Johannes Zschocke
Matthias Baumgartner
Eva Morava
Marc Patterson
Shamima Rahman
Verena Peters *Editors*

JIMD Reports

Volume 22

SSIEM

Springer

JIMD Reports
Volume 22

Johannes Zschocke
Editor-in-Chief

Matthias Baumgartner · Eva Morava ·
Marc Patterson · Shamima Rahman
Editors

Verena Peters
Managing Editor

JIMD Reports
Volume 22

Springer

SSIEM

ISSN 2192-8304 ISSN 2192-8312 (electronic)
JIMD Reports
ISBN 978-3-662-47452-5 ISBN 978-3-662-47453-2 (eBook)
DOI 10.1007/978-3-662-47453-2

Springer Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer-Verlag GmbH Berlin Heidelberg is part of Springer Science+Business Media (www.springer.com)

Contents

JIMD Reports
DOI 10.1007/8904_2014_371

RESEARCH REPORT

Innate and Adaptive Immune Response in Fabry Disease

Wladimir Mauhin • Olivier Lidove • Elisa Masat • Federico Mingozzi • Kuberaka Mariampillai • Jean-Marc Ziza • Olivier Benveniste

Received: 05 May 2014 / Revised: 18 September 2014 / Accepted: 30 September 2014 / Published online: 18 February 2015

Abstract Fabry disease is an X-linked lysosomal storage disease in which mutations of the gene (GLA) cause a deficiency of the lysosomal hydrolase α-galactosidase A (α-Gal). This defect results in an accumulation of glycosphingolipids, primarily globotriaosylceramide (Gb3) which causes a multisystemic vasculopathy. Available since 2001 in Europe, enzyme replacement therapy consists in the administration of agalsidase, a recombinant form of α-galactosidase A. Enzyme replacement therapy was shown to improve the global prognosis but allowed partial success in preventing critical events such as strokes and cardiac arrests. As in most lysosomal storage diseases, frequent immune reactions have been described in naive Fabry disease patients. Humoral immune responses following enzyme replacement therapy have also been described, with unclear consequences on the progression of the disease. While cost-effectiveness of enzyme replacement therapy in Fabry disease begins to be questioned and new therapeutic strategies arise such as chaperone or gene therapy, it appears necessary to better understand the immune responses observed in the treatment of naive patients and during enzyme replacement therapy with agalsidase. We propose a comprehensive review of the available literature concerning both innate and adaptive responses observed in Fabry disease. We particularly highlight the probable role of the toll-like receptor 4 (TLR4) and CD1d pathways triggered by Gb3 accumulation in the development of local and systemic inflammation that could lead to irreversible organ damages. We propose an immunological point of view of Fabry disease pathogenesis involving immune cells notably the invariant natural killer T cells. We finally review anti-agalsidase antibodies, their development and impact on outcomes.

Communicated by: Verena Peters

Competing interests: Declared

W. Mauhin · O. Benveniste
Internal Medicine Department, La Pitié-Salpêtrière Hospital, 47-83 boulevard de l'hôpital, 75013 Paris, France

O. Lidove (✉) · J.-M. Ziza
Internal Medicine and Rheumatology Department, La Croix Saint Simon Hospital, 125 rue d'Avron, 75020 Paris, France
e-mail: OLidove@hopital-dcss.org

W. Mauhin · O. Lidove · E. Masat · F. Mingozzi · K. Mariampillai · O. Benveniste
Inserm UMRS 974, University Pierre and Marie Curie, 47-83 boulevard de l'hôpital, 75013 Paris, France

Introduction

First described in 1898, Fabry disease (FD) is the second most common lysosomal storage disease (LSD) after Gaucher disease, with a worldwide incidence estimated between 1/40,000 and 1/117,000 live births (Meikle et al. 1999; Fuller et al. 2006). This figure is probably underestimated since late-onset variants have been described, with an expected incidence of late-onset disease as high as 1/3,100 births (Spada et al. 2006). FD is a lysosomal storage disease caused by the reduction or absence of hydrolase α-galactosidase A (α-GalA) activity in lysosomes, causing a systemic intracellular accumulation of neutral glycosphingolipids (GSL), most notably of globotriaosylceramide (Gb3). More than 600 mutations have been identified in the galactosidase alpha (GLA) gene (Xq21.3-q22) (http://www.ncbi.nlm.nih.gov/omim; Sakuraba database http://fabry-database.org/mutants). A wide

spectrum of clinical symptoms usually begins in the early childhood of FD patients and progressively enriches all along the evolution of the disease. These symptoms include acroparaesthesia, gastrointestinal disorders, angiokeratomas, heat intolerance, hearing impairment, ophthalmologic abnormalities but also proteinuria and glomerulosclerosis leading to end-stage renal disease (ESRD), cardiac hypertrophy and arrhythmia and cerebrovascular disease including transient ischemic attacks and strokes (Mehta et al. 2010). Although X-linked, FD also affects females due to the lyonization process that inactivates one X-chromosome. Affected women present mild to severe phenotype which usually appears around 5 years later than in males (Beck 2006). Before enzyme replacement therapy (ERT), life expectancy in FD patients was 58.2 years in males and 75.4 years in females (Waldek et al. 2009). Death was mostly caused by cardiovascular disease (MacDermot et al. 2001a, b; Waldek et al. 2009). In males, FD is diagnosed by demonstrating a deficiency of α-GalA in plasma or leukocytes (Winchester and Young 2006). In heterozygous females, the gold standard is the genetic analysis since enzymatic assays fail to detect one third of the patients because of the residual enzyme activity (Linthorst et al. 2010; Wang et al. 2007; Wilcox et al. 2012). Whereas "classical FD" consists in a multi-organ pathology, single organ variants are more and more evoked, following systematic investigations in specific medical conditions such as hypertrophic cardiomyopathies, cryptogenic strokes or isolated renal disease (Nakao et al. 2003; Chimenti et al. 2004; Rolfs et al. 2005). Such screenings with molecular testing are thus limited by the existence of variants of unknown significance in terms of pathogenicity and penetrance (Lukas et al. 2013; Thomas and Mehta 2013).

Another difficulty in the management of FD comes from the lack of reliable biomarkers. Historically, Gb3 levels in plasma, urine and organs, especially increased in Fabry males, have been used as potential biomarkers of the disease (Schiffmann et al. 2010). But Gb3 plasma levels are normal in the majority of Fabry females; therefore, clinical correlation appears difficult (Vedder et al. 2007). Most of the therapeutic trials have been monitored with Gb3 levels. Lyso(Gb3), the deacylated form of Gb3, is taking over this role of biomarker as it was shown to be highly increased in the plasma of both male and female patients with a relative elevation exceeding markedly that of Gb3 (Aerts et al. 2008). Moreover plasma lyso(Gb3) was shown to be an independent risk factor for white-matter lesions in males and left ventricular hypertrophy in females (Rombach et al. 2010a). Another technique was attempted using flow cytometry: as the membrane antigen CD77 corresponds to Gb3, the quantification of CD77 expression on peripheral blood mononuclear cells (PBMCs) was shown to be correlated with Gb3 accumulation and α-GalA downregulation in vitro (Thomaidis et al. 2009). Evidence-based management of FD is therefore limited by the lack of standardized and reliable biomarkers.

Concