GFM1* (elongation factor EFG1) and *TUFM* (elongation factor EFTu). Nevertheless, the *TRMU* gene emerged as the best candidate gene, because liver dysfunction was present and predominant in all cases described (Valente et al. 2007; Galmiche et al. 2012; Zeharia et al. 2009). Pathogenic *TRMU* mutations were found in every patient: c.835G>A (p.Val279Met)/c.248 + 1G>A (splicing alteration) (P1); c.835G>A/c.649G>A (p.Glu217Lys) (P2); c.697C>T (p.Leu233Phe)/c.697C>T (P3).

In spite of the similarity of the initial clinical and biochemical findings, the outcome of these patients was very different. P1 died at 6 months owing to liver failure, whereas P2 and P3 survived. At 2 and 5 years of age, respectively, they are developing normally. However, regular medical follow-up is maintained, particularly because of the persistence of liver nodules that could eventually be at risk of malignant transformation. The proportion of spontaneous clinical recovery after the acute episode in our series was similar to previously reported cases (11/15) (Zeharia et al. 2009; Uusimaa et al. 2011; Schara et al. 2011). Several hypotheses have been proposed to explain these spontaneous remissions, which are quite rare in clinical courses of mitochondrial cytopathies. Zeharia et al. suggested that a transient lack of the sulfur donor, cysteine, during the newborn period could aggravate the defect of tRNA thiouridylation and lead to mitochondrial dysfunction. Biochemical abnormalities could regress with the increase of cysteine availability during infancy, which explains the reversible phenotype (Zeharia et al. 2009). However, no experimental data support this theory. Another recent study claimed that a TRMU protein defect in fibroblast cell lines leads to a reduction of the 2-thiolation of mitochondrial tRNA but does not affect mitochondrial translation in normal conditions. Therefore, these authors proposed that the reduced level of modified mitochondrial tRNA could be a limiting factor only during early development (Sasarman et al. 2011).

We postulate that the hypothesis of a genotype and phenotype correlation could also be raised. Considering previous reported cases and our three new cases, it appears that among the five patients who died owing to *TRMU*-related hepatopathies, three carried one allele with a frameshift mutation or a splicing mutation resulting in a protein truncated before the active site Cys222: patient P1 reported here (p.Val279Met/p.Ser83ArgfsX18, c.248 + 1G>A) and two cases reported by Zeharia et al. (p.Tyr77His/p.Ser83-ArgfsX18, c.706-1G>A ; p.Val279Met/p.Ala167GlufsX36) (Zeharia et al. 2009). The other two patients who died were homozygous for a missense mutation at the first Met (p.Met1Lys/p.Met1Lys), that is predicted to be absolutely deleterious for protein activity (Zeharia et al. 2009).

On the other hand, all nine patients harboring missense mutations survived: six Yemenite Jewish patients (p.Tyr77His/p.Tyr77His in five patients; p.Leu233Phe/p.Ala10Ser for one patient), one Arab patient

(p.Gly272Asp/p.Gly272Asp), and patients P2 and P3 reported here (p.Val279Met/p.Glu217Lys; p.Leu233Phe/p.Leu233Phe).

However, two patients carrying at least one frameshift mutation recovered. One harbored a missense mutation p.Val279Met associated with a splicing mutation in the last intron c.1102-3C>G. Nevertheless, this splicing mutation may not be as harmful as those described in fatal cases since the aberrant transcript is not subject to nonsense-mediated mRNA decay and may be translated into a truncated protein that may keep a residual activity (p.Phe368SerfsX51) (Uusimaa et al. 2011). By contrast, mutations found in other patient were really deleterious: a frameshift mutation c.711_712insG (p.Gln238AlafsX14) and a nine-base–pair-in-frame insertion c.1081_1082insAGGCTGTGC (p.Arg361insAla,Val,Arg) close to a highly conserved glutamine involved in anticodon recognition (Kemp et al. 2011; Schara et al. 2011). It is worth noting that both patients received early coenzyme Q and carnitine supplementations; this could support mitochondrial functions and help to overcome the acute phase.

To sum up, in 14/16 complete genotypes described so far, patients carrying two missense mutations (except in first Met) seem to have better prognosis than patients carrying at least one frameshift or splicing mutation. Obviously, these observations between genotype and patients' outcome must be verified in a larger number of cases. However, these remarks could help evaluating prognosis or prenatal diagnosis considerations. Thus, a prenatal diagnosis was offered to P1 parents, due to clinical severity of index case. The second fetus was not carrying any mutation, the pregnancy continued and a healthy baby was born.

In conclusion, we report three unrelated cases of neonatal mitochondrial hepatopathies caused by *TRMU* mutations. The typical pattern associating deficiency of complexes I and IV and normal mtDNA copy number in liver is a strong argument for a TRMU deficiency. If liver biopsy is unavailable, we suggest that *TRMU* gene sequencing should be added to the first intention sequencing screening panel of early-onset mitochondrial liver diseases. Indeed, early molecular diagnosis could help to propose appropriate clinical managements that could perhaps facilitate remission. Further reports of additional patients with TRMU anomalies are necessary to fully elucidate prognostic factors.

One Sentence Take-Home Message

TRMU gene mutations can induce a mitochondrial liver disease in early infancy which outcome ranges from death to recovery.

Contributions of Individual Authors

GAIGNARD Pauline: identification of patients defect (biochemical analysis and genetic analysis), manuscript writing

GONZALES Emmanuel: diagnosis and follow-up of patient 3, part of the manuscript writing, revising manuscript

ACKERMANN Oanez: diagnosis and follow-up of patient 2, revising manuscript

LABRUNE Philippe: diagnosis and follow-up of patient 1, revising manuscript

CORREIA Isabelle: genetic analysis

THEROND Patrice: revising manuscript

JACQUEMIN Emmanuel: diagnosis and follow-up of patients 2 and 3, revising manuscript

SLAMA Abdelhamid: identification of patients' defect (biochemical analysis and genetic analysis), manuscript writing, and supervision

References

Chabi B, Mousson de Camaret B, Duborjal H, Issartel JP, Stepien G (2003) Quantification of mitochondrial DNA deletion, depletion, and overreplication: application to diagnosis. Clin Chem 49: 1309–1317

Coenen M, Antonicka H, Ugalde C et al (2004) Mutant mitochondrial elongation factor G1 and combined oxidative phosphorylation deficiency. N Eng J Med 351:2080–2086

Galmiche L, Serre V, Beinat M et al (2012) Toward genotype phenotype correlations in GFM1 mutations. Mitochondrion 12: 242–247

García-Cazorla A, De Lonlay P, Nassogne MC, Rustin P, Touati G, Saudubray JM (2005) Long-term follow-up of neonatal mitochondrial cytopathies: a study of 57 patients. Pediatrics 116: 1170–1177

Guan MX, Yan Q, Li X et al (2006) Mutation in TRMU related to transfer RNA modification modulates the phenotypic expression of the deafness-associated mitochondrial 12S ribosomal RNA mutations. Am J Hum Genet 79:291–302

Hagervall T, Edmonds C, McCloskey J, Björk G (1987) Transfer RNA (5-methylaminomethyl-2-thiouridine)-methyltransferase from Escherichia coli K-12 has two enzymatic activities. J Biol Chem 262:8488–8495

Kemp J, Smith P, Pyle A et al (2011) Nuclear factors involved in mitochondrial translation cause a subgroup of combined respiratory chain deficiency. Brain 134:183–195

Low E, Crushell E, Harty S, Ryan S, Treacy E (2008) Reversible multiorgan system involvement in a neonate with complex IV deficiency. Pediatr Neurol 39:368–370

Mandel H, Szargel R, Labay V et al (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat Genet 29:337–341

Naviaux R, Nguyen KV (2004) POLG mutations associated with Alpers' syndrome and mitochondrial DNA depletion. Ann Neurol 55:706–712

Rustin P, Chretien D, Bourgeron T et al (1994) Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 228:35–51

Sarzi E, Bourdon A, Chrétien D et al (2007) Mitochondrial DNA depletion is a prevalent cause of multiple respiratory chain deficiency in childhood. J Pediatr 150:531–534

Sasarman F, Antonicka H, Horvath R, Shoubridge E (2011) The 2-thiouridylase function of the human MTU1 (TRMU) enzyme is dispensable for mitochondrial translation. Hum Mol Genet 20: 4634–4643

Schara U, Von Kleist-Retzow JC, Lainka E et al (2011) Acute liver failure with subsequent cirrhosis as the primary manifestation of TRMU mutations. J Inherit Metab Dis 34:197–201

Spinazzola A, Viscomi C, Fernandez-Vizarra E et al (2006) MPV17 encodes an inner mitochondrial membrane protein and is mutated in infantile hepatic mitochondrial DNA depletion. Nat Genet 38:570–575

Umeda N, Suzuki T, Yukawa M et al (2005) Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases. J Biol Chem 280:1613–1624

Uusimaa J, Jungbluth H, Fratter C et al (2011) Reversible infantile respiratory chain deficiency is a unique, genetically heterogenous mitochondrial disease. J Med Genet 48:660–668

Valente L, Tiranti V, Marsano RM et al (2007) Infantile encephalopathy and defective mitochondrial DNA translation in patients with mutations of mitochondrial elongation factors EFG1 and EFTu. Am J Hum Genet 80:44–58

Yan Q, Bykhovskaya Y, Li R et al (2006) Human TRMU encoding the mitochondrial 5-methylaminomethyl-2-thiouridylate-methyltransferase is a putative nuclear modifier gene for the phenotypic expression of the deafness-associated 12S rRNA mutations. Biochem Biophys Res Commun 342:1130–1136

Zeharia A, Shaag A, Pappo O et al (2009) Acute infantile liver failure due to mutations in the TRMU gene. Am J Hum Genet 85: 401–407

JIMD Reports
DOI 10.1007/8904_2013_231

RESEARCH REPORT

Spondyloepiphyseal Dysplasias and Bilateral Legg-Calvé-Perthes Disease: Diagnostic Considerations for Mucopolysaccharidoses

Nancy J. Mendelsohn · Timothy Wood · Rebecca A. Olson · Renee Temme · Susan Hale · Haoyue Zhang · Lisa Read · Klane K. White

Received: 31 May 2012 / Revised: 08 March 2013 / Accepted: 10 April 2013 / Published online: 9 May 2013

Abstract Mucopolysaccharidosis type VI (MPS VI, Maroteaux-Lamy syndrome, MIM 253200) is an autosomal recessive lysosomal storage disease (LSD) caused by decreased activity of arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase) enzyme resulting in dermatan sulfate accumulation; mucopolysaccharidosis type IVA (MPS IVA, Morquio syndrome A, MIM 253000) by decreased activity of *N*-acetylgalactosamine 6-sulfatase enzyme resulting in accumulation of keratan sulfate. Clinical symptoms include coarse facial features, joint stiffness, hepatosplenomegaly, hip osteonecrosis, and dysostosis multiplex. MPS IVA symptoms are similar but with joint hypermobility.

With suspicion of MPS disease, clinicians request urine studies for quantitative and qualitative glycosaminoglycans (GAGs). Diagnosis is confirmed by decreased enzyme activity in leukocytes or cultured skin fibroblasts. Further confirmation is obtained with identification of two mutations in the *ARSB* gene for MPS VI or mutations in the *GALNS* gene for MPS IVA.

We report slowly progressing patients, one with MPS VI and two with MPS IVA, who presented with skeletal changes and hip findings resembling Legg-Calvé-Perthes disease or spondyloepiphyseal dysplasia and normal/near normal urine GAG levels. The urine analysis data presented suggest that present screening techniques for MPS are inadequate in milder patients and result in delayed or missed diagnoses. The patients presented in this paper emphasize the importance of enzymatic and molecular testing.

Communicated by: Eva Morava, MD PhD

Competing interests: None declared

N.J. Mendelsohn (✉) · R.A. Olson · R. Temme · L. Read
Department of Medical Genetics, Children's Hospitals and Clinics of Minnesota, 2525 Chicago Avenue S., CSC 560, Minneapolis, MN 55404, USA
e-mail: nancy.mendelsohn@childrensmn.org

N.J. Mendelsohn
Department of Pediatrics, Division of Genetics, University of Minnesota, Minnesota, MN, USA

T. Wood
Biochemical Genetics Lab, Greenwood Genetic Center, Greenwood, SC, USA

S. Hale
Department of Pediatrics, Division of Genetic Medicine, Biochemical Genetics Program, Seattle Children's Hospital, Seattle, WA, USA

H. Zhang
Department of Pediatrics, Medical Genetics Division, Duke University Medical Center, Durham, NC, USA

K.K. White
Department of Orthopedics and Sports Medicine, Seattle Children's Hospital, Seattle, WA, USA

Abbreviations

ARSB	Arylsulfatase B enzyme
ARSB	Arylsulfatase B gene
CDC	Center for Disease Control
ERT	Enzyme replacement therapy
GAG	Glycosaminoglycan
GALNS	*N*-acetyl-galactosamine-6-sulfatase enzyme
GALNS	*N*-acetyl-galactosamine-6-sulfatase gene
LCPD	Legg-Calvé-Perthes disease
LSD	Lysosomal storage disease
MPS	Mucopolysaccharidosis

MPS VI	Mucopolysaccharidosis type VI
MPS IVA	Mucopolysaccharidosis type IVA
OFC	Occipitofrontal circumference
SED	Spondyloepiphyseal dysplasia

Introduction

Mucopolysaccharidosis type VI (MPS VI, Maroteaux-Lamy syndrome, MIM 253200) was first described by physicians, Pierre Maroteaux and Maurice Lamy, in 1963 (Maroteaux et al. 1963). Mucopolysaccharidosis IVA (MPS IVA, Morquio syndrome, MIM 253000) was described by Luis Morquio in 1929 (Morquio 1929). Both disorders are inherited in an autosomal recessive fashion – MPS VI a deficiency of arylsulfatase B (*N*-acetylgalactosamine 4-sulfatase) and MPS IVA a deficiency of *N*-acetyl-galactosamine-6-sulfate sulfatase. The diseases are distinguished by the accumulation of dermatan sulfate in MPS VI and keratan sulfate for MPS IVA. The facial phenotype for children with MPS VI is more classic for MPS patients and severe with coarsening. The orthopedic involvement includes dysostosis multiplex, yet patients with MPS IVA have joint laxity in contrast to the other MPS diseases (Dorfman and Matalon 1976). Dermatan sulfate is not a component of the central nervous system. MPS VI patients characteristically have normal intellectual function (Valayannopoulos et al. 2010). Intelligence is normal for MPS IVA patients as well. MPS VI patients present along a spectrum of severity with younger more severe patients presenting earlier (Swiedler et al. 2005). Among 326 patients in the International Morquio A Registry, the mean age of onset of symptoms was 2.1 years, and the mean age at diagnosis is 4.7 years of age (Montano et al. 2007).

MPS diseases are rare with the overall incidence of MPS disorders ranging from 3.5 to 4.5 per 100,000 (Krabbi et al. 2012). The estimated incidence of MPS VI is 1 in 340,000 live births although it may be under recognized (Swiedler et al. 2005). The estimated incidence of MPS IV (type A) is 1 in 169,000 (Meikle et al. 1999).

Standard clinical screening for a patient suspected of an MPS disorder includes quantitative and qualitative urine studies to assess for the presence of glycosaminoglycans (GAGs). Diagnosis is established by evidence of reduced enzyme activity in isolated leukocytes or cultured skin fibroblasts (Wood et al. 2012). Further diagnostic confirmation is obtained by identification of two *ARSB* mutations for MPS VI or two *GALNS* mutations for MPS IV (Valayannopoulos et al. 2010).

We report three MPS patients who presented with skeletal changes resembling Legg-Calvé-Perthes disease (LCPD) and spondyloepiphyseal dysplasia (SED) and normal/near normal urine GAG quantitative levels. These cases emphasize the importance of diagnostic consideration of MPS disorders and address the risk of false-negative urine GAG results in this population.

Methods

Medical records were reviewed for each case. Pertinent information was collected including medical history, family history, and laboratory and DNA diagnostic analyses.

Urine Samples

Urine samples were collected during clinical evaluations with the exception of the series collected from Case 1. Samples were frozen after collection and remained stored frozen until use. No preservatives were used or mixed with the samples. Quantitative GAG testing was performed as described by de Jong (de Jong et al. 1989) and Dembure and Roesel (1991). Qualitative analysis was performed according to Hopwood and Harrison (1982). Tandem mass spectrometry measurements for urine GAGs were performed as described by Zhang et al. (2011) and Zhang (2012).

Enzyme Measurements

Measurement of GALNS and ARSB was performed as described by O.P. van Diggelen et al. (1990) and Baum et al. (1959), respectively. Measurements were performed in leukocytes, which were extracted from heparanized blood samples. A control enzyme was measured to confirm sample integrity, and a second sulfatase was measured to rule out multiple sulfatase deficiency.

Sequencing of the *ARSB* and *GALNS* genes was performed with Sanger sequencing. Primers were developed to amplify all coding exons and 15–50bp of flanking intronic sequence. Sanger sequencing of the coding regions was performed using standard protocols on an ABI 3730XL capillary sequencer. Data was analyzed using the Sequencher® version 5.0 alignment software (Gene Codes Corporation, Ann Arbor, MI).

Case Report

(See Table 1 for case presentations)

Case 1

The patient was referred at 10 years of age for evaluation for possible skeletal dysplasia. He presented with a diagnosis of bilateral LCPD (juvenile idiopathic avascular necrosis of

Table 1 Case presentations

	Case 1	Case 2	Case 3
Age at presentation	Presented to genetics clinic at 10 years	Presented to orthopedic clinic at 3 years	Presented to skeletal dysplasia clinic at 9 years
Age at diagnosis	12 years	9 years	10 years
Initial Clinical symptoms	Bilateral Legg-Calvé-Perthes	Congenital kyphosis with corrective surgery	Bilateral Legg-Calvé-Perthes
Xray Findings	Symmetric epiphyseal abnormalities of femurs, knees, ankles Flattened femoral condyles Irregularities of end plates of vertebral bodies with anterior wedging at several levels	Thoracolumbar gibbus Proximal femoral epiphyseal flattening	Mild scoliosis
1st Quantitative urine MPS	GAG 10.8 mg/mmol creatinine (normal 0-10)	GAG 7.3 mg/mmol creatinine (normal<6.8)	GAG 4.7 mg/mmol creatinine (normal<6.8)
Enzyme analysis	Leukocyte ARSB 21 nmol substrate released/h/ng protein (normal 59-369) Fibroblast ARSB enzyme 1.5 nmol/mg protein (normal control 150.1 nmol/mg protein)	GALNS 3.5 nm 4MU/h/mg/ protein (83-254)	GALNS 3.29 nm 4MU/h/mg protein (normal 83-254)
Molecular analysis	*ARSB* gene sequencing: homozygous p.Y210C (c.629A>G) missense mutation	*GALNS* gene sequencing: c.776G>A (p.R259Q) missense mutation c.901G>T (p.G301C) missense mutation	*GALNS* gene sequencing and deletion/duplication analysis: one c.901G>T (p.G301C) missense mutation

the proximal femoral head). On physical exam, growth parameters included a height of 143 cm (65% CDC growth chart), weight of 43.5 kg (92% CDC growth chart), and head circumference of 56.2 cm (95% CDC growth chart). He did not have coarse facial features, but his gums were mildly thickened. His abdomen was protuberant without hepatosplenomegaly. On forward bending, he had mild scoliosis. He had no joint abnormalities or stiffness. On neurologic exam, he had brisk reflexes at his knees and ankles with two to three beats of clonus at both ankles. Formal ophthalmologic exam was normal.

Hip x-rays revealed symmetric epiphyseal abnormalities of the femurs, knees, ankles, and flattened femoral condyles. Irregularities of the end plates of his vertebral bodies with anterior wedging at several levels were noted. An MRI of the spine showed spinal bony changes consistent with SED. MRI of the brain was normal.

Sequencing of *COL2A1* for SED and *COL11A2* for Stickler syndrome type III did not reveal deleterious mutations. Quantitative urine MPS was normal with 10.8 mg GAG/mmol creatinine (normal 0–12 mg GAG/mmol creatinine). Thin-layer chromatography identified trace amounts of dermatan sulfate.

At age 12 years, the patient continued to have hip pain with limited inward and outward rotation of his hips. He remained active despite hip discomfort. His exam was otherwise unchanged from the previous visit. Repeat urine MPS screening showed minimally elevated GAGs reported at 11.7 mg GAG/mmol creatinine (normal 0–10 mg GAG/mmol creatinine), and thin-layer chromatography again identified a trace amount of dermatan sulfate. Leukocyte ARSB enzyme activity was low (21 nmol substrate released/hr/ng protein, normal 59–369). Galactosamine-6-sulfatase activity was normal. Fibroblast activity of arylsulfatase B enzyme confirmed the deficiency at 1.5 nmol/mg protein (normal control 150.1 nmol/mg protein). *ARSB* gene sequencing revealed homozygous p.Y210C (c.629 A>G) mutation. Parents were confirmed to be carriers and reported no consanguinity. The patient's two healthy siblings are unaffected. Subsequently, quantitative and qualitative GAG analysis was performed on ten urine samples. Samples were collected at random times over 10 days. Total GAG levels ranged from 6.6 to 9 mg GAG/mmol creatinine, which was slightly lower than the previous two samples. Qualitative GAG analysis showed a mild increase in dermatan sulfate in four samples with no dermatan sulfatase detected in the remaining six samples. It is worth noting that the dermatan sulfate band corresponded to the upper dermatan band commonly noted in GAG electrophoresis. The lower band was not found in any sample. Multiple urine samples were also studied by UPLC-MS-MS using a recently developed protocol (Zhang et al. 2011; Zhang 2012). Dermatan sulfate levels were mildly elevated (see Table 2) with normal heparan sulfate levels. Chondroitin sulfate levels were mildly elevated in three of eight samples.

Further clinical evaluation included an echocardiogram, which revealed a mildly thickened aortic valve. Electrocardiogram, eye exam, hearing evaluation, and pulmonary function tests were normal. The patient completed a 3-minute stair

Table 2 The GAG concentrations of Case 1 repeat urine samples analyzed by LC-MS/MS

Sample ID	CS g/mol cr	DS g/mol cr	HS g/mol cr	Total GAGs mg GAG/mmol creatinine	Age at sample (years)
Normal range for age	**0–8.7**	**0–2.6**	**0–1.3**	**0–10**	**-**
1	8.5	3.8	0.8	7.5	13
2	8.9	3.8	0.6	6.6	13
3	10.2	3.5	0.5	9.0	13
4	7.8	3.2	0.3	8.7	13
5	7.8	4.2	0.5	7.9	13
6	5.3	3.4	0.4	8.6	13
7	7.8	3.9	1.0	7.7	13
8	4.7	2.1	0.3	8.2	13

climb and 12-min walk test without difficulty. A CT scan of the abdomen identified mild splenomegaly and normal liver volume. Growth velocity declined from the 75th percentile at age 10 to the 35th percentile at age 13. Enzyme replacement therapy (ERT) began at age 13. The dermatan sulfate concentration before ERT at age 13 was 3.7 mg GAG/mmol creatinine (average of seven time points), and decreased to 2.1 mg GAG/mmol creatinine after ERT (normal <2.8 mg GAG/mmol creatinine).

Case 2

A 2½-year-old female presented to the orthopedic clinic with "congenital kyphosis." Birth history was unremarkable with normal growth parameters: birth weight was 3,908 g (60% CDC growth chart), length 50.8 cm (63% CDC growth chart), OFC 35 cm (62% CDC growth chart). Parents reported no consanguinity, and there was no family history of skeletal dysplasia. The patient has one healthy, unaffected older sibling. By 3 years and 8 months of age, her weight was 15.1 kg (48% CDC growth chart), and her height was 96.5 cm (29% CDC growth chart). She had undergone an anterior and posterior spinal fusion at 19 months of age for progressive deformity. At 2½ years, parental concerns centered on prominent hardware, a waddling gait, and a decrease in mobility.

Her concurrent medical diagnoses included mild obstructive sleep apnea and moderate restrictive lung disease (by spirometry). She was noted, however, to have normal exercise tolerance and a normal echocardiogram.

Physical examination of the child revealed a thoracolumbar gibbus with prominent implants, pectus excavatum, and a grossly normal neurological exam. Radiographs of the spine revealed a deficient T12 vertebra with instrumentation from previous posterior fusion. The patient was followed for 9 months with evidence of progression of the kyphosis. At age 3 years, she underwent revision surgery for her kyphosis.

At age 3½ years, a genetic evaluation demonstrated the following pertinent findings: height was 96.5 cm (29% CDC growth chart), presence of a mildly high palate with normal uvula, and no evidence of corneal clouding. Additional films including chest and hand films were taken without recognition of dysostosis multiplex; however, there was evidence of proximal femoral epiphyseal flattening. An MRI scan showed a normal liver and spleen for age. A working diagnosis of SED was made. No urine GAG screen was performed at that time. Molecular testing of *COL2A1* for SED and *SEDL* for X-linked spondyloepiphyseal dysplasia tarda did not reveal deleterious mutations.

At 9 years of age, the patient returned with worsening pectus deformity and hip pain. In the interim, she had required tonsillar and adenoidal resection for obstructive apnea. Her height had fallen off the growth curve at 116.5 cm (<3% CDC growth chart). Repeat echocardiogram showed a mildly thickened mitral valve and mild tricuspid insufficiency. Spirometry revealed a vital capacity at 66% predicted. Further diagnostic testing included quantitative urine GAG levels that were mildly elevated at 7.3 mg GAG/mmol creatinine (normal <6.8). Leukocyte enzyme testing for GALNS was 3.5 nmol 4MU/hr/mg protein (83–254), consistent with a diagnosis of MPS IVA. Molecular analysis revealed c.776G>A (p.R259Q) missense and c.901G>T (p.G301C) missense mutations. Both mutations have previously been reported among individuals with MPS IVA (Kato et al. 1997; Tylki-Szymanska et al. 1998). Parental testing was not performed.

Case 3

This patient presented to skeletal dysplasia clinic at 9 years of age. His parents reported a limp since age 4 years and knee pain since age 5 years. At age 7 years, he was diagnosed with bilateral LCPD. Despite the limp and pain, he was a very active child, participating in soccer, hockey, and swimming. Family history was relevant for a father with a height of

175 cm, a history of "toxic synovitis" of the hip, which resolved (x-rays were reviewed and found to be normal), and a delayed pubescent growth spurt. Physical examination of the patient revealed a height of 125.2 cm (5.5% CDC growth chart), a weight of 27.1 kg (30% CDC growth chart), and an OFC of 54 (~70% CDC growth chart). On forward bend, he was found to have mild scoliosis but no gibbus. No other physical deformities were noted.

His quantitative urine GAG screen was normal at 4.7 mg GAG/mmol creatinine (normal <6.8). Repeat urine GAG screen was performed and was again normal at 5.9 mg GAG/mmol creatinine (normal <6.8). Molecular testing of *COL2A1* for SED, *COL11A1* for Stickler syndrome, and *SEDL* for X-linked spondyloepiphyseal dysplasia tarda did not reveal deleterious mutations. One year later, parents noted he was "developing chest wall deformity" and was having increasing complaints of hip pain. At this time, leukocyte testing for GALNS activity was 3.29 nm 4MU/hr/mg protein (normal 82–254) activity, confirming a diagnosis of MPS IVA.

Full sequencing of the coding regions of *GALNS* was completed, and one copy c.901G>T (p.G301C) was identified. Dosage studies of the *GALNS* gene were normal. Both parents had slightly decreased GALNS activities of 69.44 and 67.59 nm 4MU/h/mg protein (normal 76.1–255.1) but were not considered to be deficient.

The full sibling also had a decreased GALNS activity level of 69.44 nm 4MU/h/mg protein but did not have molecular testing. The lower level of GALNS activity in both parents and the sibling may be indicative of carrier status. Neither parent had molecular testing.

Further clinical evaluation included normal audiology and ophthalmology exams. There was no evidence of sleep apnea or upper airway obstruction. The patient also had a normal echocardiogram and electrocardiogram. MRI of the abdomen showed normal liver size. The spleen was subjectively slightly generous in size.

Discussion

Orthopedic changes including bilateral dysplastic hip disease and spondyloepiphyseal changes have long been recognized as presenting symptoms for MPS diseases (Alder 1939; Hecht et al. 1984). It is well established in the genetics metabolism community that the urine GAG levels may be inconsistent and vary with disease state or the intercurrent health of the MPS patient (de Jong et al. 1989; Gray et al. 2007). This paper describes three patients with attenuated disease and normal or near normal urine GAG levels. We discuss present urine screening methodology and suggest enzymatic testing is a crucial diagnostic test, particularly in slowly progressing patients.

Previously described attenuated cases are recognized in the literature. Dr. Victor A. McKusick described a 20-year-old patient in his textbook in 1972 with bilateral hip disease and corneal clouding who was later found to have ARSB deficiency (McKusick 1972). In 1982, Paterson et al. reported two cases of adolescents presenting with "Perthes like" appearance of the capital femoral epiphyses, noting similarities between MPS VI and spondyloepiphyseal dysplasia (Paterson et al. 1982). In 1991, Tønnesen et al. diagnosed a 33-year-old man with MPS VI who had initially presented to orthopedic surgeons at 6 years of age with bilateral hip pain, limited hip movement, limping gait, and fragmentation of the femoral heads. This patient had documented normal quantitative urine results with abnormal qualitative urine results detecting dermatan sulfate (Tønnesen et al. 1991). Gottwald et al. most recently have described an attenuated MPS VI patient who also presented with a phenotype similar to Case 1 described in this paper (Gottwald et al. 2011).

A slowly progressing individual with MPS VI has been defined clinically as a patient whose symptoms manifest primarily in a single organ system (Tønnesen et al. 1991). A cross-sectional survey of 121 untreated MPS VI patients defined slowly progressing patients to have total urine GAG levels below 100 microgram/mg creatinine and height >140 cm (Swiedler et al. 2005). Case 1 in this paper and those presented by Paterson, Tønnesen, and Gottwald in the literature meet the Swiedler criteria described as slowly progressing MPS VI.

Similarly, there are attenuated MPS IVA cases reported in the literature who have escaped diagnosis until adulthood. Fang-Kircher et al. describe a 51-year-old man diagnosed with MPS IVA who had previously been thought to have Perthes disease identified at 13 years of age (Fang-Kircher et al. 1995). Prat et al. describe a 38-year-old woman who had marked short stature, prognathism, short trunk and neck, kyphoscoliosis, genu valgum, and pes planus. She was not diagnosed until adulthood despite a history of multiple orthopedic surgical procedures as a child, including bilateral osteotomies, C1-C2 fusion, and T12-L3 fusion, a total knee replacement, and bilateral total hip replacement (Prat et al. 2008).

Dysostosis multiplex, the clinical and radiographic changes of MPS, overlap with other orthopedic conditions including bilateral LCPD and SED (Crossan et al. 1983; Andersen et al. 1988). This includes the platyspondyly, odontoid hypoplasia, and epiphyseal dysplasia (Montano et al. 2007). Common to all SED and MPS disorders are the symmetric, bilateral hip disease and spine changes described in the patients presented in this paper. The femoral head resorption seen in MPS has an appearance more in line with that seen in the epiphyseal dysplasias as compared to LCPD. In contrast to LCPD, the resorption is relentless and never reaches the reossification and remodeling stages seen in

LCPD. The onset and progression as manifested in these patients, with milder bony changes, is in keeping with their globally attenuated presentation.

Historically, there has been an appreciation of the difficulty in differentiating bilateral LCPD and epiphyseal dysplasia (Crossan et al. 1983; Andersen et al. 1988). The classic points of differentiation include timing of onset, extent of pathology, and progression of disease. In LCPD, the progression of osteonecrosis follows a fairly predictable course of stages as described by Waldenstrom: sclerosis, resorption, reossification, and remodeling (Kim 2011). The general belief is that epiphyseal dysplasia (including multiple epiphyseal dysplasia, Meyer's dysplasia, and spondyloepiphyseal dysplasia) presents in both hips simultaneously with similar stages of progression. The extent of osteonecrosis is thought to remain within the epiphysis and not involve or cross the physis. In contrast, bilateral LCPD is generally thought to present at different Waldenstrom stages of progression and often crosses the physis in the form of metaphyseal cysts. The fact that these relationships are not always consistent makes the use of these criteria in differentiating LCPD from a skeletal dysplasia suspect (Guille et al. 2002).

Patients with these skeletal changes are screened with urine GAG testing. Case 1, as well as two other MPS VI patients reported in the literature (Tønnesen et al. 1991; Gottwald et al. 2011), had unremarkable urine screening studies. Case 2 had slightly elevated quantitative urine GAG value at 7.7 mg GAG/mmol creatinine (normal <6.8). Both urine samples from Case 3 had urine GAG levels within normal range. It is widely accepted that quantitative urine GAG analyses are of limited utility as a screening tool for MPS disorders. Mucopolysaccharidosis III, MPS IVA as well as other types of MPS can yield false-negatives. It was unknown whether these problems were technical and related to the specific GAG of interest or actually reflected lower GAG levels (Stone 1998; Gray et al. 2007).

The analysis of urine from Case 1, using both dimethylmethylene-blue (DMB) dye and tandem mass spectrometry, suggests that the dermatan sulfate levels in this patient are mildly elevated, which would correlate with the milder presentation. The low levels noted here also highlight the difficulty in using urine GAG levels as screening tool with minimal elevations related to real disease.

Laboratory diagnosis of MPS IVA or MPS VI requires evidence of significantly reduced or absent *N*-acetylgalactosamine-6-sulfatase enzyme or ARSB enzyme activity, respectively, in isolated leukocytes or cultured skin fibroblasts. The presence of normal enzyme activity of a different sulfatase enzyme or sequencing analysis is important to exclude multiple sulfatase deficiency and is recommended for definitive diagnosis (Valayannopoulos et al. 2010).

Among patients with MPS VI, the p.Y210C mutation has been documented to produce approximately 3% of wild-type activity in in vitro studies, a level of activity that is much higher than other MPS VI mutations studied (Litjens et al. 1996). In agreement with its high residual activity, this mutation has been associated with an attenuated clinical phenotype and greater longevity (Karageoros et al. 2007). Despite the recognition of the p.Y210C mutation as the most common genetic change, Case 1 and that reported recently by Gottwald et al., are the only homozygous patients recognized to date (Gottwald et al. 2011). Both patients show a slowly progressing phenotype with minimal somatic involvement, mainly focused on joint and bony changes. Both patients showed borderline to normal urine GAG values with faint banding via qualitative GAG analysis (Gottwald et al. 2011). Furthermore, Case 1 is the first described patient with a homozygous p.Y210C whose urine GAGs were examined with the use of tandem mass spectroscopy which confirmed the mild elevation of dermatan sulfate. We emphasize the patients' clinical and laboratory information confirming the p.Y210C mutation in the homozygous state presents as a more mildly affected MPS VI patient and, as suggested by Gottwald et al. (2011), is likely under diagnosed.

Case 2 is a compound heterozygote for p.R259Q and p.G301C mutations. The p.R259Q mutation has previously been reported among other slowly progressing patients with MPS IVA including a two-generation MPS IVA family (Tylki-Szymanska et al. 1998). In the earlier report, parental carrier testing unexpectedly revealed p.R259Q homozygous mutations in the 33-year-old mother and two of her siblings.

Case 3 carried one copy of the p.G301C, one of the most prevalent gene mutations in MPS IVA. This change has been recognized in 6.8% of patients and is associated with severe disease manifestations (Bunge et al. 1997). The second mutation was not identified in Case 3. The lack of second mutation has previously been noted in 10–15% of MPS IVA cases (Tomatsu et al. 2005). There is either a modifier gene that has not been recognized or an intronic change that was not found by sequencing. Given his mild clinical presentation and enzyme activity level, we suggest the not yet identified second mutation is associated with an attenuated MPS IVA phenotype.

Enzyme analysis was unequivocally abnormal in all three patients presented here, providing better sensitivity than either urine GAG or molecular analysis. We suggest direct enzyme measurement may serve as the best diagnostic test for mild MPS patients, particularly when urine GAG analysis is normal or equivocal.

The patients reported here expand the literature for the slowly progressing phenotype of MPS VI and MPS IVA.

We highlight the orthopedic changes of these poorly recognized patients with MPS VI and IVA, presenting at a later age with minimal symptoms. The patients presented in this paper serve to further underscore the problems associated with using urine GAG levels as a sole method in screening for MPS diseases. Additionally, a broader understanding is needed of the sensitivity and specificity of urine GAG screening, highlighting the importance of enzymatic and molecular testing.

Take-Home Message

Attenuated MPS types VI and IVA may present with orthopedic concerns and normal or near normal quantitative GAG studies. A high index of clinical suspicion and careful enzyme analysis should be considered for early diagnosis.

Details of Contributions of Individual Authors

Nancy J. Mendelsohn, MD: Study idea conception, collection and interpretation of data, drafting and editing of manuscript

Timothy Wood, PhD, FACMG: Study idea conception, collection and interpretation of data, drafting and editing of manuscript

Rebecca A. Olson, RN, CNP, APNG: Study idea conception, collection and interpretation of data, drafting and editing of manuscript

Renee Temme, MS, CGC: Study idea conception, collection and interpretation of data, drafting and editing of manuscript

Susan Hale, MN, ARNP: Study idea conception, collection and interpretation of data, drafting and editing of manuscript

Haoyue Zhang, PhD: Quantification of GAGs, interpretation of data, drafting and editing of manuscript

Lisa Read, MPH: Collection and interpretation of data, drafting and editing of manuscript

Klane K. White, MD, MSc: Study idea conception, collection and interpretation of data, drafting and editing of manuscript

All authors read and approved of the final manuscript.

Author Who Serves as Guarantor

Nancy J. Mendelsohn, M.D.

Potential Conflict of Interest/Financial Disclosures

Nancy J. Mendelsohn received honorarium from BioMarin for travel, and receives grant funding from BioMarin. Tim Wood serves as a consultant for BioMarin. Klane White has received honoraria from BioMarin and Shire HGT, and receives grant funding from Biomarin.

Details of Funding

No outside funding was obtained for this case review.

Details of Ethics Approval

This case review did not require formal approval from an ethics committee as it did not incur any risk to the patients, and all information regarding the patients was de-identified.

Patient Consent Statement

Patient consent was not required for this study as it required only chart review and did not incur risk to the patients. All patient information was de-identified.

References

Alder A (1939) Ueber konstitutionell bedingte Granulationsveraenderungen der Leukocyten. Dtsch Arch Klin Med 183:372–378

Andersen PE Jr, Schantz K, Bollerslev J, Justesen P (1988) Bilateral femoral head dysplasia and osteochondritis. Multiple epiphyseal dysplasia tarda, spondylo-epiphyseal dysplasia tarda, and bilateral Legg-Perthes disease. Acta Radiol 29(6):705–709

Baum H, Dodgson KS, Spencer B (1959) The assay of arylsulphatases A and B in human urine. Clinica Chimica Acta 4:453–455

Bunge S, Kleijer WJ, Tylki-Szymanska A, Steglich C, Beck M, Tomatsu S et al (1997) Identification of 31 novel mutations in the N-Acetylgalactosamine-6-Sulfatase gene reveals excessive allelic heterogeneity among patients with Morquio A syndrome. Hum Mutat 10(3):223–232

Crossan JF, Wynne-Davies R, Fulford GE (1983) Bilateral failure of the capital femoral epiphysis: bilateral Perthes disease, multiple epiphyseal dysplasia, pseudoachondroplasia, and spondyloepiphyseal dysplasia congenita and tarda. J Pediatr Orthop 3:297–301

de Jong JG, Wevers RA, Laarakkers C et al (1989) Dimethylmethylene blue-based spectrophotometry of glycosaminoglycans in untreated urine: a rapid screening procedure for mucopolysaccharidoses. Clin Chem 35:1472–1477

Dembure PP, Roesel RA (1991) Screening for mucopolysaccharidoses by analysis of urinary glycosaminoglycans. In: Hommes F (ed) Techniques in diagnostic human biochemical genetics: a laboratory manual. Wiley-Liss, New York, pp 77–86

Dorfman A, Matalon R (1976) The mucopolysaccharidosis. Proc Natl Acad Sci USA 73:630

Fang-Kircher SG, Bock A, Fertschak W, Schwager W, Paschke E (1995) Morquio disease in a patient diagnosed as having Perthes disease for 38 years. J Inher Metab Dis 18:94–95

Gottwald I, Hughes J, Stewart F, Tylee K, Church H, Jones SA (2011) Attenuated mucopolysaccharidosis type VI (Maroteaux-Lamy syndrome) due to homozygosity for the pY210C mutation in the ARSB gene. Mol Genet Metab 103(3):300–302

Gray G, Claridge P, Jenkinson L et al (2007) Quantitation of urinary glycosaminoglycans using dimethylene blue as a screening

technique for the diagnosis of mucopolysaccharidoses: an evaluation. Ann Clin Biochem 44:360–363

Guille JT, Lipton GE, Tsirikos AI, Bowen JR (2002) Bilateral Legg-Calvé-Perthes disease: presentation and outcome. J Pediatr Orthop 22:458–463

Hecht JT, Scott CL Jr, Smith TK et al (Jun 1984) Mild manifestations of the Morquio syndrome. Am J Med Genet 18(2):369–371

Hopwood JJ, Harrison JR (1982) High-resolution electrophoresis of urinary glycosaminoglycans: an improved screening test for the mucopolysaccharidoses. Anal Biochem 119:120–127

Karageoros L, Brooks D, Pollard A et al (2007) Mutational analysis of 105 mucopolysaccharidosis type VI patients. Hum Mutat 28 (9):897–903

Kato Z, Fukuda S, Tomatsu S et al (1997) A novel common missense mutation G301C in the N-acetylgalactosamine-6-sulfate sulfatase gene in mucopolysaccharidosis IVA. Hum Genet 101(1):97–101

Kim HK (2011) Legg-Calvé Perthes disease: etiology, pathogenesis, and biology. J Pediatr Orthop 31(Supp 2):S141–S146

Krabbi K, Joost K, Zordania R et al (2012) The live-birth prevalence of mucopolysaccharidoses in Estonia. Genet Test Mol Biomarkers 16(8):846–849 (Epub ahead of print)

Litjens T, Brooks DA, Peters C, Gibson GJ, Hopwood JJ (1996) Identification, expression, and biochemical characterization of N-acetylgalactosamine-4-sulfatase mutations and relationship with clinical phenotype in MPS-VI patients. Am J Hum Genet 58:1127–1134

Maroteaux P, Leveque B, Marie J, Lamy M (1963) A new dysostosis with urinary elimination of chondroitin sulfate B. Presse Med 71:1849–1852

McKusick VA (1972) Heritable disorders of connective tissue. C.V. Mosby, St. Louis, MO, pp 525, 611

Meikle PJ, Hopwood JJ, Clague AE, Carey WF (1999) Prevalence of lysosomal storage disorders. JAMA 281:249–254

Montano AM, Tomatsu S, Gottesman GS, Smith M, Orii T (2007) International Morquio a registry: clinical manifestations and natural course of Morquio A disease. J Inherit Metab Dis 30:165–174

Morquio L (1929) Sur une forme de dystrophie osseuse familial. Arch de médecine des infants 32:129–135

Paterson DE, Harper G, Weston HJ et al (1982) Maroteaux Lamy syndrome, mild form – MPS VI B. Br J Radiol 55:805–812

Prat C, Lemaire O, Bret J, Zabraniecki L, Fournie B (2008) Morquio syndrome: diagnosis in an adult. Joint Bone Spine 75:495–498

Stone J (1998) Urine analysis in the diagnosis of mucopolysaccharide disorders. Ann Clin Biochem 35(Pt 2):207–225

Swiedler SJ, Beck M, Bajbouj M et al (2005) Threshold effects of urinary glycosaminoglycans and the walk test as indicators of disease progression in a survey of subjects with mucopolysaccharidosis VI (Maroteaux-Lamy syndrome). Am J Med Genet 134A:144–150

Tomatsu S, Montano AM, Nishioka T et al (2005) Mutation and polymorphism spectrum of the GALNS gene in mucopolysaccharidosis IVA (Morquio A). Hum Mutat 26:500–512

Tønnesen T, Gregersen HN, Guttler F (1991) Normal MPS excretion, but dermatan sulphaturia, combined with a mild Maroteaux Lamy phenotype. J Med Genet 28:499–501

Tylki-Szymanska A, Czartoryska B, Bunge S et al (1998) Clinical, biochemical and molecular findings in a two-generation Morquio A family. Clin Genet 53(5):369–374

Valayannopoulos V, Nicely H, Harmatz P, Turbeville S (2010) Mucopolysaccharidosis VI. Orphanet J Rare Dis 5:5

van Diggelen OP, Zhao H, Kleijer WJ et al (1990) A fluorimetric enzyme assay for the diagnosis of Morquio disease type A. Clin Chem Acta 187:131–140

Wood T, Bodamer OA, Burin MG et al (2012) Expert recommendations for the laboratory diagnosis of MPS VI. Mol Genet Metab 106(1):73–82

Zhang H (2012) Analysis of glycosaminoglycans in CSF and urine using tandem mass spectrometry: Potential for therapeutic monitoring of patients with mucopolysaccharidoses. Jrnl Amer Soc Mass Spec 22(1):66

Zhang H, Young SP, Auray-Blais C, Orchard PJ, Tolar J, Millington DS (2011) Analysis of glycosaminoglycans in cerebrospinal fluid from patients with mucopolysaccharidoses by isotope-dilution ultra-performance liquids chromatography-tandem mass spectrometry. Clin Chem 57(7):1005–1012

JIMD Reports
DOI 10.1007/8904_2013_232

CASE REPORT

Severe Neonatal Metabolic Decompensation in Methylmalonic Acidemia Caused by CblD Defect

R. Parini · F. Furlan · A. Brambilla · D. Codazzi · S. Vedovati · C. Corbetta · T. Fedeli · B. Merinero · B. Pérez · M. Ugarte

Received: 29 December 2012 / Revised: 02 April 2013 / Accepted: 10 April 2013 / Published online: 19 May 2013

Abstract CblD disorder is an autosomal recessive, rare, heterogeneous disease with variable clinical presentations, depending on the nature and location of the *MMADHC* gene mutations. Mutations in *MMADHC* lead to three distinct phenotypes: cblD-MMA, cblD-HC, and cblD-MMA/HC. To date, 18 cblD patients have been reported. Six of them were affected by cblD-MMA, but only three had a known clinical history. One of these patients presented with a metabolic decompensation at 11 months; the second one, born prematurely, was diagnosed with cblD after being treated for intracranial hemorrhage, respiratory distress syndrome, necrotizing enterocolitis, and convulsions at birth; the third one was diagnosed at 5 years of age.

Here we present a case of a cblD-MMA patient who had an acute neonatal onset with severe hyperammonemia requiring hemodiafiltration. To the best of our knowledge, this is the first cblD-MMA patient who presented acutely in the newborn period. He has developed well upon treatment with B_{12}, carnitine, and hypoproteic diet. At present time, at the age of 7, he shows normal growth and cognitive development. Thus, it is likely that the aggressive treatment of this child with hemodiafiltration might have prevented him from long-term neurological sequelae. Overall, this case shows that even severe, neonatal-onset patients may display a vitamin B_{12}-responsive MMA. Furthermore, it suggests that an early treatment with vitamins might be beneficial for patients presenting with neonatal-onset hyperammonemia regardless of the suspected disease and before receiving the biochemical diagnosis.

Abbreviations

Ado-Cbl	Adenosylcobalamin
Cbl	Cobalamin
HC	Homocystinuria
Met-Cbl	Methylcobalamin
MLS	Mitochondrial leader sequence
MMA	Methylmalonic aciduria
MMADHC	Methylmalonic aciduria cblD type with homocystinuria gene

Introduction

CblD disorder (OMIM 277410) is one of the seven known defects of the intracellular cobalamin (Cbl) metabolism which serves for the production of two coenzymes through two different pathways: one leading to the synthesis of adenosylcobalamin (Ado-Cbl), coenzyme of the mitochondrial methylmalonyl-CoA mutase (EC 5.4.99.2), and the other to the synthesis of methylcobalamin (Met-Cbl), a coenzyme of the cytosolic methionine synthase

Communicated by: Viktor Kožich

Competing interests: None declared

R. Parini (✉) · F. Furlan · A. Brambilla · T. Fedeli
Rare Metabolic Diseases Unit and Neonatal Intensive Care Unit, Fondazione MBBM, A.O. San Gerardo, Via Pergolesi 33, 20900, Monza, Italy
e-mail: rossella.parini@unimib.it

D. Codazzi · S. Vedovati
Pediatric Intensive Care Unit, A.O. Ospedali Riuniti di Bergamo, Bergamo, Italy

C. Corbetta
Laboratory for Neonatal Screening and Metabolic Diseases, Ospedale Buzzi, A.O. Istituti Clinici di Perfezionamento, Milan, Italy

B. Merinero · B. Pérez · M. Ugarte
CEDEM, CBM-SO, CIBERER, Universidad Autónoma Madrid, Madrid, Spain

(EC2.1.1.13). Only the initial steps are common to both pathways (Watkins and Rosenblatt 2011). Patients with defects in the common pathway (cblF, cblC, and cblD-MMA/HC) have methylmalonic aciduria (MMA) and homocystinuria (HC) and most frequently present with neurological and hematological problems. Patients with an aberrant Ado-Cbl pathway (cblA, cblB, and cblD-MMA) are usually characterized by methylmalonic aciduria (MMA), hyperammonemia, ketosis, and metabolic acidosis. Those ones characterized by a defective Met-Cbl pathway (cblD-HC, cblE, cblG) have only isolated homocystinuria and mainly show neurological symptoms and megaloblastic anemia (Watkins and Rosenblatt 2011).

CblD is a unique, apparently very rare, genetic condition due to mutations of a protein with two distinct functional domains, which interact with either cytosolic or mitochondrial targets (Stucki et al. 2012). Mutations of this gene, named *MMADHC* and identified in 2008 by Coelho et al. (2008), may lead to three different phenotypes on the basis of the nature and location of the mutation: those affecting the N-terminal domain of the protein result in cblD-MMA, while those affecting the C-terminal domain of the protein cause cblD-HC. Furthermore, mutations decreasing the level of the protein result in cblD-MMA/HC (Coelho et al. 2008; Miousse et al. 2009). Recently, Stucki et al. (2012) have confirmed that a single protein does exist and interacts with both cytosolic and mitochondrial substrates. Moreover, the same authors have shown that there is a delicate balance between Met-Cbl and Ado-Cbl synthesis depending on the relative strength of the mitochondrial leader sequence (MLS). In this regard, the first 61 amino acids of the protein are required to target the cblD protein to the mitochondria (MLS), whereas the synthesis of Ado-Cbl requires an intact sequence downstream from Met62, and synthesis of Met-Cbl needs only an intact sequence downstream from Met116.

Here, we report, for the first time, the case of a cblD-MMA patient who had a severe acute onset in the neonatal period and was then followed up for 7 years with a favorable evolution.

Case Report

The patient is a male, born at term (39 + 5 gestational age) after a normal pregnancy and delivery. He is the second child of healthy, first-degree cousin parents from Indian Punjab. Their first child, a girl, is healthy. Amniotic fluid at birth was meconium stained. Birth weight was 3,250 g and Apgar score 7–10.

At 4 days of life, the clinical examination revealed hypotonia, hyporeactivity, polypnea, and weight loss >10% (2,800 g). Blood tests showed metabolic acidosis (pH7.32, BE-14) and a positive C-reactive protein. In the suspicion of sepsis, intravenous treatment with ampicillin, gentamicin, ceftazidime, and bicarbonates was started. Nevertheless, his clinical conditions worsened and at 6 days he had severe respiratory distress and progressive neurological involvement with hypo-/hypertonia, myoclonic jerks, and coma. Blood tests showed severe hyperammonemia (1190 μmol/L), hyperlactacidemia (6 mmol/L), pH 7.35, BE-8, and ketonuria (Ketostix ++++). An intoxication type metabolic disease was suspected and immediately an intravenous treatment with glucolipidic calories (100 Kcal/kg) was started in combination with a loading IV dose of arginine 250 mg/kg and sodium benzoate 250 mg/kg in 2 h, followed by a maintenance of 250 mg/kg/24 h of both, associated to IV administered carnitine 100 mg/kg/day and hydroxocobalamin 1 mg/day. Hemodiafiltration was started 6 h later when plasma ammonia increased to 1348 μmol/L. After 24 h, ammonia was normalized (53 μmol/L). Specific biochemical tests were performed before hemodiafiltration during the 6 h of IV treatment and their results were received after 24 h. The plasma aminoacidogram showed depletion of threonine (54 μmol/L; ref.range 141–213), isoleucine (8 μmol/L; ref.range 31–47), phenylalanine (28 μmol/L; ref.range 45–65) and alanine (135 μmol/L; ref. range 239–345); glycine (205 μmol/L; ref.range 178–248), and glutamine (283 μmol/L; ref.range 243–809) were in the low-normal range; ornithine (523 μmol/L; ref.range 39–61), arginine (409 μmol/L; ref.range 53–71), and lysine (293 μmol/L; ref.range 107–163) were found increased. Total homocysteine was 6 μmol/L (ref.range 3–15). Urinary orotic acid was absent. Urinary organic acid analysis showed elevated urinary lactate (1157 mmol/mol creatinine) and MMA (5875 mmol/mol creatinine; ref.range <2). Treatment with carnitine and hydroxocobalamin was continued while arginine and sodium benzoate were discontinued. A hypoproteic diet through nasogastric drip feeding (4 g protein/day) was started and then continued by mouth. In the subsequent days, his neurological features improved with normalization of muscle tone, development of normal neonatal reflexes, and good suction. When the patient was discharged at 3 months of life, he was in good general conditions with a weight of 4.860 kg and a natural protein intake of 5.1 g/day (577 Kcal).

In vitro studies showed that propionate incorporation in fibroblasts was low (0.13 nmol/10 h/mg protein; ref.range 1.94 ± 1.21) and normalized after OHCbl incubation (2.30 nmol/10 h/mg protein; ref.range 2.29 ± 1.51). MMA CoA mutase activity was normal (0.89 nmol/min/mg prot; ref. range 0.89 ± 0.43) and complementation analysis excluded cblA and cblB defects. Molecular analysis of the *MMADHC* gene showed a homozygous mutation (c.57-64del8). Analysis of parents' DNA confirmed that the mutations were in separate alleles. This mutation has been

previously identified in homozygosity in two other patients and functionally characterized (Stucki et al. 2012).

Protein intake was maintained restricted with progressive slow increase on the basis of MMA plasma and urinary concentrations. MMA levels never decreased to normal values although incorporation of propionate after addition of hydroxocobalamin was normal in vitro. Hydroxocobalamin was administered daily. During the first 5 months of his life, it was given as a single IM injection once every 7 days and orally once a day in the following 6 days. After the age of 5 months, it was given orally every day. In the subsequent years, we did not go back to IM administration because the patient showed plasma B_{12} levels at periodic evaluations always between 1,800 and 4,000 pg/ml, well over the normal range (191–663).

At his last examination at the age of 7 years, weight was 29 kg (90th–97th) and height was 123 cm (50th–75th). He never showed metabolic decompensations and was never anorectic. During the first 6 years, MMA plasma values were always below 140 μmol/L (ref.range 0.04–4.00) with the exception of a value of 213 μmol/L at the age of 30 months, associated to a high urinary level of 3,000 mmol/mol creatinine (n.v. <2). In the last year, plasma and urinary values have increased mildly (Fig. 1).

At present, he is being treated with carnitine 100 mg/kg/day and hydroxocobalamin 1 mg/day orally. His natural protein intake is formally 12 g/day (0.4 g/kg/day), but we have reasons to suspect that the family does not observe a strict dietary compliance. Brain MRI performed just after diagnosis at 2 months of age showed bilateral hyperintensity of the caudate nuclei (Fig. 2a, b). At 7 years of age, neurological examination is normal, he walks, runs, and jumps; the administration of the WISC III Scale of Intelligence at 7 years of age showed normal performance subscales (performance IQ 90) and reduced verbal scores (verbal IQ 63) because of the language barrier due to the Indian origin; a nonverbal intelligence test (Leiter-R) was then administered and it showed a normal and homogeneous profile with total IQ 96. Brain MRI was repeated at 7 years and was found to be normal (Fig. 2c, d).

Discussion

To the best of our knowledge only 18 cblD patients have been reported in the literature so far (6 cblD-MMA/HC, of whom two were brothers, 6 cblD-HC and 6 cblD-MMA) (Goodman et al. 1970; Cooper et al. 1990; Suormala et al. 2004; Coelho et al. 2008; Miousse et al. 2009; Stucki et al. 2012). The clinical history of the seven most recently reported patients is not known in detail (Stucki et al. 2012). The age of diagnosis of the other 11 patients (5 cblD-MMA/HC, 3 CblD-HC, 3 cblD-MMA) ranged from 22 days, for a cblD-MMA/HC patient presenting with poor feeding and seizures, to 14 years, for another cblD-MMA/HC patient presenting with psychotic crisis. One of the three cblD-MMA patients had an acute onset with decompensation at 11 months (Cooper et al. 1990). The second one presented with self-limited metabolic acidosis in the first days of life and survived without diagnosis until 5 years of age, when he was evaluated because of vomiting and muscle weakness, and eventually diagnosed and treated (Miousse et al. 2009). The third patient (Suormala et al. 2004) had a complicated history characterized by low gestational age (32 weeks), intracranial hemorrhage, respiratory distress syndrome, necrotizing enterocolitis, and convulsions at birth; neither the age of MMA diagnosis, nor the starting age of the treatment with carnitine, hydroxocobalamin, and protein restriction are known. The treatment led to normalization of EEG traces and reduced urinary MMA. Interestingly, this patient shares with our case the same geographic origin and the same genotype. They both have a clinically isolated MMA presentation and show an apparent response to B_{12}. Another patient with the same genotype has been reported by Stucki et al. (2012), but the clinical picture is not known. These authors studied both their patient's cell lines and those of Suormala's patient and found that these cells could not normalize the adenosylcobalamin synthesis not even upon transfection with either cblD wild type or a more efficient construct (cblD_MLS_ALDH2), in which the MLS targets the mitochondria more efficiently than the wild type. This effect was negatively correlated with the levels of *MMADHC* mRNA found in the cells. Thus they speculated that in this experimental condition the shorter protein may interfere with the formation of functional complexes needed for AdoCbl synthesis or that, alternatively, it might interact with full-length protein or with binding partners located in the cytosol or at the mitochondrial membrane.

The clinical presentation of our patient was suggestive of a sepsis. The presence of meconium-stained amniotic fluid and a positive C-reactive protein further confirmed the suspicion. The very high hyperammonemia might be indicative of a urea cycle disorder, while the age of onset (4–6 days) together with the associated metabolic acidosis and ketosis were instead more suggestive of organic aciduria. Hemodiafiltration was started when plasma ammonia had not decreased after 6 h of conventional treatment and the risk of further deterioration with permanent neurological damage or death became possible. Due to the acute-onset conditions, we never formally tested this patient's responsiveness to B_{12}. However, his clinical evolution, showing no acute decompensations in 7 years, and the detection of MMA levels in plasma and urine comparable to those found in vitamin B_{12} deficiency rather than in B_{12}-unresponsive MMA (Fowler et al. 2008), led us to believe that this patient was at least partly responsive to vitamin B_{12}, in vivo. Although propionate

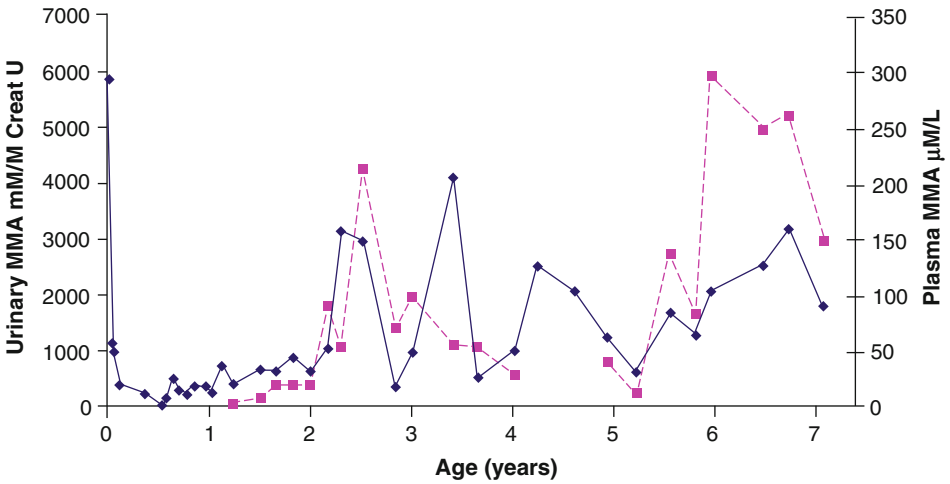

Fig. 1 Urinary (*solid line*) (n.v. < 2 mmol/mol creatinine) and plasma (n.v. 0.04–4.00 μmol/L) (*broken line*) MMA during the 7 year follow-up

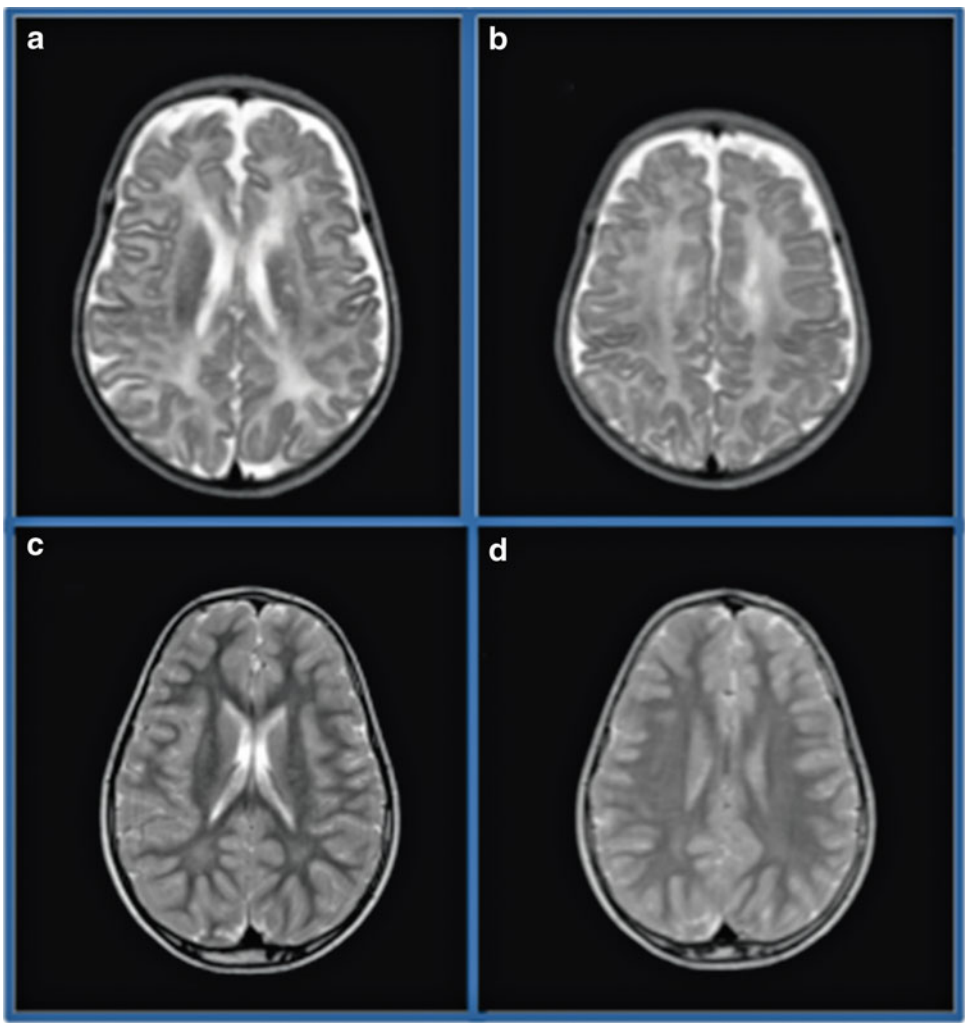

Fig. 2 Bilateral hyperintensity of the caudate nuclei in brain MRI at 2 months of life (**a**, **b**) and at 7 years (**c**, **d**). The hyperintensity of the heads (**a**) and the tails of the caudate nuclei (**b**) is absent at the age of 7 years (**c**, **d**)

incorporation was completely normalized in vitro after the addition of OHCbl, it is possible that his in vivo responsiveness was only partial thereby promoting residual MMA production. In this regard, also the patient reported by Suormala et al. (2004) was treated with protein restriction and still had a residual excretion of MMA. Likewise, also this patient's family compliance had not been optimal.

Of the three cblD-MMA patients that have been reported in the literature (Cooper et al. 1990; Suormala et al. 2004; Miousse et al. 2009), none of them presented with an acute neonatal decompensation with severe hyperammonemia and coma comparable to what we have found in our patient. They presented with a late-onset condition, probably more consistent with a vitamin-responsive disorder. In this regard, it is a common knowledge that B_{12}-responsive MMA patients have a milder later onset and very rarely present in the neonatal age (Manoli and Venditti 2010; Burlina et al. 1999). This notion might lead neonatologists to underestimate the importance of a prompt vitamin treatment of the newborn with an acute metabolic presentation, before having made a specific diagnosis. However, a number of patients with B_{12}-responsive MMA, who presented severely in the neonatal age, have also been reported (Hori et al. 2005). Thus, taken all together, this case argues in favor of starting an early vitamin treatment in patients presenting with a neonatal-onset hyperammonemia independently of the suspected disease and before having received a specific biochemical diagnosis. Neonatal screening for methylmalonic acidemia – including mutase deficiency and Cbl defects associated with increased MMA plasma levels – is performed in most European and US expanded neonatal screening programs (Loeber et al. 2012; Sun et al. 2012). The importance of such programs is once more highlighted by our findings showing a favorable and long-term outcome of a CblD-MMA patient with acute neonatal onset. It is likely that our patient could have avoided the hyperammonemic coma and hemodiafiltration, if he had undergone early screening. The evolution of this patient was quite favorable without any noticeable handicap at the age of 7 years. We strongly believe that both early B_{12} administration and aggressive treatment with hemodiafiltration prevented him from long-term neurologic sequelae.

Acknowledgments We acknowledge Fondazione Pierfranco e Luisa Mariani, Milano, for their generous support to our clinical activities and Dr. Marcello Arsura, American Business English School, Novara, Italy, for the English editing of the text.

Synopsis

CblD defect with unusual acute neonatal onset requiring hemodiafiltration.

References

Burlina AB, Bonafè L, Zacchello F (1999) Clinical and biochemical approach to the neonate with a suspected inborn error of amino acid and organic acid metabolism. Semin Perinatol 23:162–173

Coelho D, Suormala T, Stucki M et al (2008) Gene identification for the cblD defect of vitamin B_{12} metabolism. N Engl J Med 358:1454–1464

Cooper BA, Rosenblatt DS, Watkins D (1990) Methylmalonic aciduria due to a new defect in adenosylcobalamin accumulation by cells. Am J Hematol 34:115–120

Fowler B, Leonard JV, Baumgartner MR (2008) Causes of and diagnostic approach to methylmalonic acidurias. J Inherit Metab Dis 31:350–360

Goodman SI, Moe PG, Hammond KB et al (1970) Homocystinuria and methylmalonic aciduria: two cases in a sibship. Biochem Med 4:500–515

Hori D, Hasegawa Y, Kimura M et al (2005) Clinical onset and prognosis of Asian children with organic acidemias, as detected by analysis of urinary organic acids using GC/MS, instead of mass screening. Brain Dev 27:39–45

Loeber GJ, Burgard P, Cornel MC et al (2012) Newborn screening programmes in Europe; arguments and efforts regarding harmonization. Part 1 - From blood spot to screening result. J Inherit Metab Dis 35:603–611

Manoli I, Venditti CP (2010) Methylmalonic Acidemia. In: Pagon RA, Bird TD, Dolan CR, Stephens K (eds) GeneReviews [Internet]. University of Washington, Seattle, 1993–2005 [updated 2010 Sep 28]

Miousse IR, Watkins D, Coelho D et al (2009) Clinical and molecular heterogeneity in patients with the cblD inborn error of cobalamin metabolism. J Pediatr 154:551–556

Stucki M, Coelho D, Suormala T et al (2012) Molecular mechanisms leading to three different phenotypes in cblD defect of intracellular cobalamin metabolism. Hum Mol Genet 21(6):1410–1418

Sun A, Lam C, Wong DA (2012) Expanded newborn screening for inborn errors of metabolism: overview and outcomes. Adv Pediatr 59:209–245

Suormala T, Baumgartner MR, Coelho D et al (2004) The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. J Biol Chem 279:42742–42749

Watkins D, Rosenblatt DS (2011) Inborn errors of cobalamin absorption and metabolism. Am J Med Genet C Semin Med Genet 157:33–44

JIMD Reports
DOI 10.1007/8904_2013_233

RESEARCH REPORT

Socio-emotional Problems in Children with CDG

K.F.E. van de Loo • L. van Dongen • M. Mohamed • T. Gardeitchik • T.W. Kouwenberg • S.B. Wortmann • R.J.T. Rodenburg • D.J. Lefeber • E. Morava • C.M. Verhaak

Received: 27 February 2013 / Revised: 05 April 2013 / Accepted: 12 April 2013 / Published online: 4 June 2013

Abstract *Background*: Congenital disorders of glycosylation (CDG) form a group of inherited metabolic diseases. Although the clinical presentation shows extreme variability, the nervous system is frequently affected. Several parents of our patients diagnosed with CDG reported behavioral problems, including mood swings, depressive behavior, and anxiety. This raised the question whether patients with CDG have an increased risk for socio-emotional problems.

Methods: We evaluated 18 children with confirmed CDG. The Child Behavior Checklist (CBCL) was used to screen for socio-emotional problems. To determine the disease progression and severity in CDG, the Nijmegen Paediatric CDG Rating Scale (NPCRS) was used. Results were compared to "norm scores" and to children with mitochondrial disorders and children with other chronic metabolic disorders with multisystem involvement.

Results: Results showed a high prevalence of socio-emotional problems in children with CDG. Mean total scores, scores on withdrawn/depressed behavior, social problems, and somatic complaints were significantly increased. More than two thirds of our CDG patients have abnormal scores on CBCL. The mean score on social problems was significantly higher compared to our two control groups of patients with other chronic metabolic disorders.

Conclusions: Patients with CDG have an increased risk of developing socio-emotional problems. A standard screening for psychological problems is recommended for the early detection of psychological problems in CDG patients.

Communicated by: Verena Peters
Competing interests: None declared

Authors van de Loo, van Dongen contributed equally;
Authors Morava and Verhaak contributed equally
We state no conflict of interest and the authors are all in agreement with the submission of the manuscript

K.F.E. van de Loo · C.M. Verhaak
Department of Medical Psychology, Radboud University Medical Centre, Nijmegen, The Netherlands

L. van Dongen · M. Mohamed · T. Gardeitchik · T.W. Kouwenberg · S.B. Wortmann · R.J.T. Rodenburg
Department of Pediatrics, Institute for Genetic and Metabolic Disease, Radboud University Medical Centre, Nijmegen, the Netherlands

M. Mohamed · T. Gardeitchik · T.W. Kouwenberg · S.B. Wortmann · R.J.T. Rodenburg · D.J. Lefeber · E. Morava
Laboratory of Genetic Endocrine and Metabolic Diseases, Institute for Genetic and Metabolic Disease, Radboud University Medical Centre, Nijmegen, the Netherlands

D.J. Lefeber
Department of Neurology, Institute for Genetic and Metabolic Disease, Radboud University Medical Centre, Nijmegen, the Netherlands

E. Morava (✉)
Department of Pediatrics, Institute for Genetic and Metabolic Disease, Hayward Genetics Centre, Tulane Medical School, New Orleans, Louisiana, USA
e-mail: emoravakozicz@tulane.edu

E. Morava
Department of Pediatrics, Radboud University Medical Centre, Nijmegen, the Netherlands

Abbreviations

CBCL	Child Behavior Checklist
CDG	Congenital disorders of glycosylation
MCD	Mitochondrial disease
NPCRS	Nijmegen Paediatric CDG Rating Scale
NPMDS	Newcastle Paediatric Mitochondrial Disease Scale

Introduction

Glycosylation is a biologically essential enzymatic multi-step process, which has impact on the folding, secretion, transport, and function of proteins and lipids (Freeze 2006;

Jaeken 2010). Defects in the glycosylation cause congenital disorders of glycosylation (CDG), which is now approaching 50 different defects (Jaeken 2010; Jaeken et al. 2009; Theodore and Morava 2011). Each disorder is called after the corresponding gene symbol followed by "-CDG".

PMM2-CDG (CDG-Ia), MPI-CDG (CDG-Ib), and ALG6-CDG (CDG-Ic) are the most common reported types of CDG, with over 700 patients, over 20 patients, and over 30 patients affected worldwide, respectively. Other CDG subtypes are rare and in some cases only reported in a single patient (Mohamed et al. 2011; Sparks and Krasnewich 2005).

Because of the ubiquitous requirement of glycoproteins, CDGs usually present as a multisystem disease. Neurologic involvement, failure to thrive, liver dysfunction, coagulation abnormalities, and dysmorphic features are frequent. Typical CDG type I features include inverted nipples, fat pads, low serum thyroxin-binding globulin, and strabismus. Neurological involvement is common and differs in severity in CDG-I patients. Some CDG subtypes present with specific symptoms. For example, MPI-CDG is characterized by hepatic-intestinal problems (Liem et al. 2008), and ATP6V0A2-CDG (cutis laxa type IIA) and COG7-CDG patients often show cutis laxa (Morava et al. 2007, 2009a, b).

In medical literature, patients with CDG are generally described as very social extrovert children, of warm character (Grünewald 2009). However, this hypothesis is based on observations and has never been studied in diverse forms of CDG. Until now, there is no literature available regarding the socio-emotional functioning in patients with CDG. In our own patient group, however, socio-emotional problems, including mood swings, depressive behavior, or possible anxiety, have been reported by parents. This raised the question whether patients with CDG have an increased risk of socio-emotional problems like mood disorders and social problems.

The prevalence of socio-emotional problems in children with chronic diseases is higher compared to healthy children. In the Netherlands, at least 14 % of the children suffer from a chronic disease (Mokkink et al. 2007). Literature shows that children with chronic diseases show more internalizing problems like socio-emotional problems and depressive symptoms (Boekaerts and Röder 1999; Evans et al. 2005). This is also described in metabolic and endocrine diseases. Children with a genetic cause of hyperandrogenism, phenylketonuria, and type 1 diabetes show more socio-emotional problems than healthy controls (Mueller et al. 2010; Northam et al. 2006; Waisbren and White 2010). Endocrine disorders like hypothyroidism can manifest with depression as a first symptom (Davis and Tremont 2007). Furthermore, children with mitochondrial disorders show a higher rate of withdrawn, depressive behavior, compared to norm scores and children with other metabolic disorders (Koene et al. 2009; Morava et al. 2010). These results suggest that depression is more likely the result of the mitochondrial disorder itself, rather than due to the fact that this metabolic disorder is a chronic disease.

The aim of this study is to assess if children with CDG have more socio-emotional problems compared to norms and if these problems are inherent to CDG. Based on the literature and observations in the clinical practice, it is expected that children with CDG show more internalizing problems compared to healthy children and compared to children with other chronic diseases. To test this hypothesis, we compared children with CDG to population norm scores and to children with other chronic metabolic diseases with multisystem presentation, to evaluate whether the symptoms can be partially due to a chronic disease. We also compared the children with CDG to patients with mitochondrial disorders, who have been shown to present with inherent psychological problems.

Methods

For this study, we included patients with a biochemically and genetically confirmed diagnosis of congenital disorders of glycosylation aged 1 to 18 years. Patients with different subtypes of CDG were included in the study (Table 1). The study was conducted upon initiative from several parents attending a CDG patient/parent information day. Subsequently, CBCL screening and psychological evaluation and consult were offered to 26 of our patients of pediatric age. Eighteen patients responded to the interviews. In all 18 patients, a psychological screening was performed using the Child Behavior Checklist (CBCL) to screen for socio-emotional problems (Achenbach and Rescorla 2000; Achenbach and Rescorla 2001). The primary caretaker was invited to answer the CBCL interview questions. Because intellectual disability is common in CDG patients, we decided to also include intellectually disabled patients in our study. Patients were scored on IQ by WPPSI/WISC and an IQ of 70 was used as cut-off score for intellectual disability. In patients younger than 2.6 years, a developmental assessment was performed using the BSID. In the group of 18 patients, 8 children (44%) (5 girls and 3 boys) are intellectually disabled.

Clinical Studies

To determine the severity of the metabolic disorder, two of our investigators scored the patients with the Nijmegen Paediatric CDG Rating Scale (NPCRS) (Achouitar et al. 2011) by reviewing the medical history and clinical examination. Three different questionnaires are available for three age groups: 0–24 months, 2–11 years, and 12–18 years (Achouitar et al. 2011). These questionnaires consist of three sections. Section I (Current function) includes seven questions regarding seven general functions (vision, hearing, communication, feeding, self-care, mobility, and

Table 1 Characteristics, NPCRS, and CBCL scores of CDG patients

Id	Sex	Age	IQ	Diagnose	Visual involvement	Communication problems	Gastrointestinal	Muscle hypotonia, weakness	Liver involvement	Growth retardation	Impaired development	Strabismus	Ataxia	CDG score	Total CBCL score	CBCL T-score internalizing	CBCL T-score externalizing	CBCL T-score anx/dep	CBCL T-score with/dep	CBCL T-score social problems	CBCL T-score somatic complaints
1	M	15	<70	ALG6-CDG	-	+	-[b]	+	-	+	+	+	+	22	64	47	63	50	53	70	58
2	M	17	>70	ALG13-CDG	-	-	-	-	-	+	+	+	-	4	56	52	57	50	54	63	61
3	F	10	<70	ATP6V0A2-CDG	-	+	+[b]	+	+	-	+	+	-	18	57	61	53	60	60	59	57
4	M	15	>70	PMM2-CDG	-	-	-	+	+	-	+	+	-	8	67	69	63	65	70	66	61
5	F	13	>70	PMM2-CDG	-	+	+	+	+	+	+	-	+	11	70	75	63	78	66	78	76
6	M	14	<70	ALG6-CDG	-	+	+	+[c]	-	+	+	+	+	27	70	65	66	50	66	70	72
7	F	6	>70	SRD5A3-CDG	-	+	-	+	+	-	+	+	-	16	50	39	47	50	52	59	50
8	F	1	<70	PMM2-CDG	+	+	+[b]	+	+	-	+	+	-	22	50	62	39	50	60		70
9	M	17	<70	PMM2-CDG	+[a]	+	-	+[c]	-	-	+	+	+	30	47	34	43	50	50	67	50
10	F	12	<70	PMM2-CDG	+[a]	+	+[b]	+[c]	-	+	+	+	+	33	66	53	65	50	57	73	59
11	F	2	>70	PMM2-CDG	+	+	+[b]	+	+	+	+	+	-	23	49	56	44	51	63		50
12	F	3	>70	PMM2-CDG	-	-	-	+	+	-	+	+	+	14	36	29	42	50	50		50
13	F	14	>70	ATP6V0A2-CDG	-	-	-	-	-	-	+	-	+	6	69	69	63	67	66	87	68
14	F	8	<70	ALG6-CDG	-	-	+[b]	+[c]	-	-	+	+	-	19	73	67	64	63	68	77	64
15	M	8	>70	PMM2-CDG	+	+	+[b]	+[c]	+	+	+	+	+	28	72	69	58	54	68	77	74
16	M	14	>70	PMM2-CDG	-	+	-	+	-	-	+	+	+	13	40	40	34	50	53	51	50
17	F	10	<70	MPI-CDG	-	+	+[b]	+	+	+	+	-	+	18	71	52	71	50	64	72	57
18	M	8	>70	MPI-CDG	-	+	+[b]	+	+	+	+	+	+	19	65	59	59	54	60	57	61

Gray: increased score for social problems

All patients had impaired educational achievement

[a] Also hearing problems

[b] Also feeding problems

[c] Wheelchair use

educational achievement). The scale for 0–24 months only has five questions in this section (vision, hearing, communication, feeding, and mobility). Section II (System Specific Involvement) consists of questions to review central nervous system, blood, gastrointestinal, endocrine, respiratory, cardiovascular, renal, and liver functions over the preceding six months. Section III (Current Clinical Assessment) includes nine questions related to the current status of the patient (growth, development, vision, strabismus and eye movement, myopathy, ataxia, pyramidal, extrapyramidal, and neuropathy) (Achouitar et al. 2011). All items have four answer options: normal (0), mild (1), moderate (2), and severe (3), except the assessment of development. On this item, a score of 0–7 is possible. The total scale consists of 26 questions and the maximum score is 82.

Psychological Studies

Primary caretakers of the CDG patients completed the CBCL concerning the child. The CBCL is a widely used screening questionnaire for behavioral, emotional, and social functioning in children aged 1.5–18 years (Achenbach and Rescorla 2000, 2001). In this study, the CBCL was filled in by one parent.

We were interested in the total score of the CBCL as a measure of overall problems, as well as the internalizing and externalizing score, for children aged 1.5–18 years. Furthermore, of the syndrome profile, we used the subscales anxious/depressed (1.5–18 years) and withdrawn/ depressed (1.5–18 years) to investigate depressed behavior, social problems (only in CBCL 6–18 years), and somatic complaints (1.5–18 years). The remaining subscales were also analyzed to screen for possible other behavioral problems.

The raw scores were analyzed and assigned to T-scores using the ASEBA Windows software Assessment Data Manager (ADM) (Achenbach and Rescorla 2000, 2001). For the total, internalizing, and externalizing scores, we used T-score of 60 (93rd percentile) as cut-off point. For the subscales, we used a T-score of 65 (93rd percentile) as cut-off point.

Control Group 1

Our first control group consisted of 18 children with other inborn errors of metabolism (of other metabolic disorders), namely, mitochondrial disorders. These disorders also have a multisystem nature, in which organs are affected in variable severity. The patients are under regular outpatient follow-up at our hospital. Psychological screening using the CBCL is performed as routine care in this group of patients. We selected 18 patients for our control group, matched with the CDG group based on age, sex, and IQ. Equal to the

CDG group, in this group 8 children are intellectually disabled (IQ < 70) (5 girls and 3 boys). Comparable to the CDG group, we determined the severity of the metabolic disorders in this control group using the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) (Phoenix et al. 2006). The NPCRS, used in the CDG group, is derived from this scale. Most questions overlay and the scoring system is equal. The scale also consists of three sections, 26 items, and the maximum score is 82.

Control Group 2

Our second control group consisted of 18 patients with inborn errors of metabolism, other than CDG and mitochondrial disorders. We selected these children due to the multisystem nature of their disease. This group included children diagnosed with amino acidopathies (phenylketonuria, homocysteinemia), carbohydrate metabolism disorder (glycogen storage disease, galactosemia), organic acidemia (isovaleric acidemia, methylmalonic acidemia, propionic acidemia), urea cycle disorder (carbamyl phosphate synthetase deficiency), and fatty acid oxidation disorders (short-chain acyl-CoA dehydrogenase deficiency). Psychological screening using the CBCL has been performed as routine care in this group of patients. From this database we selected 18 consecutive patients, also matched on age, sex, and IQ. Seven out of the eighteen children are intellectual disabled, 4 girls and 3 boys.

Analysis

For the data analysis, SPSS version 18 software has been used. Significance was set at a p-value <0.05. To determine normal distribution, we used the Kolmogorov-Smirnov test. This test confirmed normal distribution on all scores on the CBCL and on the NPCRS and NPMDS. We compared the CBCL scores, including the total, internalizing, and externalizing scores and the subscores anxious/depressed, withdrawn/depressed, social problems, and somatic complaints, of our study group to norm scores of the American population using the one sample *T*-test, in absence of the Dutch norms. We compared the CBCL scores of our study group to the control groups with multiple independent sample T-ests. We evaluated possible correlations between clinical symptoms, severity and disease progression, and socio-emotional problems based on disease severity and progression (NPCRS scores) and the CBCL total, internalizing, and externalizing scores, using the Pearson Correlation.

Results

CDG Group

The CDG group consists of 18 patients with confirmed congenital disorders of glycosylation, 8 boys and 10 girls. The mean age is 10 years and 10 months.

The severity of the disease was scored by the NPCRS, as described above (Achouitar et al. 2011). Mean scores were 7.3 (1–15) (Section I), 2.4 (0–4) (Section II), 8.7 (3–16) (Section III), and 18.4 (4–33) (Total). The clinical features and CBCL scores of the CDG patients are demonstrated in Table 1.

Out of the 18 patients, 13 (72%) had an abnormal score on the CBCL. Ten children (56%) scored above the cut-off point on the total score, 8 (44%) children on the internalizing scale, and 8 (44%) children on the externalizing scale. On the subscales, three children (17%) scored above the cut-off point on anxious/depressed, 6 (33%) on withdrawn/depressed, and 5 (28%) on somatic complaints. Because the subscale social problems is not included in the CBCL 1.5–5 years, data of only 15 patients is available on this subscale. Ten of these 15 children (67%) showed social problems.

All of the children with CDG with an IQ of <70 (100%) show problems on one or more measures of the CBCL compared to 50% of the children with CDG with a normal IQ.

More than half of the PMM2-CDG patients have an aberrant CBCL score (6 out of 9). With four children showing total and internalizing problems, three showed external problems. Five of them have an abnormal score on the subscale social problems, two on anxious/depressed behavior, three on withdrawn/depressed behavior, and three on somatic complaints. The two patients with MPI-CDG showed an abnormal total score on the CBCL. One of them also had abnormal scores on externalizing problems and social problems. All three patients with ALG6-CDG in our study group have abnormal total and externalizing scores and abnormal scores on social problems. Two of them also have abnormal scores on internalizing scores and withdrawn/depressed behavior. One of them also had an abnormal score on somatic complaints. One of two children diagnosed with ATP6V0A2-CDG in our study group had abnormal scores on all four subscales, except social problems, and on total, internalizing, and externalizing scores. The other child had only an abnormal score on internalizing problems on the CBCL. The two children diagnosed with ALG13-CDG and SRD5A3 had normal CBCL scores.

Table 2 Characteristics, NPMDS, and CBCL scores of MCD patients

Id	Sex	Age	IQ	Diagnose/Complex deficiency*	Visual involvement	Communication problems	Gastrointestinal	Muscle hypotonia, weakness	Liver involvement	Growth retardation	Impaired development	Ptosis	Ataxia	MCD score	Total CBCL score	CBCL T-score internalizing	CBCL T-score externalizing	CBCL T-score anx/dep	CBCL T-score with/dep	CBCL T-score social problems	CBCL T-score somatic complaints
1	F	9	>70	C III and IV def.	+	+	-[a]	+	-	-	+	-	-	18	72	78	70	68	70	67	80
2	M	18	>70	C I and III def.	+	-	-[a]	+	-	+	+	+	+	27	60	57	56	57	57	67	54
3	F	5	>70	C I def.	-	+	-[a]	+	-	-	+	+	-	15	60	62	51	50	67		62
4	M	7	<70	C I, II and III def.	-	-	+[a]	+	-	-	+	-	-	12	68	52	64	50	66	62	50
5	F	3	>70	C I, II and III def.	-	-	+	+	-	-	+	-	-	9	67	72	58	63	76		74
6	F	11	<70	C I and III def.	-	+	+[a]	+	-	-	+	-	+	15	64	64	62	66	66	54	50
7	M	11	<70	C V def.	-	+	+[a]	+[b]	+	+	+	+	-	25	47	52	33	50	62	56	57
8	M	12	>70	C III def.	-	+	-	-	+	-	+	+	-	10	67	70	58	68	70	82	64
9	M	17	<70	C I def.	-	+	-	-	-	-	+	-	-	8	57	67	46	54	86	58	54
10	F	9	>70	C I, III and V def.	+	-	-	+	-	+	+	-	-	14	46	52	34	52	56	51	53
11	M	12	>70	C I def.	-	+	-	-	-	-	+	-	-	3	52	69	40	63	70	54	64
12	F	17	>70	C V def.	-	-	+	+	-	-	+	-	-	8	29	33	34	50	50	50	50
13	M	6	>70	C I and III def.	-	-	-	-	-	-	+	-	-	10	53	65	48	57	54	53	72
14	F	19	<70	C I and IV def.	+	+	+[a]	+[b]	-	+	+	-	-	27	59	58	49	50	60	61	68
15	F	16	<70	C I and IV def.	+	+	+[a]	+[b]	-	+	+	-	-	29	70	70	66	57	85	54	68
16	F	2	<70	C I def.	+	+	-[a]	+	-	-	+	-	-	20	57	58	59	50	63		53
17	F	6	<70	C II and III def.	-	-	+[a]	+	+	+	+	-	-	16	77	70	80	57	77	77	68
18	M	6	>70	C III def.	-	-	+[a]	-	-	+	+	-	-	9	53	41	50	50	50	58	53

Gray: increased score for social problems

*All patients, except 5, 10, and 11, had impaired educational achievement. The diagnosis is made based on measurements in a muscle biopsy according to established diagnostic procedures (Janssen et al. (2006) Chemistry 52: 860)

[a] Also feeding problems

[b] Wheelchair use

Control Group 1

The first control group consisted of 18 patients with a mitochondrial disease, 8 boys and 10 girls, with a mean age of 10 years and 8 months. The severity of the disease was scored by the NPMDS (see Methods) (Phoenix et al. 2006). Mean scores on Section I, II, and III are, respectively, 6.2 (0–17), 1.7 (0–4), and 7.4 (2–16) and the mean total score was 15.3 (3–29). Independent T-tests show no significant differences in scores between the CDG group and the MCD group. Clinical features and the CBCL scores of the MCD patients are demonstrated in Table 2. Twelve (67%) out of the 18 patients had an abnormality on the CBCL. Nine children (50%) scored above the cut-off point on the total score, 10 (56%) on the internalizing, and 5 (28%) on the externalizing scale. Three children (17%) had a score above the cut-off point on the subscale anxious/depressed, 10 (56%) on withdrawn/depressed, and 6 (33%) children on the subscale somatic complaints. Four out of 15 children (27%) showed social problems.

Control Group 2

The second control group consists of 18 patients with inborn errors of metabolism, including amino acidopathies, carbohydrate metabolism disorder, organic academia, urea cycle disorder, and fatty acid oxidation disorders. Mean age was 10 years and 4 months, with ages varying from 3 years and 9 months to 18 years and 5 months. No instrument was available to score disease severity. CBCL scores of the second control group are demonstrated in Table 3.

Eleven (61%) out of the 18 patients had an abnormality on the CBCL. Eight children (44%) scored above the cut-off point on the total score, six (33%) on the internalizing scale, and 5 (28%) on the externalizing scale. Three children (17%) showed problems on the subscale anxious/depressed, two (11%) on withdrawn/depressed, and five (28%) on somatic complaints. Five out of 15 children (33%) showed social problems.

Correlation Clinical Severity and Organ Involvement with CBCL Scores

No correlations were found between the CBCL total score and the NPCRS total score (Pearson Correlation = 0.068, $p = 0.790$), NPCRS section I (Pearson Correlation = 0.088, $p = 0.728$), or NPCRS section III (Pearson Correlation = −0.143, $p = 0.572$). There is a small positive correlation between Section II on the NPCRS and total score on CBCL in CDG as measured by the Pearson Correlation (Pearson Correlation = 0.482, $p = 0.043$). However, regarding the small sample sizes we do not consider this result as statistically significant.

Table 3 Characteristics and CBCL scores of the patients of control group 2

Id	Sex	Age	IQ	Diagnose	Total CBCL score	CBCL T-score internalizing	CBCL T-score externalizing	CBCL T-score anx/dep	CBCL T-score with/dep	CBCL T-score social problems	CBCL T-score somatic complaints
1	M	4	<70	Methylmalonic aciduria	55	51	59	50	56		50
2	M	10	>70	Glycogenosis type Ia	71	71	65	66	76	69	67
3	M	7	<70	Propionic acidemia	50	41	44	50	54	56	50
4	M	9	>70	Glycogenosis type IX	64	58	66	57	66	65	50
5	M	6	>70	Carbamoyl phosphate synthetase I deficiency	62	58	58	57	62	53	53
6	M	12	<70	Glycogenosis type Ia	58	62	53	63	53	58	67
7	F	15	>70	Cobalamin C deficiency	67	58	64	50	63	70	62
8	F	18	<70	Cobalamin C deficiency	62	64	51	62	57	61	65
9	F	10	<70	Isovaleric aciduria	69	64	70	66	64	62	53
10	F	9	<70	Glycogenosis type IX	53	48	47	50	50	51	61
11	F	5	>70	Isovaleric aciduria	42	49	39	56	51		50
12	F	8	>70	Ketotic hypoglycemia	53	56	44	54	52	54	61
13	F	10	>70	Glycogenosis type IX	55	61	51	57	50	51	68
14	M	3	<70	Glycogenosis type IX	48	55	44	56	60		53
15	M	10	>70	Glycogenosis type IX	36	45	33	50	50	50	57
16	F	7	>70	Phenylketonuria	62	58	64	54	64	57	53
17	F	18	<70	Galactosemia	52	58	34	55	63	73	53
18	F	15	>70	Methylmalonic aciduria	68	74	59	72	60	70	84

Gray: increased score for social problems

Table 4 Mean behavior T-scores in the patients with CDG compared to norm scores. $N = 18$

	Total score (CBCL 1.5–18 years)	Internalizing score (CBCL 1.5–18 years)	Externalizing score (CBCL 1.5–18 years)	Anxious/ depressed (CBCL 1.5–18 years)	Withdrawn/ depressed (CBCL 1.5–18 years)	Social problems (CBCL 6–18 years)	Somatic complaints (CBCL 1.5–18 years)
Mean T-score	59.6	55.4	55.2	55.1	60.0	68.4	60.4
Mean diff. (95% CI)	9.6 (3.7–15.4)	5.4 (–1.2–12.1)	5.2 (–0.2–10.7)	1.1 (–2.9–5.2)	6.0 (2.7–9.3)	14.4 (9.1–19.7)	6.4 (2.1–10.8)
p-value	**0.003**	0.101	0.059	0.571	**0.001**	**0.000**	**0.006**

For the standard CBCL scales, a subscale score of <65 is normal, 65–69 scores are borderline, and from the score >70 indicate a clinical problem (normative ranges are different for girls and for boys) (Achenbach and Rescorla 2000, 2001)

CDG Patients Scores Compared to Norm Scores

Mean CBCL total score is significantly higher in children with CDG compared to norm scores ($p = 0.003$; Table 4). The internalizing and externalizing scales were not significantly higher ($p = 0.101$ and $p = 0.059$, respectively). The mean score of anxious, depressive behavior in our study group was not statistically significantly increased ($p = 0.571$).

Compared to the norms scores, in children with CDG the mean score was significantly higher on withdrawn/ depressed behavior ($p = 0.001$), social problems ($p = 0.000$), and on somatic complaints ($p = 0.006$). Furthermore, we screened the other subscales for possible problems. Results showed more thought problems ($p = 0.044$), attention problems ($p = 0.004$), aggressive behavior ($p = 0.011$), affective problems ($p = 0.003$), anxiety problems ($p = 0.032$), somatic problems ($p = 0.031$), ADHD problems ($p = 0.008$), and conduct problems ($p = 0.016$) in the CDG group compared to the norms.

Table 5 shows the mean differences between CDG patients with an IQ of > 70 and an IQ <70 and the norm scores. In CDG patients with a normal IQ, results still show a significantly increased mean score on the

Table 5 Mean behavior T-scores in the patients with CDG and an IQ of >70 and < 70 compared to norm scores

	Total score (CBCL 1.5–18 years)	Internalizing score (CBCL 1.5–18 years)	Externalizing score (CBCL 1.5–18 years)	Anxious/ depressed (CBCL 1.5–18 years)	Withdrawn/ depressed (CBCL 1.5–18 years)	Social problems (CBCL 6–18 years)	Somatic complaints (CBCL 1.5–18 years)
Patients with CDG and an IQ of >70. $N = 10$							
Mean T-score	57.4	55.7	53.0	56.9	60.2	67.3	60.1
Mean diff. (95% CI)	7.4 (–2.0–16.8)	5.7 (–5.4–16.8)	3.0 (–4.5–10.5)	2.9 (–4.1–9.9)	6.2 (0.9–11.5)	13.3 (3.0–23.5)	6.1 (–1.1–13.3)
p-value	0.108	0.275	0.386	0.371	**0.027**	**0.019**	0.089
Patients with CDG and an IQ of <70. $N = 8$							
Mean T-score	62.3	55.1	58.0	52.9	59.8	69.7	60.9
Mean diff. (95% CI)	12.3 (4.0–20.5)	5.1 (–4.1–14.3)	8.0 (–1.8–17.8)	–1.1 (–5.6–3.4)	5.8 (0.5–11.0)	15.7 (10.5–20.9)	6.9 (0.7–13.0)
p-value	**0.010**	0.229	0.094	0.573	**0.035**	**0.000**	**0.033**

For the standard CBCL scales, a subscale score of <65 is normal, 65–69 scores are borderline, and from the score >70 indicate a clinical problem (normative ranges are different for girls and for boys) (Achenbach and Rescorla 2000, 2001)

Table 6 Mean behavior T-scores in the patients with CDG compared to T-scores in the control groups

	Total score (CBCL 1.5–18 years)	Internalizing score (CBCL 1.5–18 years)	Externalizing score (CBCL 1.5–18 years)	Anxious/ depressed (CBCL 1.5–18 years)	Withdrawn/ depressed (CBCL 1.5–18 years)	Social problems (CBCL 6–18 years)	Somatic complaints (CBCL 1.5–18 years)
Mean behavior T-scores in the patients with CDG compared to T-scores in control group 1. $N = 18$							
Mean T-score CDG group	59.6	55.4	55.2	55.1	60.0	68.4	60.4
Mean T-score control group 1	58.8	60.6	53.2	56.2	65.8	60.3	60.8
Mean diff. (95% CI)	0.8 (–7.0–8.6)	–5.1 (–13.5–3.3)	2.0 (–6.1–10.1)	–1.1 (–6.2–3.9)	–5.8 (–11.9–0.2)	8.1 (1.1–15.2)	–0.3 (–6.5–5.8)
p-value	0.841	0.226	0.621	0.658	0.058	**0.026**	0.913
Mean behavior T-scores in the patients with CDG compared to T-scores in control group 2. $N = 18$							
Mean T-score CDG group	59.6	55.4	55.2	55.1	60.0	68.4	60.4
Mean T-score Control group 2	57.1	57.3	52.5	56.9	58.4	60.0	58.7
Mean diff. (95% CI)	2.5 (–4.7–9.7)	–1.8 (–9.4–5.8)	2.7 (–4.8–10.3)	–1.8 (–6.8–3.2)	1.6 (–3.1–6.3)	8.4 (1.9–14.9)	1.7 (–4.3–7.8)
p-value	0.487	0.626	0.469	0.461	0.489	**0.013**	0.565

For the standard CBCL scales, a subscale score of <65 is normal, 65–69 scores are borderline, and from the score >70 indicate a clinical problem (normative ranges are different for girls and for boys) (Achenbach and Rescorla 2000, 2001)

subscales withdrawn/depressed behavior ($p = 0.027$) and social problems ($p = 0.019$) compared to norms, even though less patients are included ($n = 10$). Children with CDG with an IQ of <70 show more total problems ($p = 0.010$), withdrawn/depressed behavior ($p = 0.035$), somatic complaints ($p = 0.033$), and social problems ($p = 0.000$) compared to the norms.

Comparison Between CDG Group and the Control Groups

The mean CBCL total, externalizing, and internalizing scores in our study group was not significantly aberrant from both control groups (Table 6). The same was true for the subscales anxious/depressed, withdrawn/depressed behavior, and somatic complaints.

The occurrence of social problems was significantly higher in our study group compared to children with mitochondrial disorders ($p = 0.026$) and children with other metabolic disorders ($p = 0.013$).

Conclusions

The aim of this study was to investigate whether children with CDG have more socio-emotional problems compared to the norm group and compared to children with other chronic metabolic diseases with multisystem presentation. As observed in our clinic, results showed that children with CDG show more overall problems compared to norms. Furthermore, they show more withdrawn/depressive behavior, social problems, somatic complaints, affective problems, and anxiety problems compared to the norms. As previously described, this is in line with children with other metabolic diseases who also show more socio-emotional problems compared to healthy controls (Koene et al. 2009; Morava et al. 2010; Mueller et al. 2010; Northam et al. 2006; Waisbren and White 2010). Compared to children with other metabolic diseases, children with CDG, however, show more social problems. This could mean that social problems are inherent to CDG. However, regarding the small sample sizes, more research is needed to draw such a firm conclusion.

One might hypothesize that a more severely affected child would have higher scores on the CBCL. However this was not the case, there was no correlation found between total score on NPCRS and the scores on the CBCL. Results suggest that socio-emotional problems are more a result of the disease itself rather than of the disease severity or progression.

In our study, all of the children, with CDG with an IQ below 70, show one or more socio-emotional problems. This is even higher as described in literature, which shows that the prevalence of socio-emotional problems is three to five times higher in the intellectually disabled compared to the normal population (Došen 2008). Therefore, children with CDG who are also intellectually disabled have an even higher risk of socio-emotional problems. According to Koskentausta et al. (2004), the CBCL is reliable in mild intellectually disabled children, but is less reliable in children with moderate, severe, or profound intellectual disability. A study of Borthwick-Duffy et al. (1997) showed, however, that CBCL is reliable in children with an intellectual disability and supports the use of the CBCL in these children. Furthermore, Masi et al. (2002) report that the CBCL overall measure shows a high correlation with other measures of socio-emotional problems in mentally retarded children and could therefore be used as a reliable screenings instrument. A limitation is that this study did not make a subdivision in IQ values below 70 due to small sample sizes; in future studies, this is recommended to draw firm conclusions.

By evaluating the relatively small CDG subpopulation with normal intelligence, the scores are still significantly higher on subscales withdrawn/depressed behavior and social problems compared to the population (Table 5). Therefore, the occurrence of withdrawn/depressed behavior and social problems in children with CDG seems to be independent of intelligence.

Surprisingly, we found no differences regarding anxious/depressed behavior in children with CDG compared to the population. Even in the intellectually disabled children, there were no differences found, while according to the literature, mentally retarded children are more vulnerable to socio-emotional problems, especially anxiety and depression (Masi et al. 1999, 2000). According to Masi et al. (2002) the anxious/depressed subscale of the CBCL, however, does not correlate with other depression measures in questionnaires developed solely for mentally retarded children. So it is questionable if the results regarding the anxious/depressed subscale could be partially explained by the questionnaire used. Future studies must take mental retardation into account and use multiple diagnostic instruments to investigate depression.

Results in our mixed cohort showed that in almost all types of CDG, children showed one or more socio-emotional problems. PMM2-CDG patients are described as happy children and no mood disorders have been reported yet. Nevertheless in our study, six out of the nine PMM2-CDG patients showed abnormal scores on CBCL. PMM2-CDG is the largest CDG group, with over 700 patients reported worldwide. Furthermore, all three ALG6-CDG patients showed significant psychological problems. In literature, observations regarding behavior in this patient group are lacking. However, these results should be interpreted with caution regarding the small sample size of the group. Therefore, future research is recommended to investigate whether the prevalence, degree, and nature of psychological problems are variable between different subtypes of CDG.

This study suggests that CDG patients could suffer from untreated psychological problems. However, one should be cautious with drug therapy. CDG effects liver function and most drugs used in psychiatric treatment are eliminated by the liver. The effect of these drugs on glycosylation is not yet evaluated. Because of the possible side effects of psychopharmaca in inborn errors of metabolism, psychological therapy is of first choice in children with metabolic disorders and socio-emotional problems.

Congenital disorders of glycosylation form a relatively young but growing group of inborn errors of metabolism. Future studies are needed to further elucidate the psychological aspects of the disease and are essential for optimal interpretation and treatment of socio-emotional problems in children with CDG.

In summary, our study results show an increased prevalence of socio-emotional problems in patients diagnosed with CDG compared to norm scores. Mean total scores, scores on withdrawn/depressed behavior, social problems and somatic complaints were significantly increased. More than two thirds of our CDG patients have abnormal scores on CBCL. Compared to children with other metabolic disorders, CDG patients show more social problems. Therefore, it can be concluded that patients with CDG have an increased risk of developing socio-emotional problems. Based on our results, a standard screening for psychological problems is recommended in CDG patients for the early detection of psychological problems and to provide adequate treatment at an early stage.

Synopsis

A standard screening for psychological problems is recommended for the early detection of psychological problems in CDG patients.

Contributors

Eva Morava and Chris Verhaak designed and supervised the study and commentated on the text. Lotte van Dongen and Kim van de Loo managed the data, the statistical analyses, and wrote the article.

M. Mohamed, T. Gardeitchik, T.W. Kouwenberg, S.B. Wortmann and D.J. Lefeber - were involved in the conception and design, and reviewed the manuscript.

R.J.T. Rodenburg- was involved in the conception and design, and responsible for the laboratory diagnostic analysis of the mitochondrial patients and reviewed the manuscript.

Guarantor

E. Morava

Role of Funding Source

No financial support was used by this study.

Ethics Approval

There was no ethics approval required.

Patient Consent Statement

No patient consent was required.

Conflicts of Interest

The authors disclose any financial conflict of interest.

References

Achenbach TM, Rescorla LA (2000) Manual for the ASEBA preschool forms and profiles. University of Vermont Department of Psychiatry, Burlington, VT

Achenbach TM, Rescorla LA (2001) Manual for the ASEBA school-age forms and profiles. University of Vermont, Research Center for Children, Youth, and Families, Burlington, VT

Achouitar S, Mohamed M, Gardeitchik T et al (2011) Nijmegen paediatric CDG rating scale: a novel tool to assess disease progression. J Inherit Metab Dis 34:923–927

Boekaerts M, Röder I (1999) Stress, coping, and adjustment in children with a chronic disease: a review of the literature. Disabil Rehabil 21:311–337

Borthwick-Duffy SA, Lane KL, Widaman KF (1997) Measuring problem behaviors in children with mental retardation: dimensions and predictors. Res Dev Disabil 18:415–433

Davis JD, Tremont G (2007) Neuropsychiatric aspects of hypothyroidism and treatment reversibility. Minerva Endocrinol 32:49–65

Došen A (2008) Psychische stoornissen, gedragsproblemen en verstandelijke handicap. Koninklijke van Gorcum, Assen

EvansDL CDS, Lewis L et al (2005) Mood disorders in the medically ill: scientific review and recommendations. Biol Psychiatry 58:175–189

Freeze HH (2006) Genetic defects in the human glycome. Nat Rev Genet 7:537–551

Grünewald S (2009) The clinical spectrum of phosphomannomutase 2 deficiency (CDG-Ia). Biochim Biophys Acta 1792:827–834

Jaeken J (2010) Congenital disorders of glycosylation. Ann N Y Acad Sci 1214:190–198

Jaeken J, Hennet T, Matthijs G, Freeze HH (2009) CDG nomenclature: time for a change! Biochim Biophys Acta 1792:825–826

Koene S, Kozicz TL, Rodenburg RJ et al (2009) Major depression in adolescent children consecutively diagnosed with mitochondrial disorder. J Affect Disord 114:327–332

Koskentausta T, Iivanainen M, Almqvist F (2004) CBCL in the assessment of psychopathology in Finnish children with intellectual disability. Res Devel Disabil 25:341–354

Liem YS, Bode L, Freeze HH, Leebeek FW, Zandbergen AA, Paul Wilson J (2008) Using heparin therapy to reverse protein-losing enteropathy in a patient with CDG-Ib. Nat Clin Pract Gastroenterol Hepatol 5:220–224

Masi G, Brovedani P, Mucci M, Favilla L (2002) Assessment of anxiety and depression in adolescents with mental retardation. Child Psychiatry Hum Deve 32:227–237

Masi G, Favilla L, Mucci M (2000) Generalized anxiety disorder in adolescents and young adults with mild mental retardation. Psychiatry 63:54–64

Masi G, Mucci M, Favilla L, Poli P (1999) Dysthymic disorder in adolescents with intellectual disability. J Intellect Disabil Res 43:80–87

Mohamed M, Guillard M, Wortmann SB et al (2011) Clinical and diagnostic approach in unsolved CDG patients with a type 2 transferrin pattern. Biochim Biophys Acta 1812:691–698

Mokkink LB, van der Lee JH, Grootenhuis MA, Offringa M, van Praag BMS, Heymans HSA (2007) Omvang en gevolgen

van chronische aandoeningen bij kinderen. Tijdschr Kindergeneeskd 75:154–158

Morava E, Gardeitchik T, Kozicz T et al (2010) Depressive behaviour in children diagnosed with a mitochondrial disorder. Mitochondrion 10:528–533

Morava E, Guillard M, Lefeber DJ, Wevers RA (2009a) Autosomal recessive cutis laxa syndrome revisited. Eur J Hum Genet 17:1099–1110

Morava E, Wevers RA, Willemsen MA, Lefeber D (2009b) Cobblestone-like brain dysgenesis and altered glycosylation in congenital cutis laxa Debré type. Neurology 73:1164

Morava E, Zeevaert R, Korsch E et al (2007) A common mutation in the COG7 gene with a consistent phenotype including microcephaly, adducted thumbs, growth retardation, VSD and episodes of hyperthermia. Eur J Hum Genet 15:638–645

Mueller SC, Ng P, Sinaii N et al (2010) Psychiatric characterization of children with genetic causes of hyperandrogenism. Eur J Endocrinol 163:801–810

Northam EA, Rankins D, Cameron FJ (2006) Therapy insight: the impact of type 1 diabetes on brain development and function. Nat Clin Pract Neurol 2:78–86

Phoenix C, Schaefer AM, Elson JL et al (2006) A scale to monitor progression and treatment of mitochondrial disease in children. Neuromuscul Disord 16:814–820

Sparks SE, Krasnewich DM (2005) Congenital disorders of glycosylation overview. In: Pagon RA, Bird TD, Dolan CR, Stephens K (eds) GeneReviews [Internet]. University of Washington, Seattle (WA)

Theodore M, Morava E (2011) Congenital disorders of glycosylation: sweet news. Curr Opin Pediatr 23:581–587

Waisbren S, White DA (2010) Screening for cognitive and social-emotional problems in individuals with PKU: tools for use in the metabolic clinic. Mol Genet Metab 99:96–99

JIMD Reports
DOI 10.1007/8904_2013_235

RESEARCH REPORT

Metabolic Profiling of Total Homocysteine and Related Compounds in Hyperhomocysteinemia: Utility and Limitations in Diagnosing the Cause of Puzzling Thrombophilia in a Family

Sally P. Stabler • Mark Korson • Reena Jethva • Robert H. Allen • Jan P. Kraus • Elaine B. Spector • Conrad Wagner • S. Harvey Mudd

Received: 08 December 2011 / Revised: 25 May 2012 / Accepted: 13 June 2012 / Published online: 4 June 2013

Abstract We describe a family illustrating the diagnostic difficulties occurring when pyridoxine-responsive cystathionine beta-synthase (CBS) deficiency presents with thrombotic disease without associated ocular, skeletal, or CNS abnormalities, a situation increasingly recognized. This family had several thromboembolic episodes in two generations with apparently inconstant elevations of plasma total homocysteine (tHcy). When taking (sometimes even low amounts) of pyridoxine, the affected family members had low-normal tHcy and normal values for cystathionine, methionine, and cysteine. Withdrawal of vitamin therapy was necessary before lower cystathionine, elevated methionine, and decreased cysteine became apparent, a pattern suggestive of CBS deficiency, leading to the finding that the affected members were each compound heterozygotes for *CBS* p.G307S and p.P49L. To assist more accurate diagnosis of adults presenting with thrombophilia found to have elevated tHcy, the patterns of methionine-related metabolites in CBS-deficient patients are compared in this article to those in patients with homocysteine remethylation defects, including inborn errors of folate or cobalamin metabolism, and untreated severe cobalamin or folate deficiency. Usually serum cystathionine is low in subjects with CBS deficiency and elevated in those with remethylation defects. *S*-Adenosylmethionine and *S*-adenosylhomocysteine are often markedly elevated in CBS deficiency when tHcy is above 100 umol/L. We conclude that there are likely other undiagnosed, highly B6-responsive adult patients with CBS deficiency, and that additional testing of cystathionine, total cysteine, methionine, and *S*-adenosylmethionine will be helpful in diagnosing them correctly and distinguishing CBS deficiency from remethylation defects.

Communicated by: Ivo Bari

Competing interests: S.P. Stabler and R.H. Allen

S.P. Stabler (✉) · R.H. Allen
Division of Hematology, Department of Medicine, University of Colorado School of Medicine, 12700 E. 19th Avenue, Room 9122, Bldg. RC2, Campus Box B170, Aurora, CO, USA
e-mail: Sally.Stabler@ucdenver.edu

M. Korson
Division of Genetics and Metabolism, Department of Pediatrics, Tufts Medical Center, Boston, MA, USA

R. Jethva
Section of Neurology, Department of Pediatrics, St. Christopher's Hospital for Children, Philadelphia, PA, USA

J.P. Kraus · E.B. Spector
Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO, USA

C. Wagner
Department of Biochemistry, Vanderbilt University, Nashville, TN, USA

S.H. Mudd
Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, MD, USA

Abbreviations

5-MTHF	5-methyltetrahydrofolate
AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
B6	Pyridoxine
Cbl	Cobalamin
CBS	Cystathionine B-synthase
CGL	Cystathionine gamma-lyase
Cysta	Cystathionine

MAT	Methionine adenosyltransferase
Me-Cbl	Methyl-cobalamin
Met	Methionine
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
PLP	Pyridoxal phosphate
SAHH	S-adenosylhomocysteine hydrolase
tCys	Total Cysteine
tHcy	Total homocysteine

Introduction

In this article, we report the clinical and metabolic findings for a family with four members in two generations with elevated levels of tHcy among whom more than three thromboembolic events occurred before it was found that they are each compound heterozygotes for mutations in the gene that encodes CBS. This history raises two important interrelated issues: (a) Why did these patients go so long without a specific diagnosis? (b) What steps might be taken to improve the ability to determine the cause of abnormally high tHcy? To address these issues, after presenting the facts about the family reported upon, we discuss the several limitations that played a role in delaying their diagnoses, present a compilation of the results of assays of not only tHcy and methionine, but also cystathionine, *S*-adenosylmethionine (AdoMet), and *S*-adenosylhomocysteine (AdoHcy) in a variety of patients with elevated levels of tHcy, then discuss how these compiled metabolite results may be useful in providing criteria for the differential diagnosis of elevated tHcy.

Figure 1 outlines the relevant pathways of homocysteine and methionine metabolism (Mudd 2011). Homocysteine is at a branch point and can be remethylated to methionine by a folate and cobalamin (vitamin B12)-dependent enzyme, methionine synthase (MS, EC 2.1.1.13), or condensed with serine to form cystathionine by the pyridoxal phosphate (B6)-dependent enzyme CBS (EC 4.2.1.22). Cystathionine is cleaved by cystathionine gamma-lyase (EC 4.4.1.1) to form cysteine. Methionine can be converted to AdoMet, the donor in many important transmethylation reactions, including the formation of *N*-methylglycine (sarcosine). The resulting AdoHcy is hydrolyzed to homocysteine by *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1). The alternate remethylation of homocysteine using betaine as a donor of the methyl group, is not shown, but may be exploited as an effective homocysteine lowering treatment (Mudd 2011).

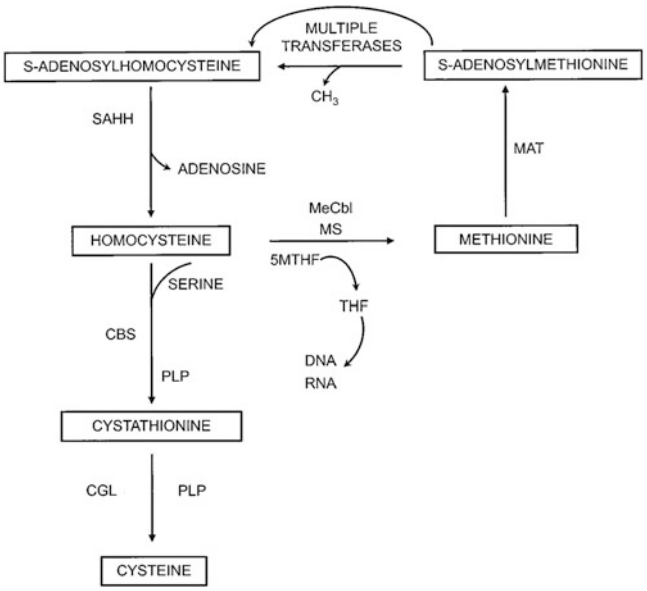

Fig. 1 Pathways of methionine and homocysteine metabolism are shown. Homocysteine is methylated to methionine by methionine synthase (*MS*) with methyl-cobalamin (*Me-Cbl*). 5-methyltetrahydrofolate (*5-MTHF*) is demethylated to tetrahydrofolate (*THF*) in this reaction. Methionine is activated to S-adenosylmethionine by methionine adenosyltransferase (*MAT*). Multiple transferases utilize S-adenosylmethionine producing S-adenosylhomocysteine in the reactions, which can be hydrolyzed to homocysteine and adenosine by S-adenosylhomocysteine hydrolase (*SAHH*). Homocysteine can be condensed with serine by the pyridoxal phosphate (PLP)-dependent enzyme cystathionine beta-synthase (*CBS*) to form cystathionine. Cystathionine is cleaved by another PLP-dependent enzyme, cystathionine gamma-lyase (*CGL*) forming cysteine

Case Report

A 55-year-old female of Northern European and Irish ancestry had presented with a deep venous thrombosis at age 18 while on oral contraceptives. She had had multiple pulmonary emboli by age 45. For more than 10 years, tHcy had been known to be at times elevated, ranging from 16 to 186 umol/L, assayed in clinical labs and on varying vitamin regimens. Both methylmalonic acid and methionine had been assayed and said to be normal on several occasions. The subject had no ocular, skeletal, or other physical or cognitive findings suggestive of CBS deficiency. Fibroblast assay had been negative for severe methylenetetrahydrofolate reductase deficiency in the past. Her brother, age 52 years and asymptomatic, according to historical detail had been found to have elevated tHcy of 80 umol/L. He also had normal values on unknown vitamin regimens. His daughter, the 26-year-old niece of the proband, had a sagittal sinus thrombosis with left parietal infarct at age 20 while on oral contraceptives. Her tHcys had been as high as 137 umol/L in the past. Her brother, the 21-year-old nephew of the proband, was asymptomatic but had a documented tHcys value of

166 umol/L. Values of methionine metabolites and vitamin treatments, if known, are shown in Table 1. As will be covered in more detail in the "Discussion," only when the proband and her brother discontinued all vitamin supplements did metabolite patterns indicative of CBS deficiency become apparent. The four affected family members and the wife of the brother, the mother of the affected niece and nephew, were then analyzed for *CBS* mutations. The proband, brother, niece, and nephew were each compound heterozygotes for *CBS* mutations p.G307S and p.P49L; the mother of the niece and nephew, a p.G307S carrier. A pedigree of the case family is shown in Fig. 2.

Methods

Methylmalonic acid, 2-methylcitric acid, tHcy, cystathionine, methionine, and total cysteine were assayed in serum or plasma by capillary stable isotope dilution gas chromatography/mass spectrometry as previously described (Stabler et al. 1988, 1993). AdoMet and AdoHcy were assayed in serum or plasma either by stable isotope dilution liquid chromatography/mass spectrometry (Stabler and Allen 2004) or by HPLC separation of naphthalene dialdehyde derivatives with fluorescent detection (Capdevila and Wagner 1998).

The values for metabolites shown in Tables 2–4 and the figures were compiled from samples assayed for diagnosis and treatment of inborn errors and for previously reported cohorts of patients with CBS deficiency or remethylation defects (Stabler et al. 1988; Stabler et al. 1993; Allen et al. 1993; Savage et al. 1994; Tangerman et al. 2000; Mudd et al. 2001; Yaghmai et al. 2002; Stabler et al. 2002; Maclean et al. 2002; Orendäc et al. 2003; Stabler and Allen 2004; Guerra-Shinohara et al. 2007; Strauss et al. 2007; Keating et al. 2011). The group with remethylation defects includes inborn errors of cobalamin metabolism, such as cblC or cblD disorders, defects of methionine synthase or methionine synthase reductase, or severe deficiency of methylenetetrahydrofolate reductase (MTHFR) (not the thermolabile C.677C>T polymorphism) (47 samples), or clinically symptomatic cobalamin or folate-deficient patients with megaloblastic anemia and/or neurologic disease (85 samples). All subjects with cobalamin deficiency or cblC defects also had elevated methylmalonic acid. Many subjects had multiple sample determinations on various treatment regimens such as methionine-restricted diets, with or without cysteine replacement, B6 and/or other vitamin supplements, or administration of betaine. For patients with CBS deficiency or the inborn errors of remethylation, data both for periods of treatment and for no treatment are shown in the Results section, whereas for subjects with cobalamin or folate deficiency, values are shown only for periods of no treatment. The different studies were reviewed by relevant institutional review boards (see references above) as well as the University of Colorado School of Medicine. The numbers of values for CBS-deficient samples were as follows: tHcy – 117, cystathionine – 116, methionine – 118, total cysteine – 100, AdoMet – 40, and AdoHcy – 37. The numbers of values for remethylation defects were as follows: tHcy – 132, cystathionine – 131, methionine – 121, total cysteine – 119, AdoMet – 28, and AdoHcy – 28. All six values were available for 49 samples from adult controls (Stabler and Allen 2004).

The testing for CBS mutations in the case family was performed by the University of Colorado DNA Diagnostics Laboratory by PCR amplification and bidirectional DNA sequencing of all 16 coding exons and the exon/intron borders. The reference sequence was NM 000071.1. Primer sequences are available upon request.

Statistical analysis was performed with SPSS Version 19 and < 0.05 was considered significant. To compare two groups, the independent samples Mann-Whitney *U* test was used. For more than two groups, ANOVA was used with the Scheffe post hoc test for significance.

Results

Family Metabolite Values

As shown in Table 1, when taking supplemental vitamin B6, the proband and the three other members of her family with genetically proven CBS deficiency each had normal values for methionine and methionine metabolites. B6 doses as low as 2 mg/day were enough, on occasion, to normalize tHcy. When the dose of B6 for the proband was decreased from 100 to 2 mg/day, tHcys rose from 11.7 to 49.1 umol/L and methionine doubled from 23 to 47 umol/L. When she was withdrawn from all B6 supplements, tHcy rose after almost 2 months to 222 umol/L. By that time her other metabolites had developed a pattern very suggestive of CBS deficiency with cystathionine relatively low, total cysteine below the normal range, and methionine elevated. Serum *S*-adenosylmethionine (AdoMet) and *S*-adenosylhomocysteine (AdoHcy) rose to 1,282 and 1,539 nmol/L, 7.6 and 50 times the upper limits of their reference ranges. When the brother was taken off vitamins his tHcy initially rose, as did methionine, AdoMet, and AdoHcy, but cystathionine and total cysteine remained normal. Subsequently, for reasons that are not clear, the elevations of tHcy and methionine diminished.

Table 1 Metabolic values, vitamin treatment, and symptoms in case family members

Index case											
	tHcy	Met	Cysta	tCys	AdoMet	AdoHcy	Vitamin treatment				
Age (y)	(umol/L)	(umol/L)	(nmol/L)	(umol/L)	(nmol/L)	(nmol/L)	B6	B12	Folate	Comments to treatment	Symptoms
42	(102.1)*									Unknown	DVT age 18
	(32.9)									Unknown	
43	(186)									Unknown	
44	(67.8)									Unknown	
	(20.4)							Post B12 injection 24 h			
	(23.1)							Post B12 injection 48 h			
	(68)										PE age 45
46	(12.2)	(21)								Unknown	
50	(9.4)	(21)									
51	(18)										
	6	20	74	311	118	8	100 mg	100 mcg	400 mcg	MVI also	
52	(176.8)									Unknown	
	(124.2)									Unknown	
53	(184.4)									Unknown	
	(33.6)									Unknown	
	(66.6)									Unknown	
	(35.8)									Unknown	
	(92.9)									Unknown	
	11.7	22.5	128	363	69	38	2 mg			Decrease B6 from 100 to 2 mg × 2 days	
	49.1	47.2	210	327	137	20	2 mg			2 mg × 7 days (from 100 mg)	
	(71.1)	(39)								Off all vitamins × 26 days	
	67.8	35	139	253	169	33.9				Off all vitamins × 45 days	
	222	135	135	133	1282	1539				Off all vitamins × 53 days	
	(10.4)	(13.4)					100 mg				

Brother											
43	(7.9)	(31)								Unknown	None
	(12.2)	(34)									
48	8.6	28.9	136	245	98	8	2 mg	15 mcg	400 mcg	MVI	
51	72.3	63.3	295	245	657	78				Off all vitamins × 55 days	
	15.7	27.8	208	326	115	21				Off all vitamins × 62 days	
	22.1	42.5	204	317	210	61				Off all vitamins × 70 days	
52	(14)						100 mg				
Nephew											
16	(166)									Unknown	None
17	(59)										
	6.1	26.1	102	236	96	7	?	1 mg	1 mg		
Niece											
21	5.8	27.7	99	242	62	12	100 mg	500 mcg	1 mg		Sagittal sinus thrombosis and left parietal infarction at age 20 of treatment
24	(137)									None	
	(13)						50 mg				
25	(24)						100 mg				

Reference range (5–14) (13–45) (44–342) (200–361) (71–168) (8–26)

DVT deep venous thrombosis, *PE* pulmonary embolism, *MVI* multivitamin

* Values in parentheses assayed elsewhere, usually commercial clinical laboratory

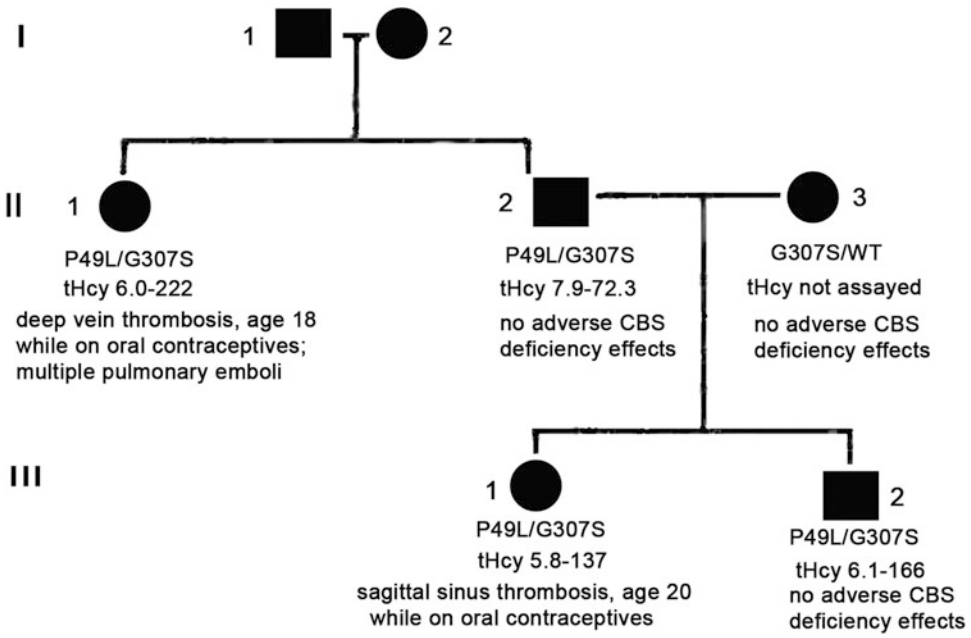

Fig. 2 The pedigree is shown for the case family. The proband is shown as II-1; her brother, II-2; and brother's wife, II-3. The proband's niece is III-1 and nephew, III-2

Table 2 Median (range) metabolites* in untreated vs. treated CBS deficiency

		Untreated CBS $N = 25$	Treated CBS $N = 93$	Sig**
tHcy (umol/L)	(Median)	125.0	119.0	NS
	(Range)	(15.7–281.4)	(4.8–312)	
Cystathionine (nmol/L)	(Median)	78	25	0.004
	(Range)	(25–295)	(25–357)	
Methionine (umol/L)	(Median)	135	160	NS
	(Range)	(27.8–1,595)	(11.7–2,823)	
Total cysteine	(Median)	124	138	NS
	(Range)	(40–326)	(40–363)	
AdoMet (nmol/L)	(Median)	804	446	NS
	(Range)	(115–1,282)	(62–1,130)	
AdoHcy (nmol/L)	(Median)	160	56	NS
	(Range)	(21–1,539)	(7–530)	
AdoMet/AdoHcy (ratio)	(Median)	3.7	3.8	NS
	(Range)	(0.8–8.6)	(1.5–16.4)	

* See methods for actual number of values of each metabolite

** Independent samples Mann-Whitney U test <0.05 was significant

Compiled Metabolite Values for Other Patients with Either CBS Deficiency or Remethylation Defects

To extend the information available on the metabolites relevant to abnormally elevated tHcy, values obtained for patients assayed in association with previous reports from the authors' laboratories were gathered and combined into Tables 2–4 and Figs. 3–7. In Table 2 median (range) values for the metabolites are shown for the CBS-deficient samples obtained off treatment ($N = 25$) as compared to these obtained on standard treatments ($N = 93$). The only statistically different value was that for cystathionine which, unexpectedly, was higher in the untreated group.

Table 3 Metabolite values* (median, range) in congenital disorders of remethylation ($N = 47$) vs. acquired vitamin B_{12} or folate deficiency ($N = 85$)

			Congenital remethylation defects				
			Treated				
		Total	No ($N = 8$)	Yes ($N = 39$)	Sig**	Acquired vitamin deficiency	Sig[a]
tHcy (umol/L)	(Median)	66.0	130.5	62.7	0.008	80.8	NS
	(Range)	(21.3–303)	(55–241)	(21.3-303)		(17.1–254.4)	
Cystathionine (nmol/L)	(Median)	1,032	1,192	1,005	NS	656	0.004
	(Range)	(304–3,439)	(434–3,439)	(304–1,963)		(145–7923)	
Methionine (umol/L)	(Median)	18.0	14.8	19.8	NS	18.7	NS
	(Range)	(3.7–88.0)	(6.0–27.0)	(3.7–88.0)		(6.5–315)	
Total cysteine (umol/L)	(Median)	184	142	190	NS	242	<0.001
	(Range)	(75–328)	(91–213)	(75–328)		(78–816)	
AdoMet (nmol/L)	(Median)	108	86	124	0.020	100	NS
	(Range)	(49–421)	(49–186)	(93–421)		(65–234)	
AdoHcy (nmol/L)	(Median)	26	22	28	NS	44	0.009
	(Range)	(15–80)	(16–51)	(15–80)		(26–66)	
AdoMet/AdoHcy (ratio)	(Median)	4.1	3.7	4.9	NS	2.5	0.005
	(Range)	(1.2–16.8)	(1.5–5.8)	(1.2–16.8)		(1.5–4.0)	

NS Not significant

* See methods for actual number of values since fewer results were available for some metabolites

** Independent samples Mann-Whitney U test <0.05 was significant for treated vs. untreated congenital remethylation defects

[a] Independent samples Mann-Whitney U test <0.05 was significant for congenital remethylation defects vs. acquired vitamin deficiency

Table 3 shows values in the congenital remethylation disorders comparing the untreated ($N = 8$) vs. treated ($N = 39$). THcy was higher and AdoMet was lower without treatment, but cystathionine and methionine were not different. Table 3 also shows that values in the group with acquired vitamin deficiency were very similar to those with congenital defects.

Because there were either no or only minor differences in these metabolites due to treatment of the genetic remethylation defects, and because the differences between the remethylation defects and acquired vitamin deficiencies were also minor, these groups were all combined in Table 4 as a single remethylation group for comparison with values from controls and from the case family members when tHcy was elevated (>14 umol/L). The results for each metabolite are as follows:

tHcy

The median tHcys was higher in CBS deficiency than in those with remethylation defects, 124.8 vs. 79.4 umol/L, but the 25–75 % interquartile ranges were very similar, that is, 68.9–166.8 vs. 53.0–108.4 umol/L, respectively. Thus, any specific value of tHcy cannot be used to distinguish between these two types of defects.

Cystathionine

The median cystathionine for CBS-deficient persons was 40 compared to 839 nmol/L for those with remethylation defects (0.001). As shown in Fig. 3, serum cystathionine showed an almost complete separation between these conditions despite the treated status of many of the patients. Figure 3 shows also that about 50 % of those with CBS deficiency had cystathionine values too low to be accurately measured by the method used. Such values are shown arbitrarily as 25 nmol/L, the value used in the statistical calculations. Even the two CBS-deficient subjects (not case family members) with tHcy in the reference range had cystathionine too low to be reliably measured. There were 19 subjects with remethylation defects who presented with elevated tHcys and who also had simultaneous cystathionine values in the reference range, but the median value was markedly higher at 241 nmol/L than the median for the 102 CBS-deficient subjects with elevated tHcy, 25 nmol/L (<0.001).

Table 4 Median (interquartile range) of metabolites and ratios in CBS deficiency vs. remethylation defects, controls, and case family

		CBS Def $N = 118$*	Remethylation defects $N = 132$*	Sig**	Controls $N = 49$	ANOVA***	Family $N = 6$[b]	Sig[a]
tHcy (umol/L)	Median	124.8	79.4	<0.001	7.4	<0.001	58.4	NS
	(25–75 %)	(68.9–166.8)	(53.0–108.4)		(6.3–9.2)		(15.7–222)	
Cysta (nmol/L)	Median	40[c]	839	<0.001	157[c]	<0.001	206	<0.001
	(25–75 %)	(25–76)	(471–1,248)		(121–198)		(135–295)	
Met (umol/L)	Median	160	18.6[c]	<0.001	22.4[c]	<0.001	44.8	0.004
	(25–75 %)	(47–813)	(13.4–28.7)		(18.6–27)		(27.8–135.0)	
tCys (umol/L)	Median	136	221	<0.001	289	<0.001	285	NS
	(25–75 %)	(77–201)	(177–262)		(262–318)		(133–327)	
AdoMet (nmol/L)	Median	488	104[c]	<0.001	107[c]	<0.001	189	0.011
	(25–75 %)	(155–868)	(93–159)		(93–121)		(115–1,282)	
AdoHcy (nmol/L)	Median	73	38[c]	0.008	14[c]	<0.001	48	NS
	(25–75 %)	(24–382)	(25–49)		(12–18)		(20–1,539)	
AdoMet/AdoHcy (ratio)	Median	3.8[c]	3.2[c]	NS	7.1	0.015	5.2	0.039
	(25–75 %)	(1.8–8.9)	(2.3–4.3)		(6.4–9.0)		(0.8–8.41)	
tHcy/Cysta (ratio)	Median	2.8	0.1[c]	<0.001	0.05[c]	<0.001	0.2	NS
	(25–75 %)	(1.1–4.8)	(0.06–0.18)		(0.04–0.06)		(0.08–1.60)	
tHcy/Met (ratio)	Median	0.5[c]	3.8	<0.001	0.4[c]	<0.001	1.2	0.004
	(25–75 %)	(0.2–1.5)	(2.0–7.6)		(0.3–0.4)		(0.5–1.9)	
Cysta/Met (ratio)	Median	0.2[c]	46.0	<0.001	6.6[c]	<0.001	4.6	<0.001
	(25–75 %)	(0.05–1.0)	(19.7–85.7)		(5.6–9.3)		(1.0–7.5)	
Met/tCys (ratio)	Median	1.5	0.09[c]	<0.001	0.08[c]	<0.001	0.1	0.013
	(25–75 %)	(0.2–6.9)	(0.06–0.13)		(0.06–0.09)		(0.09–1.0)	
tHcy/tCys (ratio)	Median	1.0	0.4	<0.001	0.03	<0.001[d]	0.2	NS
	(25–75 %)	(0.3–1.7)	(0.2–0.5)		(0.02–0.03)	0.019[d]	(0.5–1.7)	
tCys/Cysta (ratio)	Median	2.6	0.2	<0.001	1.8	<0.001	1.6	<0.001
	(25–75 %)	(1.6–4.2)	(0.1–0.4)		(1.5–2.4)		(0.8–1.8)	

* See Methods for actual number of values of each metabolite since some metabolites had missing values comparing CBS deficiency vs. remethylation defects

** Independent samples Mann-Whitney U test <0.05 was significant for CBS deficiency vs. remethylation defects

***,[c] ANOVA – For a given metabolite or ratio, values sharing a superscript are not significantly different from each other. (Case family values not included due to small "N"). Significance for the multiple comparisons shown for CBS deficiency vs. remethylation defects vs. controls. Post hoc test shown; Scheffe

[a] Independent samples Mann-Whitney U test <0.05 was significant for remethylation defects vs. the family when tHcy ≥14umol/L

[b] Median and actual range of values from samples when tHcys ≥14 umol/L

[d] Significance for CBS deficiency vs. remethylation defects vs. controls <0.001, for remethylation defects vs. controls, 0.019

Methionine

The median methionine value was much higher in the CBS-deficient individuals than in those with remethylation defects, 160 vs. 18.6 umol/L (<0.001). However, as shown in the plot of methionine against tHcy (Fig. 4), when tHcy is low there is some overlap between the two groups. As tHcy increases to over 100 umol/L, the methionine values in CBS-deficient subjects increase markedly and overlap little with the levels for remethylation disorders.

Total Cysteine

Median total cysteine was almost 50 % lower in CBS-deficient patients compared to those with remethylation defects, 136 vs. 221 umol/L (<0.001). A plot of total cysteine against tHcy (Fig. 5) shows that, in general, patients with remethylation defects had normal total cysteine although occasional subjects had low values, and that most CBS-deficient subjects had values below the reference range, even when treated.

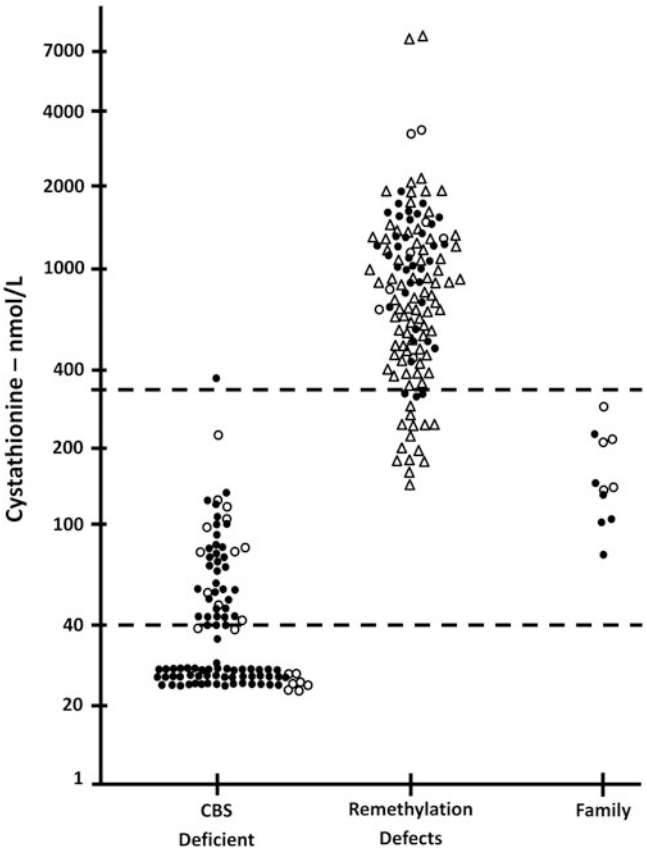

Fig. 3 Values of serum or plasma cystathionine are shown for CBS-deficient patients, those with remethylation defects and the case family subjects. A previously determined reference range is shown by the *dotted lines* (44–342 nmol/L). Many subjects with CBS deficiency had cystathionine levels below the limits of detection and they are arbitrarily shown as 25 nmol/L. *Closed symbols* denote treated patients, *open symbols* untreated patients. For remethylation disorders, triangles denote acquired cobalamin or folate deficiency, *circles* denote congenital remethylation defects

AdoMet

Median AdoMet was higher in the CBS-deficient individuals compared to those with remethylation defects: 488 vs. 104 nmol/L (<0.001). A plot of AdoMet against tHcy (Fig. 6) shows that AdoMet values for those with CBS deficiency were generally higher than those for subjects with remethylation defects, especially when tHcy was greater than 100 umol/L, and that those with remethylation defects were generally in the normal range or slightly elevated, rather than low. An AdoMet above 425 nmol/L was found only in CBS deficiency.

AdoHcy

Those with CBS deficiency had a higher median value of AdoHcy than did those with remethylation defects: 73 vs. 38 nmol/L (0.005). As with AdoMet, the values for AdoHcy became markedly elevated in CBS-deficient individuals when tHcy was > 100 umol/L (Fig. 7). An AdoHcy value above 100 nmol/L was seen only in CBS deficiency.

Ratios Between Metabolites

Ratios between different metabolites were analyzed to see whether a more clear distinction between CBS deficiency and remethylation defects would be apparent (Table 4). With the exception of the AdoMet/AdoHcy ratio, all the metabolite ratios analyzed were markedly different between these two groups (0.001). Compared to controls, those with CBS deficiency had statistically different ratios for tHcy/Cysta, Met/tCys. tHcy/tCys, and tCys/Cysta; those with remethylation defects, different ratios for tHcy/Met, Cysta/Met, tHcy/tCys, and tCys/Cysta compared to controls.

Discussion

B6 Responsiveness of the Case Family Members

There was a remarkable response to pyridoxine in this family, with the tHcy levels falling to as low as 6.0, 8.6, and 5.8 umol/L in the proband, her brother, and niece. Among the published values for tHcy during treatment of CBS deficiency there have been few this low (Gaustadnes et al. 2002; Kelly et al. 2003; Ducros et al. 2006; Bermúndez et al. 2006; Weiss et al. 2006; Wilcken et al. 2006; Varlibas et al. 2009; Skovby et al. 2010; Novy et al. 2010; Kanzelmeyer et al. 2011). The definition of pyridoxine responsiveness in CBS deficiency is usually that the posttreatment values of tHcy are less than 50–60 umol/L (Kluijtmans et al. 1999; Yap et al. 2001). In view of the fact that the p.G307S mutation in the homozygous state is clearly B6-nonresponsive, and that even compound heterozygotes carrying p.G307S and the highly responsive p.I278T mutation or any one of at least six other point mutations are nonresponsive (evidence summarized in (Mudd 2011), the unusual responsiveness in the case family is apparently conferred by the second mutation, p.P49L. Compound heterozygotes carrying p.P49L as well as either deletion ΔG151-A159 (de Franchis et al. 1998), or point mutation p.E144K or p.R125Q are B6-responsive (Gaustadnes et al. 2002; Cozar et al. 2011). Expressed in *E. coli* and assayed either without or with addition of pyridoxal phosphate, the activity of the p.P49L mutant was found to be 2 % and 71 %, respectively, of wild type (Cozar et al. 2011) or 91 % and 82 % of wild type (Majtan et al. 2010). The difference between the relative activities without added pyridoxal phosphate may perhaps be due to the use of a stronger promoter and expression with a fusion

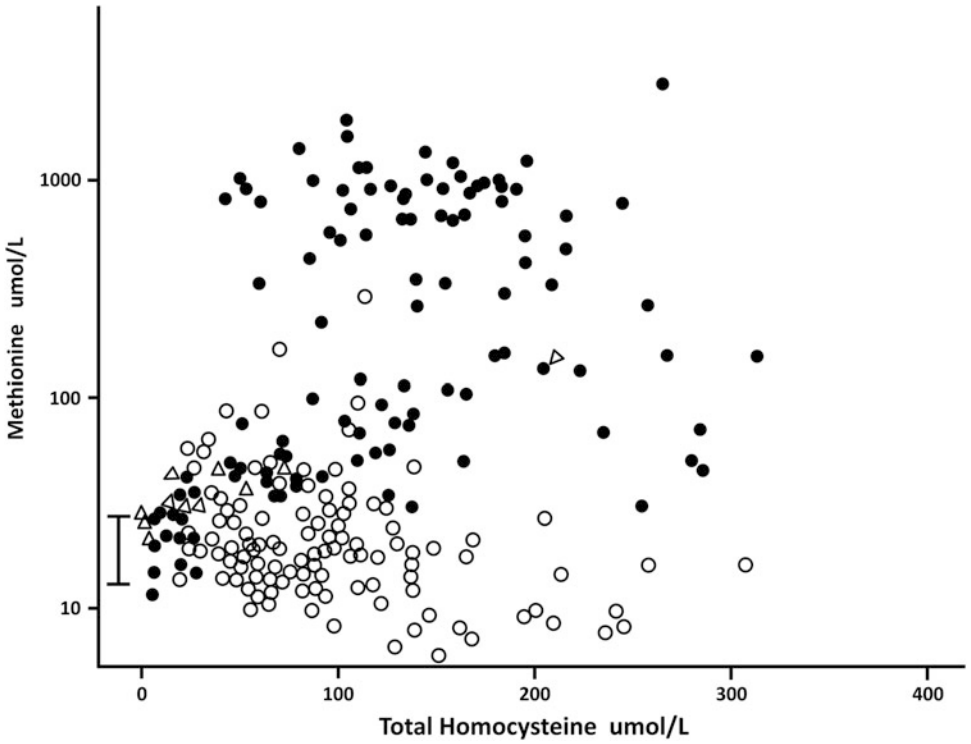

Fig. 4 The serum or plasma total homocysteine is plotted against the serum or plasma methionine in three groups of patients. The reference range of serum methionine is shown by the bar, 14–43 umol/L. Patients with CBS deficiency are shown as *closed circles*, patients with remethylation defects are *open circles*. The case family members are shown as *open triangles*

partner in the studies of Majtan and coworkers. The close to wild-type activity of p.P49L in both studies fits with the relatively low tHcy values on B6 of compound heterozygotes carrying this mutation.

Limitations in the Current Diagnosis of Elevated tHcy

That the diagnosis of CBS deficiency was made for the case family only as long as 37 years after the proband had had a deep vein thrombosis and 13 years after she was found to have elevated tHcy may be attributed to several limitations of current diagnostic and management practices. Because these difficulties may be relevant for other patients, they will be discussed in the sections that follow.

Expectation That CBS-Deficient Patients Will Have Clinical Abnormalities in Addition to Vascular Problems

Based on the initial 20 or more years experience with homocystinuria due to CBS deficiency, it was apparent that this condition often presents with some combination of mental retardation, dislocated optic lenses, skeletal manifestations, and thrombotic disease (Mudd et al. 1985). However, in recent years it has become increasingly evident that it may also present by means of thromboembolic episodes only, without the other abnormalities (Skovby et al. 2010; Mudd 2011). At least 29 patients with thrombotic presentation in adolescence or adulthood without ectopic lenses, major skeletal or CNS abnormalities, have been reported prior to this article (Newman and Mitchell 1984; Marchal et al. 1989; Cochran et al. 1990; Favre et al. 1992; Lu et al. 1996; Gaustadnes et al. 2002; Gaustadnes et al. 2000a, b; Maclean et al. 2002; Kelly et al. 2003; Linnebank et al. 2003; Sueyoshi et al. 2004; Ducros et al. 2006; Wilcken et al. 2006; Chauveheid et al. 2008; Skovby et al. 2010; Novy et al. 2010; Magner et al. 2011; Cozar et al. 2011), but the true prevalence of such individuals remains unknown. Of those 29 patients, 18 were proven to be B6-responsive, and 6 may be presumed to be responsive because they were homozygotic for the p.I278T mutation. For those responders with data reported, the mean tHcy was 209.2 umol/L when not on B6 (range 58–285 umol/L), and 21.1 umol/L while on B6 (range 8–49 umol/L). For four patients, B6 responsiveness was questionable, but none were clearly not responsive. The available evidence further suggests that perhaps the predominant portion of B6 responders such as p.I278T homozygotes may be clinically normal, without even thromboembolic episodes (Skovby et al. 2010; Magner et al. 2011). Thus, underdiagnosis of CBS deficiency is clearly possible if the diagnosis is made only on the basis of clinical abnormalities.

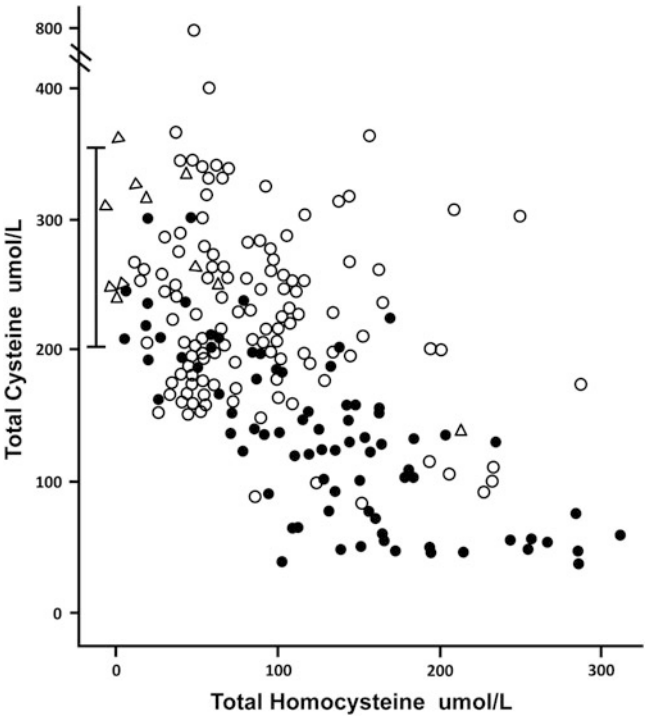

Fig. 5 The serum or plasma total homocysteine is shown plotted against the serum or plasma total cysteine in the three groups of patients. The patients with CBS deficiency are shown as *closed circles*, the patients with remethylation defects are *open circles*, and the case family members are shown as *open triangles*. The reference range for total cysteine is shown by the bar, 200–361 umol/L

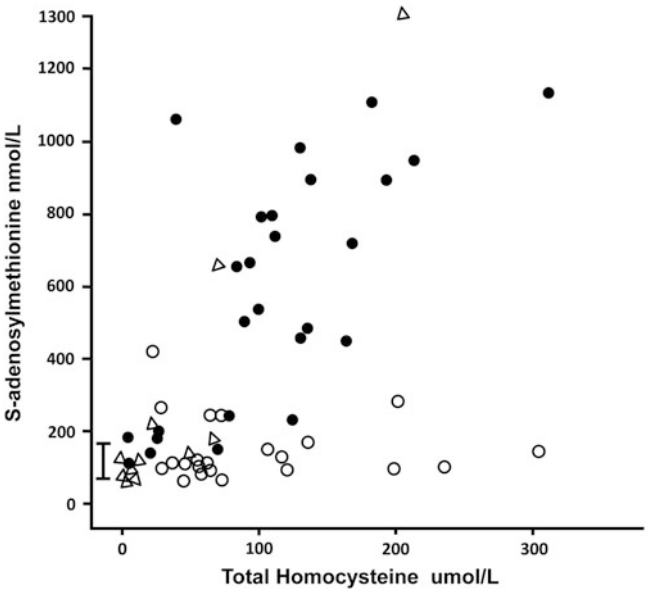

Fig. 6 The serum or plasma total homocysteine is shown plotted against the plasma or serum S-adenosylmethionine values. The reference range is shown by the bar, 71–168 nmol/L. The patients with CBS deficiency are shown as *closed circles*, the patients with remethylation disorders as *open circles*, and the case family members are shown as *open triangles*

Failure of Newborn Screening for Elevated Methionine to Detect B6-Responsive CBS Deficiency

Newborn screening for CBS deficiency, usually based on detection of blood methionine elevation, started as early as 1968 in Massachusetts, and is now mandatory throughout the United States (http://genes-r-us.uthscsa.edu/nbsdisorders.pdf). However, it is widely acknowledged that newborn screening for elevated methionine detects mainly B6 nonresponsive forms of CBS deficiency (Mudd et al. 1985; Peterschmitt et al. 1999; Sokolova et al. 2001; Gan-Schreier et al. 2010). Although worldwide such forms are estimated to have prevalences of 1 in 58,000 to 1,000,000 (Mudd 2011). The results of molecular genetic screening predict that CBS deficiency may often be far more common. For example, homozygosity for the p.I278T CBS mutation may occur as often as 1:20,400 in Denmark (Gaustadnes et al. 1999), and the deficiency due to the most common mutations may be as high as 1:6400 in Norway (Refsum et al. 2004), or 1:15,500 in the Czech Republic (Sokolova et al. 2001; Janosík et al. 2009).

B6-responsive patients when taking even minimal doses of B6 may have metabolite values that are normal or so close to normal as to prevent diagnosis.

To establish CBS deficiency and to distinguish that abnormality from other causes *of elevated tHcy, assays of tHcy must be accompanied by assays of other relevant metabolites*

The results for the initial assays carried out at the University of Colorado for each member of the case family were entirely normal. Indeed the initial tHcy values were so low that lab error was considered as a possibility. In retrospect, this was the situation because each was taking vitamins including B6 at the time. After withdrawal of vitamin supplementation, the metabolite patterns of the proband and her brother became diagnostic of CBS deficiency. Vitamin B6 is commonly added to energy bars, drinks, diet supplements, etc., and is present in multi-vitamins in the United States and Canada. It seems possible that vitamin intake of which neither the patients nor the physicians were aware may have caused the apparently erratic variations in the metabolite levels in the proband and her brother and thus played a role in obscuring the diagnosis. Despite this marked vitamin responsiveness, and the lack of ocular, CNS, or skeletal manifestations, two members of the family had had life-threatening thrombotic events. Of note, both had such events while on oral contraceptives, an additional risk factor for thrombotic events.

Taking into account the newly compiled metabolite data presented in this report, the relative contributions of the various metabolites toward establishing the diagnosis for

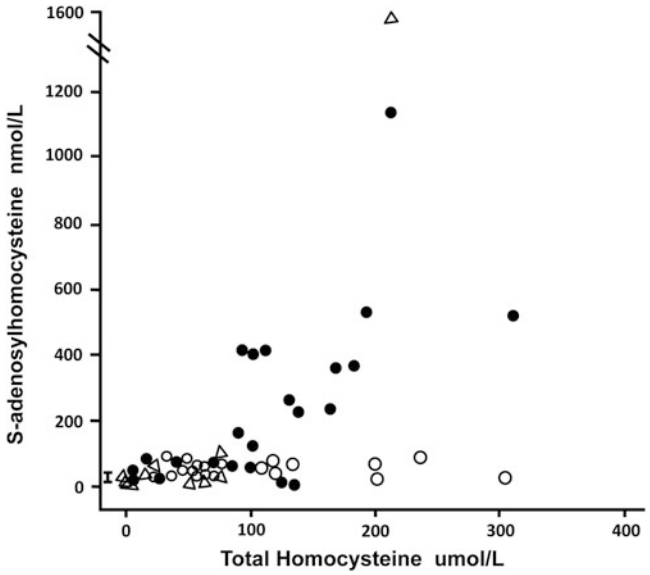

Fig. 7 The serum or plasma total homocysteine is shown plotted against the plasma or serum S-adenosylhomocysteine values in the three groups of patients. The reference range is shown by the bar, 8–26 nmol/L. The patients with CBS deficiency are shown as *closed circles*, those with remethylation defects with *open circles*, and the case family members as *open triangles*

the case family and distinguishing between CBS deficiency and remethylation defects may be characterized as follows:

Very helpful diagnostically for this family at times of elevated tHcy were the elevations of AdoMet (Fig. 6) that occurred in the brother even when methionine was barely elevated (Table 1), as well as the rise of AdoHcy (Fig. 7), although that occurred most markedly only in the proband (Table 1). Taken together with the sparse data in the literature about AdoMet and AdoHcy values in CBS deficiency and remethylation disorders (Maclean et al. 2002; Stabler et al. 2002; Orendäc et al. 2003; Guerra-Shinohara et al. 2007), our data indicate that in remethylation defects serum or plasma AdoMet is usually normal to mildly elevated, and AdoHcy is modestly increased over the reference range. In contrast, in CBS deficiency AdoMet and AdoHcy may be strikingly elevated, increasing as the tHcy rises above 100 umol/L (Figs. 6 and 7). Thus, assays of AdoMet and AdoHcy may have strong diagnostic utility in separating CBS deficiency from remethylation defects. A limitation is that sample handling, storage, and preparation variables can cause major loss of AdoMet and increases in AdoHcy, factors that have limited their utility in the study of archived samples (Stabler and Allen 2004).

Elevations of methionine are expected in CBS deficiency and are used for screening for that condition (Mudd 2011), and monitoring compliance with methionine-restricted diets, an important part of the treatment of B6 nonresponsive patients. Such elevations, when present, were very useful in establishing the diagnosis for the case family. However, it is not uncommon for patients who present with only thrombotic manifestations of CBS deficiency to have methionines that are within the reference range or only mildly elevated (Lu et al. 1996; Ducros et al. 2006; Novy et al. 2010). Inborn errors of remethylation usually lead to normal or low serum methionine, but methionine values are dependent on the intake of methionine, and methionine was elevated in some of those with remethylation defects (Fig 4), possibly due to liver disease. Acquired cobalamin or folate deficiencies rarely have low methionine (Stabler et al. 1988). Together, these considerations indicate that homocystinuria due to CBS deficiency should not be ruled out based on a normal or only slightly elevated serum methionine value.

Another potentially useful (but rarely employed) diagnostic maneuver is the methionine loading test. However, the results in highly responsive CBS-deficient patients on pyridoxine have not been well studied. Renal insufficiency, common in adults with vascular disease, also causes elevated post-load tHcy and could lead to confusing results.

The results compiled in Fig. 3 show that assay of serum cystathionine may be very useful in distinguishing CBS deficiency (tendency to low cystathionine) from remethylation defects (tendency to elevated cystathionine), even while on vitamin therapy, yet caution is necessary, as shown by the results for the case family whose cystathionine values were generally in the normal range and overlapped the lower end of the range of patients with remethylation defects.

As already mentioned in "Results," total cysteine tends to be lower in CBS deficiency than in remethylation defects, but there is overlap, even when tHcy is high. For the case family, only the proband's total cysteine was below the reference range at a time the tHcy was extremely elevated.

To determine if, in addition to being helpful in distinguishing CBS-deficient individuals from those with remethylation defects, ratios in the family members might be suggestive even when the metabolite absolute values were in the normal range, the ratios from the family members in the six samples in which tHcy was elevated were analyzed (Table 4) and compared to the group with remethylation defects. The most striking differences were that the cysta/met ratio was tenfold less, and the tCys/Cysta ratio was eightfold higher in the family vs. remethylation defects.

Issues Relative to the Diagnosis and Management of Individuals with Elevated tHcy

Management issues arise when an undiagnosed patient with CBS deficiency sustains a thrombotic event in adult life. Assay of tHcy is part of the thrombophilia diagnostic panels in many clinical laboratories and hospitals. The most common cause of an elevated homocysteine value in

folate-fortified populations is cobalamin deficiency. Appropriately, methylmalonic acid is assayed and, if normal, as in the proband's case, can eliminate vitamin B12 deficiency. The availability of secondary metabolite testing for an adult general medical patient found to have elevated tHcy is quite limited. Cystathionine assays can be very valuable (Fig. 3), and are available from commercial reference labs in the United States. It is important that sensitive and specific quantitative assays be employed because the standard amino acid analyzer–based methods often define cystathionine normals as <1,000 nmol/L (occasionally 5–10 umol/L), levels that would not distinguish remethylation defects from CBS deficiency. Figure 3 shows that only a minority of subjects with remethylation defects have cystathionine >1,000 nmol/L. Mutation analysis of CBS can be used to confirm the suspected diagnosis in patients with elevated homocysteine although the yield will be better if they are preselected for elevated methionine and lack of clinical features consistent with methylation defects.

Primary care providers and hematologists reviewing homocysteine data frequently employ an empiric treatment approach with high-dose folic acid, vitamin B12, and occasionally vitamin B6, yet vitamin administration to patients with elevated tHcy prior to further testing may be inadvisable, especially for a patient presenting with a thrombotic episode only. Such patients (if similar to the case family members) are the ones most likely to completely normalize tHcys, and other methionine metabolites when taking vitamin supplements, so that to subsequently obtain an accurate diagnosis, the consulting service must decide whether to discontinue the vitamins and re-assay after a 1–2 month "washout period." This does put the patient at some risk for a thrombotic event, although many such subjects will be on anticoagulation treatment and perhaps have less risk. It was only after all vitamin supplements had been discontinued in the proband and her brother that the metabolic pattern diagnostic of CBS deficiency became apparent, and was then confirmed by molecular genetic studies. A definite diagnosis is important for optimal disease management, prognosis assessment, and reproductive counseling. Because the family members were so responsive to vitamin B6, other typical measures (such as betaine- or methionine restriction) were not instituted but do remain as therapeutic options in the future. Many patients benefit from these additional treatment measures.

Summary

We conclude that there are likely individuals with highly responsive CBS deficiency presenting in adult life, particularly with thrombotic disease, who could be diagnosed by assays of a complete panel of methionine and related metabolites, especially before vitamin therapy is started or after its withdrawal. The cystathionine level has strong discriminatory value in distinguishing hyperhomocysteinemia due to CBS deficiency from remethylation defects and is commercially available with the caveat that only assays sensitive in the low nanomolar range are useful. Serum or plasma AdoMet may be markedly elevated in CBS deficiency and may be diagnostically useful, even when methionine is barely elevated. However, commercial assay is not widely available at present.

Further research on the prevalence of highly responsive CBS deficiency in thrombophilia clinics is clearly needed since CBS deficiency is one of the few causes of thrombophilia that requires therapy other than anticoagulation. It is also important to screen asymptomatic family members.

Acknowledgments We would like to acknowledge the expert technical assistance of Carla Ray, Linda Farb, and Bev Raab. Preparation of the manuscript was assisted by Theresa M. Martinez.

Take-Home Message

In connection with the puzzling history of a family with B6-responsive cystathionine B-synthase deficiency without decreased cystathionines, and usually with normal methionines, extensive data are presented on values of relevant metabolites that may facilitate the differential diagnosis of elevated total homocysteine.

Contributions of Authors

SPS, RHA, and SHM were responsible for planning the details in the manuscript. SPS, MK, RJ, RHA, JPK, EBS, CW, and SHM were all involved in the conduct of obtaining the clinical and laboratory measures described in the manuscript. SPS, JPK, CW, and SHM wrote the bulk of the manuscript with contributions from the other authors.

Guarantor for Article

Sally P. Stabler serves as the guarantor for this article, accepting full responsibility for the work. She had access to all the data and controlled the decision to publish.

Competing Interest Statement

Two of the authors, Sally P. Stabler and Robert H. Allen and the University of Colorado have competing interests. A patent on the use of homocysteine, methylmalonic acid, and cystathionine had been obtained by the University

of Colorado and the two authors, which is now expired. A company has been formed at the University of Colorado to assay homocysteine, methylmalonic acid, and cystathionine. The other authors do not have competing interests.

Funding for Research

JPK was supported by the American Heart Association Grant NA09GRNT2110159. The authors confirm independence from the sponsors; the content of this article has not been influenced by the sponsors.

Ethics Approval

The compiled data came from studies previously reported which were under the purview of Institutional Review Boards as listed in the References. In addition, Protocol # 00–664 – "Methionine Metabolites in Inborn Errors" – was approved by the Colorado Multiple Institutional Review Board. Personal patient information for the case family is not included in the manuscript.

References

Allen RH, Stabler SP, Lindenbaum J (1993) Serum betaine, N, N-dimethylglycine and N-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. Metabolism 42:1448–1460

Bermúndez M, Frank N, Bernal J, Urreizti R, Briceño I, Begoña M, Perez-CerdÄ C, Ugarte M, Grinberg D, Balcells S, Kraus JP (2006) High prevalence of *CBS* p.T191M mutation in homocystinuric patients from Columbia. Hum Mutat 27:296–302

Capdevila A, Wagner C (1998) Measurement of plasma *S*-adenosylmethionine and *S*-adenosylhomocysteine as their fluorescent isoindoles. Anal Biochem 264:180–184

Chauveheid M-P, Lidove O, Papo T, Laissy J-P (2008) Adult-inset homocystinuria arteriography mimics fibromuscular dysplasia. Am J Med 121:e5–e6

Cochran FB, Sweetman L, Schmidt K, Barsh G, Kraus J, Packman S (1990) Pyridoxine-unresponsive homocystinuria with an unusual clinical course. Am J Med Genet 35:519–522

Cozar M, Urreizti R, Vilarinho L, Grosso C, Dodelson de Kremer R, Asteggiano CG, Dalmau J, Garcia AM, Vilaseca MA, Grinberg D, Balcells S (2011) Identification and functional analyses of CBS alleles in Spanish and Argentinian homocystinuric patients. Hum Mutat 32:835–842

de Franchis R, Sperandeo MP, Sebastio G, Andria G, The Italian Collaborative Study Group on Homocystinuria (1998) Clinical aspects of cystathionine β-synthase deficiency: how wide is the spectrum? Eur J Pediatr 157(Suppl 2):S67–S70

Ducros V, Rousset J, Garambois K, Boujet C, Rolland MO, Valenti K, Bouillet L, Jaillard A, Favier A (2006) Severe hypermethioninemia revealing homocystinuria in two young adults with mild phenotype. Revue de Medécine Interne 27:140–143

Favre JP, Becker F, Lorcerie B, Dumas R, David M (1992) Vascular manifestations in homocystinuria. Ann Vasc Surg 6:294–297

Gan-Schreier H, Kebbewar M, Fang-Hoffman J, Wilrich J, Abdoh G, Ben-Omran T, Shahbek N, Bener A, Al Rifai H, Al Khal AL, Lindner M, Zschocke J, Hoffman GF (2010) Newborn population screening for classic homocystinuria by determination of total homocysteine from Guthrie cards. J Pediatr 156:427–432

Gaustadnes M, Ingerslev J, Rütiger N (1999) Prevalence of congenital homocystinuria in Denmark. N Engl J Med 340:1513

Gaustadnes M, Rüdiger N, Rasmussen K, Ingerslev J (2000a) Intermediate and severe hyperhomocysteinemia with thrombosis: a study of genetic determinants. Thromb Haemost 83:554–558

Gaustadnes M, Rüdiger N, Rasmussen K, Ingerslev J (2000b) Familial thrombophilia associated with homozygosity for the cystathionine beta-synthase 833T - C mutation. Arterioscler Thromb Vasc Biol 20:1392–1395

Gaustadnes M, Wilcken B, Oliveriusova J, McGill J, Fletcher J, Kraus JP, Wilcken DEL (2002) The molecular basis of cystathionine β-synthase deficiency in Australian patients: genotype-phenotype correlations and response to treatment. Hum Mutation 20:117–126

Guerra-Shinohara EM, Morita OE, Pagliusi RA, Blaia-d'Avila VL, Allen RH, Stabler SP (2007) Elevated serum *S*-adenosylhomocysteine in cobalamin-deficient megaloblastic anemia. Metabolism 56:339–347

Janosík M, SokolovÄ J, JanosíkovÄ B, Krijt J, KlatovskÄ V, Kozich V (2009) Birth prevalence of homocystinuria in Central Europe: frequency and pathogenicity of mutation c.1105C>T (p.R369C) in the cystathionine beta-synthase gene. J Pediatr 154:431–437

Kanzelmeyer N, Tsikas D, Chobanyan-Jürgens K, Beckmann B, Vaske B, Illsinger S, Das AM, Lücke T (2011) Asymmetric dimethyhlarginine in children with homocystinuria or phenylketonuria. Amino Acids. doi:DOI 10.1007/s00726-011-0892-4

Keating AK, Freehauf C, Jiang H, Brodsky GL, Stabler SP, Allen RH, Graham DK, Thomas JA, Van Hove JLK, Maclean KN (2011) Constitutive induction of pro-inflammatory and chemotactic cytokines in cystathionine beta-synthase deficient homocystinuria. Mol Genet Metab 103:330–337

Kelly PJ, Furie KL, Kistler JP, Barron M, Picard EH, Mandell R, Shih VE (2003) Stroke in young patients with hyperhomocysteinemia due to cystathionine beta-synthase deficiency. Neurology 60:275–279

Kluijtmans LAJ, Boers GHJ, Kraus JP, van den Heuvel LPWJ, Cruysberg JRM, Trijbels FJM, Blom HJ (1999) The molecular basis of cystathionine β-synthase deficiency in Dutch patients with homocystinuria: effect of CBS genotype on biochemical and clinical phenotype, and on response to treatment. Am J Hum Genet 65:59–67

Linnebank M, Junker R, Nabavi DG, Linnebank A, Koch HG (2003) Isolated thrombosis due to the cystathionine β-synthase mutation c.833T>C (I278T). J Inherit Metab Dis 26:509–511

Lu C-Y, Hou J-W, Wang P-J, Chiu H-H, Wang T-R (1996) Homocystinuria presenting as fatal common carotid artery occlusion. Pediatr Neurol 15:159–162

Maclean KN, Gaustadnes M, Oliveriusova J, Janosik M, Kraus E, Kozich V, Kery V, Skovby F, Rüdiger N, Ingerslev J, Stabler SP, Allen RH, Kraus JP (2002) High homocysteine and thrombosis without connective tissue disorders are associated with a novel class of cystathionine β-synthase (CBS) mutations. Hum Mutation 19:641–655

Magner M, KrupkovÄ L, Honzik T, Zeman J, HyÄnek J, Kozich V (2011) Vascular presentation of cystathionine beta-synthase deficiency in adulthood. J Inherit Metab Dis 34:33–37

Majtan T, Liu L, Carpenter JF, Kraus JP (2010) Rescue of cystathionine beta-synthase (CBS) mutants with chemical chaperones: purification and characterization of eight CBS mutant enzymes. J Biol Chem 285:15866–15873

Marchal G, Giroud M, Nivelon A, Saudubray JM, Becker F, Martin F, Dumas R (1989) Révélation tardive d'une homocystinurie par

une aphasie et un spasme des artères iliaques externes. Ann Med Interne 140:520–522

Mudd SH (2011) Hypermethioninemias of genetic and non-genetic origin: a review. Am J Med Genet Part C 157:3–32

Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, Andria G, Boers GHJ, Bromberg IL, Cerone R, Fowler B, Grobe H, Schmidt H, Schweitzer L (1985) The natural history of homocystinuria due to cystathionine β-synthase deficiency. Am J Hum Genet 37:1–31

Mudd SH, Cerone R, Schiaffino MC et al (2001) Glycine N-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninemia. J Inherit Metab Dis 24:448–464

Newman G, Mitchell JRA (1984) Homocystinuria presenting as multiple arterial occlusions. Q J Med 210:251–258

Novy J, Ballhausen D, Bonafé L, Cairoli A, Angelillo-Scherrer A, Bachmann C, Michel P (2010) Recurrent postpartum cerebral sinus vein thrombosis as a presentation of cystathionine beta-synthase deficiency. Thromb Haemost 103:871–873

Orendäc M, Zeman J, Stabler SP, Allen RH, Kraus JP, Bodamer O, Stöckler-Ipsiroglu S, Kvasnicka J, Kožich V (2003) Homocystinuria due to cystathionine β-synthase deficiency: novel biochemical findings and treatment efficacy. J Inherit Metab Dis 26:761–773

Peterschmitt MJ, Simmons JR, Levy HL (1999) Reduction of false negative results in screening of newborns for homocystinuria. N Engl J Med 341:1572–1576

Refsum H, Fredriksen A, Meyer K, Ueland PM, Kase BF (2004) Birth prevalence of homocystinuria. J Pediatr 144:830–832

Savage D, Gangaidzo I, Lindenbaum J, Kiire C, Mukiibi JM, Moyo A, Gwanzura C, Mudenge B, Bennie A, Sitima J, Stabler SP, Allen RH (1994) Vitamin B_{12} deficiency is the primary cause of megaloblastic anaemia in Zimbabwe. Br J Haematol 86:844–850

Skovby F, Gaustadnes M, Mudd SH (2010) A revisit to the natural history of homocystinuria due to cystathionine β-synthase deficiency. Mol Genet Metab 99:1–3

Sokolova J, Janosikova B, Terwilliger JD, Freiberger T, Kraus JP, Kozich V (2001) Cystathionine beta-synthase deficiency in Central Europe: discrepancy between biochemical and molecular genetic screening for homocystinuric alleles. Hum Mutation 18:548–549

Stabler SP, Allen RH (2004) Quantification of serum and urinary S-adenosylmethionine and S-adenosylhomocysteine by stable-isotope-dilution liquid chromatography-mass spectrometry. Clin Chem 50:365–372

Stabler SP, Marcell PD, Podell ER, Allen RH, Savage DG, Lindenbaum J (1988) Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography–mass spectrometry. J Clin Invest 81:466–474

Stabler SP, Lindenbaum J, Savage DG, Allen RH (1993) Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. Blood 81:3404–3413

Stabler SP, Steegborn C, Wahl MC, Oliveriusova J, Kraus JP, Allen RH, Wagner C, Mudd SH (2002) Elevated plasma total homocysteine in severe MAT I/III deficiency. Metabolism 51:981–988

Strauss KA, Morton DH, Puffenberger EG, Hendrickson C, Robinson DL, Wagner C, Stabler SP, Allen RH, Chwatko G, Jakubowski H, Niculescu MD, Mudd SH (2007) Prevention of brain disease from severe 5,10-methylenetetrahydrofolate reductase deficiency. Mol Genet Metab 91:165–175

Sueyoshi E, Sakamoto I, Ashizawa K, Hayashi K (2004) Pulmonary and lower extremity vascular lesions in a patient with homocystinuria: radiologic findings. Am J Radiology 182:830–831

Tangerman A, Mudd SH, Wilcken B, Boers GHJ, Levy HL (2000) Methionine transamination metabolites in patients with homocystinuria due to cystathionine β-synthase deficiency. Metabolism 49:1071–1077

Varlibas F, Cobanoglu O, Ergin B, Tireli H (2009) Different phenotypy in three siblings with homocystinuria. Neurologist 15:144–146

Weiss N, Demeret S, Sonneville R, Guillevin R, Bolgert F, Pierrot-Deseilligny C (2006) Bilateral internal carotid artery dissection in cystathionine beta-synthase deficiency. Eur Neurol 55:177–178

Wilcken DEL, Wang J, Sim AS, Green K, Wilcken B (2006) Asymmetric dimethylarginine in homocystinuria due to cystathionine β-synthase deficiency: Relevance of renal function. J Inherit Metab Dis 29:30–37

Yaghmai R, Kashani AH, Geraghty MT, Okoh J, Pomper M, Tangerman A, Wagner C, Stabler SP, Allen RH, Mudd SH, Braverman N (2002) Progressive cerebral edema associated with high methionine levels and betaine therapy in a patient with cystathionine β-synthase (CBS) deficiency. Am J Med Genet 108:57–63

Yap S, Boers GHJ, Wilcken B, Wilcken DEL, Brenton DP, Lee PJ, Walter JH, Howard PM, Naughten ER (2001) Vascular outcome in patients with homocystinuria due to cystathionine β-synthase deficiency treated chronically. A multicenter observational study. Arterioscler Thromb Vasc Biol 21:2080–2085

JIMD Reports
DOI 10.1007/8904_2013_236

ORIGINAL ARTICLE

Fatty Acid Oxidation Disorders in a Chinese Population in Taiwan

Yin-Hsiu Chien · Ni-Chung Lee · Mei-Chyn Chao · Li-Chu Chen · Li-Hsin Chen · Chun-Ching Chien · Hui-Chen Ho · Jeng-Hung Suen · Wuh-Liang Hwu

Received: 14 April 2013 / Revised: 14 April 2013 / Accepted: 25 April 2013 / Published online: 23 May 2013

Abstract *Background:* Fatty acid oxidation (FAO) disorders are a heterogeneous group of inborn errors in the transportation and oxidation of fatty acids. FAO disorders were thought to be very rare in the Chinese population. Newborn screening for FAO disorders beginning in 2002 in Taiwan may have increased the diagnosis of this group of diseases.

Materials and Methods: Till 2012, the National Taiwan University Hospital Newborn Screening Center screened more than 800,000 newborns for FAO disorders. Both patients diagnosed through screening and patients detected after clinical manifestations were included in this study.

Results: A total of 48 patients with FAO disorders were identified during the study period. The disorders included carnitine palmitoyltransferase I deficiency, carnitine acylcarnitine translocase deficiency, carnitine palmitoyltransferase II deficiency, very long-chain acyl-CoA dehydrogenase deficiency, medium-chain acyl-CoA dehydrogenase deficiency, multiple acyl-CoA dehydrogenase deficiency, short-chain defects, and carnitine uptake defect. Thirty-nine patients were diagnosed through newborn screening. Five false-negative newborn screening cases were noted during this period, and four patients who were not screened were diagnosed based on clinical manifestations. The ages of all patients ranged from 6 months to 22.9 years (mean age 6.6 years). Except for one case of postmortem diagnosis, there were no other mortalities.

Conclusions: The combined incidence of FAO disorders estimated by newborn screening in the Chinese population in Taiwan is 1 in 20,271 live births. Newborn screening also increases the awareness of FAO disorders and triggers clinical diagnoses of these diseases.

Abbreviations

CACT	Carnitine acylcarnitine translocase
CPT I	Carnitine palmitoyltransferase 1
CPT II	Carnitine palmitoyltransferase 2
CUD	Systemic carnitine uptake defect
FAO	Fatty acid oxidation
LCHAD/mTFP	Long-chain 3-hydroxy acyl-CoA dehydrogenase/mitochondrial trifunctional protein
MAD deficiency/ GA II	Multiple acyl-CoA dehydrogenase deficiency/glutaric aciduria type II (synonym)
MCAD	Medium-chain acyl-CoA dehydrogenase
MS/MS	Tandem mass spectrometry
NBS	Newborn screening
SCAD	Short-chain acyl-CoA dehydrogenase
VLCAD	Very-long-chain acyl-CoA dehydrogenase

Communicated by: Jerry Vockley

Competing interests: None declared

Y.-H. Chien · N.-C. Lee · W.-L. Hwu (✉)
Department of Medical Genetics and Pediatrics, National Taiwan University Hospital, Taipei, Taiwan
e-mail: hwuwlntu@ntu.edu.tw

M.-C. Chao
Division of Genetics, Endocrinology and Metabolism, Department of Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

M.-C. Chao
Department of Genome Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

L.-C. Chen · L.-H. Chen · C.-C. Chien
Department of Medical Genetics, National Taiwan University Hospital, Taipei, Taiwan

H.-C. Ho · J.-H. Suen
Taipei Institute of Pathology, Taipei, Taiwan

Introduction

Fatty acid oxidation (FAO) disorders are a heterogeneous group of inborn errors involving the transportation or oxidation of fatty acids, including the uptake and activation of fatty acids, the carnitine cycle, and the β-oxidation spiral (Roe and Ding 2001). Long-chain fatty acids, activated by being bound to their CoA esters in the cytosol, are shuttled across the barrier of the inner mitochondrial membrane by the carnitine cycle (Brivet et al. 1999). This cycle is composed of four steps mediated by a plasma membrane carnitine transporter, two carnitine palmitoyltransferases (CPT I and CPT II), and a carnitine-acylcarnitine translocase (CATC). In the mitochondria, β-oxidation of fatty acids occurs and requires a sequential action of very-long-chain acyl-CoA dehydrogenase (VLCAD), long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD), medium-chain acyl-CoA dehydrogenase (MCAD), and short-chain acyl-CoA dehydrogenase. Those dehydrogenases transfer electrons to ubiquinone via two flavoproteins—electron transfer flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QO). Impaired transfer of electrons affects multiple dehydrogenation reactions and is thus termed multiple acyl-CoA dehydrogenase (MAD) deficiency. FAO disorders can present with life-threatening symptoms, such as hypoketotic hypoglycemia, Reye-like syndrome in infants (Brivet et al. 1999; Baruteau et al. 2009; Spiekerkoetter 2010), acute encephalopathy (Gregersen et al. 2001; Spiekerkoetter 2010), cardiomyopathy (Bonnet et al. 1999; Spiekerkoetter et al. 2009a), myolysis (Spiekerkoetter et al. 2009a; van Adel and Tarnopolsky 2009), and liver dysfunction (Clayton 2003). Currently more than 15 distinct FAO disorders have been elucidated based on enzymatic and/or molecular analyses (Gregersen et al. 2008). Among them, MCAD deficiency is the most common disease in Caucasians of northern European origin (Roe and Ding 2001). Before newborn screening for these disorders, FAO disorders were characterized by high morbidity and mortality (Wanders et al. 1999) and required strict treatment measures. The mortality rate of FAO disorders was approximately 48% but could be higher in specific diseases such as VLCAD deficiency (60%) or CATC deficiency (92%) (Baruteau et al. 2012).

After the development of tandem mass spectrometry (MS/MS) analysis of acylcarnitines (Millington et al. 1990), the number of disorders that could be detected by newborn screening greatly increased (Huang et al. 2006), and most aminoacidopathies, FAO disorders, and organic acidurias became diagnosable. The combined incidence of FAO disorders reaches 1:9,000 in some countries, and half of the detected patients are affected by MCAD deficiency (Zytkovicz et al. 2001; Lindner et al. 2010). Early institution of high-glucose infusion, a low-fat diet, and avoidance of fasting can prevent the occurrence of metabolic decompensation (Baruteau et al. 2012). Therefore, MS/MS newborn screening (NBS) has been shown to improve the outcome of patients, and this screening is also cost effective (Pandor et al. 2004). Currently, different sets of FAO disorders are screened in different countries. The American College of Clinical Genetics proposed 29 core and 25 secondary conditions for screening, which include most of the FAO disorders (2006), while only 5 FAO disorders are included in screening in Germany (Lindner et al. 2011). In Taiwan, MS/MS was introduced in 2001 (Huang et al. 2006); however, only MCAD deficiency is recommended by the Bureau of Health Promotion, Department of Health, and all other FAO disorders can be screened only after the parents' consent (Niu et al. 2010).

The incidence of FAO disorders in the Chinese population was thought to be very low, and no patients except those with carnitine uptake defects (CUD) had been diagnosed before the initiation of NBS. However, the under-diagnosis of FAO disorders is likely, and NBS provides an opportunity to learn their true incidences. Unfortunately, data from previously published NBS pilot studies were not comprehensive (Huang et al. 2006; Niu et al. 2010); for example, the high incidence of CUD in the Chinese population was only realized at a later time (Lee et al. 2010; Chen et al. 2013). In this report, we present single-center longitudinal data over a 10-year period for the diagnosis and treatment of FAO disorders from both NBS and clinical recognition.

Materials and Methods

MS/MS NBS was performed at the National Taiwan University Hospital (NTUH) Newborn Screening Center. Dried blood spot (DBS) samples were obtained from newborn babies 48 to 72 h after birth, and acylcarnitine profiles were analyzed by MS/MS NBS as described (Huang et al. 2006). The results of the screening were available 24–48 h after the DBS samples were received. Two cutoff values were set for each metabolite (Table 1). Newborns with an initial screening value exceeding the diagnostic cutoff were requested for a confirmatory test at our hospital. Newborns with an initial screening value not exceeding the diagnostic cutoff but equal to or exceeding the screening cutoff (the inconclusive cases) were requested for a second screening. If the second screening results were still abnormal, the babies were requested for a confirmatory test. The targeted FAO disorders were carnitine palmitoyltransferase I (CPT I) deficiency, carnitine palmitoyltransferase II (CPT II) deficiency, carnitine acylcarnitine translocase (CACT) deficiency, very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, long-chain 3-hydroxyacyl-CoA

Table 1 Cutoff values for individual acylcarnitines

Concentration (μM)	Screening cutoff	Diagnostic cutoff*
C0	≤ 6.44	
C03	≥ 4.74	≥ 8.8
C03DC	≥ 0.42	
C04	≥ 0.79	
C04DC	≥ 0.6	
C05	≥ 0.4	≥ 1.1
C05DC	≥ 0.23	≥ 0.23
C05OH	≥ 0.56	≥ 2.2
C06	≥ 0.3	
C08	≥ 0.28	≥ 1.1
C08:1	≥ 0.56	
C10	≥ 0.26	
C10:1	≥ 0.25	
C12	≥ 0.73	
C12:1	≥ 0.3	
C14	≥ 0.52	
C14:1	≥ 0.52	
C14OH	≥ 0.18	
C16	≥ 6.4	≥ 8.8
C16:1	≥ 0.61	
C16OH	≥ 0.18	
C18	≥ 1.78	
C18:1	≥ 2.75	≥ 3.3
C18:1OH	≥ 0.16	
C0/(C16+C18)	≥ 50	

*Diagnostic cutoff was set for selected acylcarnitines

dehydrogenase (LCHAD) deficiency, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, short-chain defects, multiple acyl-CoA dehydrogenase (MAD) deficiency, and carnitine uptake defect (CUD). The confirmatory test includes enzyme assays and/or mutation analyses by accredited clinical laboratories when available (information provided upon request). Immediately after receiving a positive confirmation test, the patients were put on treatment. Short chain defects are generally benign conditions (Gallant et al. 2012), so babies positive for them were not put on treatment. The treatment and follow up of CUD patients have been described previously (Lee et al. 2010; Chen et al. 2013). In brief, the CUD patients were all given carnitine supplements, and none of them experienced metabolic decompensation or cardiac problems after treatment. For other FAO disorders, a low-fat diet (equal to or less than 30% of energy from fat) was the main treatment; for long-chain FAO disorders, this could be combined with a medium-chain triglyceride (MCT) supplement, and for secondary carnitine deficiency, this could be combined with a carnitine supplement.

Table 2 Case numbers and incidences of FAO defects in Taiwan

Disease	Case number	Incidence (per 100 000 newborns)
CPT I deficiency	1	0.13
CPT II/CACT deficiency	2	0.25
VLCHAD deficiency	2	0.25
LCHAD deficiency	0	0.00
MCAD deficiency	3	0.38
Short-chain defects	8	1.01
MAD deficiency	1	0.13
CUD	22	2.78
Total	39	4.93

Between January 1, 2003, and December 31, 2012, 790,569 newborns were screened by the NTUH Newborn Screening Center. Cutoff values were not changed during the study period except for CUD diagnosis. Approximately 80,000 newborns screened before 2003 were excluded from the study because the cutoff values were not set at that time. Both NBS-confirmed cases and patients diagnosed through clinical manifestations were included in the study. Data analyses included screening results, clinical manifestations, genotypes, treatments, and outcomes. Because differential diagnoses of short-chain defects were not completed, and those patients did not need treatment, we did not include them in the outcome analysis.

Results

Incidences Determined from NBS

During the study period, 39 newborns were identified by screening to have FAO disorders, with an incidence of 1 in 20,271 live births or 4.53 cases per 100,000 live births (Table 2). In addition, we also identified 12 newborns whose mothers had CUD (the maternal CUD cases); those newborns only had transient carnitine deficiency and were not included in the current study. Among all FAO disorders, CUD was the most prevalent disease, with an incidence of 1 in 35,935 or 2.78 cases per 100,000 live births.

Initial Clinical Manifestations

Two patients were symptomatic at the time of diagnosis through NBS. One patient with VLCAD deficiency presented with hypoglycemia shortly after birth and was found to have cardiomegaly at 10 days of age. The other patient had CACT deficiency that presented with hyper-

Table 3 Initial screening results of acylcarnitine levels in the four false-negative cases

Patient	FN-1	FN-2	FN-3		FN-5	
Disease	CUD	MAD deficiency	CPT II deficiency		MAD deficiency	
Mutation / Marker (μM)	F17L/?	A84T/ F128S	F383Y/ G601fsX62 1	Cutoffs at another center*	R99C/ L127H	Cutoffs at NTUH*
Free carnitine	**3.620**	38.60	19.9	<70.0	24.99	<52.79
C2-carnitine	5.090	24.80	**5.23**	>7.0	41.07	<53.59
C3-carnitine	0.200	1.43	0.18	<6.0	1.46	<4.74
C4-carnitine	0.210	0.55	0.09	<1.5	0.44	<1.4
C5-carnitine	0.030	0.26	0.05	<0.6	0.36	<0.57
C5DC-carnitine	0.000	0.137	0.08	<0.3	0.07	<0.23
C5OH-carnitine	0.210	0.15	0.05	<0.8	0.16	<0.78
C6-carnitine	0.110	0.48	0.03	<0.5	0.58	<0.62
C8-carnitine	0.070	**0.71**	0.1	<0.5	0.55	<0.56
C10-carnitine	0.000	**1.30**	0.38	<0.5	**1.29**	<0.53
C12-carnitine	0.000	0.92	NA	NA	2.25	<0.73
C14-carnitine	0.160	0.72	**1.37**	<0.8	**1.16**	<0.83
C14:1-carnitine	0.000	**1.20**	0.24	<0.6	**1.55**	<0.59
C16-carnitine	1.610	2.93	7.1	<8.0	3.73	<6.41
C18-carnitine	0.220	0.94	**2.39**	<2.0	0.79	<1.78
C18:1-carnitine	0.480	1.53	3.25	<3.5	1.54	<2.75

Bold characters indicate abnormal data

*FN-1, FN-2, and FN-3 were screened by another newborn screening center, and FN-5 was screened by the National Taiwan University Hospital (NTUH), so two different sets of cutoff values were presented

ammonemia and hypoglycemia after birth. Others patients were asymptomatic at the time that screening results were obtained.

The False-Negative Cases

Five known false-negative cases that received screening from either our screening center or other centers were noted and listed in Tables 2 and 3. FN-1 was found to be affected by CUD after her younger brother was diagnosed. In her first DBS test, her free carnitine level was low, but low free carnitine levels were not reported in the initial pilot MS/MS screening. FN-2 was referred for hypotonia, an elevated serum creatine kinase (CK) level, and hyperammonemia after a fever episode at 1 year of age. She had experienced two previous uneventful episodes of acute bronchiolitis at the ages of 3 and 5 months. Her DBS acylcarnitine profile was typical of MAD deficiency but was not picked up by the screening algorithm (Table 3), and *ETFDH* gene p.A84T/p.F128S mutations were later confirmed (Er et al. 2011). FN-3 was referred for hepatomegaly and liver failure at 6 months of age; CPT II deficiency was suspected and confirmed by mutation analysis, which found two *CPT2* gene mutations, p.F383Y and p.G601fsX621. FN-4 was diagnosed because her younger brother was found to have MAD deficiency. Data from her first screening was not available because it was performed by another screening center. She had no symptoms until the end of this study.

Recent studies suggest a high incidence of MAD deficiency in South China. Therefore, we retrospectively evaluated our newborn screening data using the Region 4 post-analytic tool (Marquardt et al. 2012). One newborn (FN-5) was found to have a high MAD deficiency score, and the diagnosis was later confirmed by mutation analysis.

The Clinical Cases

Four cases who did not receive NBS for FAO disorders came to our attention. Case 1 was a 20-year-old female patient who suffered from intermittent abdominal pain since the age of 15 years (Table 4). Chronic pancreatitis was suspected by magnetic resonance imaging (MRI) results, but a laparoscope examination at 19 years of age did not confirm pancreatitis; the laparoscopy showed many tiny stones in the gall bladder and resulted in a cystectomy. A diagnosis of CPT I deficiency was finally made when she was 20 years old. Case 3 was a 2.5-year-old female patient who presented with recurrent hypoglycemia, elevated liver enzymes, and hepatomegaly. She was put on a carnitine supplement, but still experienced three more attacks with consciousness disturbance after gastroenteritis. The final diagnosis made was CPT II deficiency when she was 3 years old. Case 9 died of poor activity and jaundice at 1 year of age. Her younger brother was later found to have VLCAD deficiency by NBS. Case 17 presented with proximal muscle weakness, dyspnea, and rhabdomyolysis

Table 4 List of all patients except those with short-chain defect or CUD

Case No.	Defective enzyme	Clinical presentation	Specific enzyme activity*	Genotype**	Diet fat intake
1	CPT I	At age of 15 years Recurrent acute pancreatitis	0.080 (Normal > 0.42)	**c.1367C>T (p.S456L)/c.1433C>T (p.A478V)**	30%
2	CPT I	NBS	0.110 (Normal 0.36 – 0.88)	**c.727C>T (p.L91P)/c.497G>T (p.S166I)**	30%
3	CPT II	At age of 3 years Hypoglycemia, hepatitis, hepatomegaly	0.5 (Normal 15.8 ± 4.2)	NA	30%
4 (FN-3)	CPT II	At age of 6 months Hypoglycemia, metabolic acidosis, impaired liver function, hepatomegaly	0.02 (Normal 0.4 – 1.85)	c.1148 T>A (p.F383Y)/ **c.1803delC (p.G601fsx621)**	25%
5	CPT-II?	NBS	0.65 (Normal 0.4 – 1.85)	c.1102 G>A (p.V368I) He	30%
6	CACT	NBS (hyperammonemia, metabolic acidosis)	NA	NA	NA
7	VLCAD	NBS	NA	**c.1273 G>A (p.A425T)**/c.1349 G>A (p.R450H)	40%
8	VLCAD	NBS (hypoglycemia, cardiac hypertrophy)	NA	**c.1751+1 G>A/c.961 A>G (p.N321D)**	<30%
9	VLCAD	Elevated liver enzymes, hyperammonemia, expired	NA	NA	NA
10	MCAD	NBS	NA	**c.446_449delTGAC (p.T150RfsX4)**/ c.580A>G (p.N194D)	<30%
11	MCAD	NBS	NA	c.580A>G (p.N194D)/c.580A>G (p.N194D)	NA
12	MCAD	NBS	NA	NA	NA
13 (FN-2)	MAD	At age of 12 months Hypotonia, elevated liver enzymes, hyperammonemia	Fibroblast assay: elevated C6-C12, C14, and C16-carnitines, very low C2 carnitine	c.250G>A (p.A84T)/c.383T>C (p.**F128S)**	<30%
14	MAD	NBS	Fibroblast assay: elevated C6-C16-carnitines, very low C2 carnitine	**c. 295C>G (p.R99G)**/?	Normal
15 (FN-4)	MAD	At age of 2.9 years Sibling screening	NA	**c. 295C>G (p.R99G)**/?	Normal
16 (FN-5)	MAD	NBS	NA	**c.295C>T (p.R99C)**/c.380T>A (p.L127H)	NA
17	MAD	At age of 17 years Weakness and rhadomyolysis	NA	c.250G>A (p.A84T)/c.250G>A (p.A84T)	<30%

NA data not available, *FN* false-negative cases, *NBS* detected by newborn screening

*Specific enzyme activity: nmol/min/mg protein for CPT I and CPT II activity; fibroblast beta-oxidation study by acyl-^{3}H-carnitine for MAD deficiency; normal ranges in parenthesis

**Genotype: CPT1A gene for CPT I deficiency; CPT2 gene for CPT II deficiency; ACADM gene for MCAD deficiency; ACADVL gene for VLCAD deficiency; ETFDH gene for MADD (Bold characters indicate novel mutations; ? indicates mutation not found)

at the age of 17 years. His DBS acylcarnitine profile was typical of MAD deficiency, and *ETFDH* gene p.A84T homozygous mutations were later found.

Treatments and Courses

At the end of this study period, the mean age of all patients was 6.6 years (6 months to 22.9 years). A low fat diet was prescribed for 10 of the 12 (83%) patients with known dietary histories, and concomitant carnitine was prescribed for 3 (27%) patients. During the study period, no patients needed intensive care or prolonged hospitalization.

For the NBS cases, occasionally patients with CPT I, CPT II, VLCAD, or MAD deficiencies were put on oral or intravenous calorie supplementation because of concurrent illness. The two VLCAD deficiency patients experienced mild metabolic instability during follow up. Rhadomyolysis occurred in Case 7 (VLCAD deficiency) when she was infected by a Noro virus at the age of 2.5 years. One episode of elevation of CK (from 188 IU/L to 1838 IU/L) occurred in Case 8 (VLCAD deficiency) when he contracted an upper respiratory tract infection at the age of 3 months. None of these events were repeated.

Among the false-negative and clinically diagnosed patients, the girl affected by CPT I deficiency (Case 1) had poor compliance to the diet, and she continued to suffer from intermittent abdominal pain and muscle pain after stringent exercise. Case 3 (CPT II deficiency) was put on a low-fat diet, but she could not tolerate the MCT supplement and had approximately one episode of rhadomyolysis per year. Case 4 (FN-3, CPT II deficiency) started a low-fat diet, MCT supplement, and carnitine supplement after diagnosis. Although she received an intravenous calorie supply two times during concurrent illness, there was no metabolic decompensation. Case 13 (FN-2, MAD deficiency) was put on riboflavin, carnitine, and a low-fat, low-protein diet, but she had facial eczema and frequently needed hospitalization for fear of metabolic decompensation. Case 17 was put on riboflavin and a low-fat, low-protein diet, and he recovered from respiratory failure 1 month after the attack.

Discussion

Incidence of the FAO Disorders in the Chinese Population

Our NBS program disclosed an incidence of FAO disorders as 1 in 20,271. Han et al. have screened for FAO disorders by MS/MS NBS analysis for patients suspected to have inborn errors and found only a few cases (Han et al. 2007). Therefore, underdiagnosis before NBS was a serious issue. The incidence of FAO disorders in the Chinese population could be even higher if we included the false-negative cases. For example, CUD can only be detected by NBS in half of affected newborns because of carnitine transportation through the placenta from the mother (Wilcken et al. 2001; Lahjouji et al. 2004). CUD disease severity decreased in patients detected by NBS. Most of the mothers affected by CUD were asymptomatic (Chen et al. 2013). The carrier rate of the more severe CUD mutation in the Southern Chinese population, p.R254X, is approximately 1 in 125 (Tang et al. 2002). This mutation represented 50% of all mutations in clinically detected cases of CUD but represented only 30% of all mutations in NBS-detected cases (Lee et al. 2010), suggesting a decrease in the severity of the disease in the latter group of patients.

The Occurrences of False-Negative Cases in NBS

The occurrence of false-negative cases in NBS can be either technically or physiologically related. The acylcarnitine profile for MAD deficiency is composed of mild but multiple elevations of a group of acylcarnitines. Screening for MAD deficiency by analyzing the level of a single acylcarnitine results in low sensitivity. The detection rate of MAD deficiency may be improved by applying more sophisticated algorithms weighing several acylcarnitines together. Using an improved interpretive tool (Marquardt et al. 2012), we retrospectively detected one patient and confirmed the increased sensitivity of this tool. This is especially important because the incidence of the riboflavin-responsive form of MAD deficiency is high in Taiwan (Liang et al. 2009; Lan et al. 2010) and Southern China (Wang et al. 2011) owing to a high incidence of the p.A84T mutation in the *ETFDH* gene (a carrier rate of 1 in 74), and the treatment with carnitine and riboflavin is easy and effective.

Patients with FAO disorders may have normal acylcarnitines at birth. As we have discussed in the previous section, newborns with CUD can be missed by NBS because of normal free carnitine levels at birth (Wilcken et al. 2001; Lahjouji et al. 2004). A looser cut-off value can increase the sensitivity of the tests, but will also increase the rate of false-positives. Repeat screening for an inconclusive NBS result can also be dangerous. In many FAO disorders (Illsinger et al. 2008), especially VLCAD deficiency (Ficicioglu et al. 2010; Spiekerkoetter et al. 2010b; Sahai et al. 2011), repeat tests can have normal results in FAO patients, resulting in a false-negative screening. To solve these diagnostic issues, we have developed second-tier molecular tests for diseases in which common mutations occur (Wang et al. 2013).

Treat or Not

NBS detects significantly more patients with MCAD and short-chain acyl-CoA dehydrogenase (SCAD) deficiency than are detected by previous clinical manifestations (Wilcken et al. 2003; Gallant et al. 2012). Patients with MCAD deficiency can present with acute decompensation followed by a poor outcome, and, therefore, early diagnosis and treatment are important. However, some MCAD deficiency cases detected by NBS revealed novel mutations with no known clinical meaning. It may be prudent to follow such MCAD patients to avoid fasting during concurrent illnesses, but for SCAD deficiency the disease has been classified as a benign condition not needing treatment (Gallant et al. 2012).

In CUD, it can be difficult to predict the phenotype of asymptomatic patients detected by NBS; most of the mothers with CUD are also asymptomatic. However, we have encountered two mothers with CUD that were symptomatic: one died suddenly and one had undiagnosed cardiomegaly (Chen et al. 2013). Although phenotype prediction can be difficult (Rose et al. 2012), treatment for CUD is simple and safe. Therefore, it is prudent to maintain normal blood free carnitine levels for all patients with CUD (El-Hattab et al. 2010).

Clinical manifestations of VLCAD deficiency also vary. Patients with null mutations or other severe mutations (Andresen et al. 1996) should be immediately placed on a low-fat diet with supplemental calories provided by MCT supplementation (Solis and Singh 2002; Spiekerkoetter et al. 2009b). For patients with missense mutations or asymptomatic mothers with VLCAD deficiency detected through a false-positive NBS in their babies (McGoey and Marble 2011), a looser diet may be applicable (Spiekerkoetter et al. 2009b), but phenotype prediction for novel mutations may not be possible.

The role of low-fat diets in FAO disorders depends on the type and severity of the disease. For asymptomatic patients with long-chain defects, a normal diet may be tolerable. Intake of MCT oil prior to exercise can be effective to prevent myopathic symptoms (Spiekerkoetter et al. 2010a). L-carnitine supplementation in patients with long-chain defects can be dangerous (Spiekerkoetter et al. 2010a); however, we found secondary carnitine deficiency in 27% of our cases, and L-carnitine was prescribed to these patients to maintain a normal free carnitine level.

Conclusion

FAO disorders are not rare in Taiwan and the combined incidence is higher than 1 in 20,271 after NBS. CUD and MAD deficiency are the most common FAO disorders in Taiwan. NBS also increased the awareness of FAO disorders resulting in an increase in clinical diagnoses after the initiation of NBS. Early diagnosis and early initiation of treatment have led to a more favorable outcome for patients with FAO disorders as demonstrated in the current study.

Acknowledgment We thank Dr. Nicola Longo from the University of Utah for performing the mutation analysis for patients of CPT I deficiency and CPT II deficiency. This study was partially support by grants (DOH94-HP-2203, DOH95-HP-2207) from the Department of Health, Taiwan.

References

American College of Medical Genetics Newborn Screening Expert Group (2006) Newborn screening: toward a uniform screening panel and system–executive summary. Pediatrics 117(5 Pt 2): S296–307

Andresen BS, Bross P, Vianey-Saban C et al (1996) Cloning and characterization of human very-long-chain acyl-CoA dehydrogenase cDNA, chromosomal assignment of the gene and identification in four patients of nine different mutations within the VLCAD gene. Hum Mol Genet 5(4):461–472

Baruteau J, Levade T, Redonnet-Vernhet I, Mesli S, Bloom MC, Broue P (2009) Hypoketotic hypoglycemia with myolysis and hypoparathyroidism: an unusual association in medium chain acyl-CoA desydrogenase deficiency (MCADD). J Pediatr Endocrinol Metab 22(12):1175–1177

Baruteau J, Sachs P, Broue P, et al (2012) Clinical and biological features at diagnosis in mitochondrial fatty acid beta-oxidation defects: a French pediatric study of 187 patients. J Inherit Metab Dis 2012 Oct 3 [Epub ahead of print]

Bonnet D, Martin D, De Pascale L et al (1999) Arrhythmias and conduction defects as presenting symptoms of fatty acid oxidation disorders in children. Circulation 100(22):2248–2253

Brivet M, Boutron A, Slama A et al (1999) Defects in activation and transport of fatty acids. J Inherit Metab Dis 22(4):428–441

Chen YC, Chien YH, Chen PW et al (2013) Carnitine uptake defect (primary carnitine deficiency): risk in genotype-phenotype correlation. Hum Mutat 34(4):655

Clayton PT (2003) Diagnosis of inherited disorders of liver metabolism. J Inherit Metab Dis 26(2–3):135–146

El-Hattab AW, Li FY, Shen J et al (2010) Maternal systemic primary carnitine deficiency uncovered by newborn screening: clinical, biochemical, and molecular aspects. Genet Med 12(1):19–24

Er TK, Chen CC, Liu YY et al (2011) Computational analysis of a novel mutation in ETFDH gene highlights its long-range effects on the FAD-binding motif. BMC Struct Biol 11:43

Ficicioglu C, Coughlin CR 2nd, Bennett MJ, Yudkoff M (2010) Very long-chain acyl-CoA dehydrogenase deficiency in a patient with normal newborn screening by tandem mass spectrometry. J Pediatr 156(3):492–494

Gallant NM, Leydiker K, Tang H et al (2012) Biochemical, molecular, and clinical characteristics of children with short chain acyl-CoA dehydrogenase deficiency detected by newborn screening in California. Mol Genet Metab 106(1):55–61

Gregersen N, Andresen BS, Corydon MJ et al (2001) Mutation analysis in mitochondrial fatty acid oxidation defects: exemplified by acyl-CoA dehydrogenase deficiencies, with special focus on genotype-phenotype relationship. Hum Mutat 18(3): 169–189

Gregersen N, Andresen BS, Pedersen CB, Olsen RK, Corydon TJ, Bross P (2008) Mitochondrial fatty acid oxidation defects–remaining challenges. J Inherit Metab Dis 31(5):643–657

Han LS, Ye J, Qiu WJ, Gao XL, Wang Y, Gu XF (2007) Selective screening for inborn errors of metabolism on clinical patients using tandem mass spectrometry in China: a four-year report. J Inherit Metab Dis 30(4):507–514

Huang HP, Chu KL, Chien YH et al (2006) Tandem mass neonatal screening in Taiwan–report from one center. J Formos Med Assoc 105(11):882–886

Illsinger S, Lucke T, Peter M et al (2008) Carnitine-palmitoyltransferase 2 deficiency: novel mutations and relevance of newborn screening. Am J Med Genet A 146A(22):2925–2928

Lahjouji K, Elimrani I, Lafond J, Leduc L, Qureshi IA, Mitchell GA (2004) L-Carnitine transport in human placental brush-border membranes is mediated by the sodium-dependent organic cation transporter OCTN2. Am J Physiol Cell Physiol 287(2):C263–C269

Lan MY, Fu MH, Liu YF et al (2010) High frequency of ETFDH c.250G>A mutation in Taiwanese patients with late-onset lipid storage myopathy. Clin Genet 78(6):565–569

Lee NC, Tang NL, Chien YH et al (2010) Diagnoses of newborns and mothers with carnitine uptake defects through newborn screening. Mol Genet Metab 100(1):46–50

Liang WC, Ohkuma A, Hayashi YK et al (2009) ETFDH mutations, CoQ10 levels, and respiratory chain activities in patients with riboflavin-responsive multiple acyl-CoA dehydrogenase deficiency. Neuromuscul Disord 19(3):212–216

Lindner M, Hoffmann GF, Matern D (2010) Newborn screening for disorders of fatty-acid oxidation: experience and recommendations from an expert meeting. J Inherit Metab Dis 33(5):521–526

Lindner M, Gramer G, Haege G et al (2011) Efficacy and outcome of expanded newborn screening for metabolic diseases–report of 10 years from South-West Germany. Orphanet J Rare Dis 6:44

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

McGoey RR, Marble M (2011) Positive newborn screen in a normal infant of a mother with asymptomatic very long-chain Acyl-CoA dehydrogenase deficiency. J Pediatr 158(6):1031–1032

Millington DS, Kodo N, Norwood DL, Roe CR (1990) Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism. J Inherit Metab Dis 13(3):321–324

Niu DM, Chien YH, Chiang CC et al (2010) Nationwide survey of extended newborn screening by tandem mass spectrometry in Taiwan. J Inherit Metab Dis 33(Suppl 2):S295–S305

Pandor A, Eastham J, Beverley C, Chilcott J, Paisley S (2004) Clinical effectiveness and cost-effectiveness of neonatal screening for inborn errors of metabolism using tandem mass spectrometry: a systematic review. Health Technol Assess 8(12): iii, 1–121

Roe CR, Ding J (2001) Mitochondrial fatty acid oxidation disorders. In: Scriver C, Beaudet A, Sly W, Valle D (eds) The metabolic and molecular bases of inherited disease New York. McGraw-Hill, New York, pp 2297–2326

Rose EC, di San Filippo CA, Ndukwe Erlingsson UC, Ardon O, Pasquali M, Longo N (2012) Genotype-phenotype correlation in primary carnitine deficiency. Hum Mutat 33(1):118–123

Sahai I, Bailey JC, Eaton RB, Zytkovicz T, Harris DJ (2011) A near-miss: very long chain acyl-CoA dehydrogenase deficiency with normal primary markers in the initial well-timed newborn screening specimen. J Pediatr 158(1):172, author reply 172–173

Solis JO, Singh RH (2002) Management of fatty acid oxidation disorders: a survey of current treatment strategies. J Am Diet Assoc 102(12):1800–1803

Spiekerkoetter U (2010) Mitochondrial fatty acid oxidation disorders: clinical presentation of long-chain fatty acid oxidation defects before and after newborn screening. J Inherit Metab Dis 33 (5):527–532

Spiekerkoetter U, Lindner M, Santer R et al (2009a) Management and outcome in 75 individuals with long-chain fatty acid oxidation defects: results from a workshop. J Inherit Metab Dis 32 (4):488–497

Spiekerkoetter U, Lindner M, Santer R et al (2009b) Treatment recommendations in long-chain fatty acid oxidation defects: consensus from a workshop. J Inherit Metab Dis 32(4):498–505

Spiekerkoetter U, Bastin J, Gillingham M, Morris A, Wijburg F, Wilcken B (2010a) Current issues regarding treatment of mitochondrial fatty acid oxidation disorders. J Inherit Metab Dis 33(5):555–561

Spiekerkoetter U, Haussmann U, Mueller M et al (2010b) Tandem mass spectrometry screening for very long-chain acyl-CoA dehydrogenase deficiency: the value of second-tier enzyme testing. J Pediatr 157(4):668–673

Tang NL, Hwu WL, Chan RT, Law LK, Fung LM, Zhang WM (2002) A founder mutation (R254X) of SLC22A5 (OCTN2) in Chinese primary carnitine deficiency patients. Hum Mutat 20(3):232

van Adel BA, Tarnopolsky MA (2009) Metabolic myopathies: update 2009. J Clin Neuromuscul Dis 10(3):97–121

Wanders RJ, Vreken P, den Boer ME, Wijburg FA, van Gennip AH, Ijlst L (1999) Disorders of mitochondrial fatty acyl-CoA beta-oxidation. J Inherit Metab Dis 22(4):442–487

Wang ZQ, Chen XJ, Murong SX, Wang N, Wu ZY (2011) Molecular analysis of 51 unrelated pedigrees with late-onset multiple acyl-CoA dehydrogenation deficiency (MADD) in southern China confirmed the most common ETFDH mutation and high carrier frequency of c.250G>A. J Mol Med (Berl) 89(6): 569–576

Wang L-Y, Chen N-I, Chen P-W et al (2013) Newborn screening for citrin deficiency and carnitine uptake defect using second-tier molecular tests. BMC Med Genet 14:24

Wilcken B, Wiley V, Sim KG, Carpenter K (2001) Carnitine transporter defect diagnosed by newborn screening with electrospray tandem mass spectrometry. J Pediatr 138(4): 581–584

Wilcken B, Wiley V, Hammond J, Carpenter K (2003) Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 348(23):2304–2312

Zytkovicz TH, Fitzgerald EF, Marsden D et al (2001) Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. Clin Chem 47(11):1945–1955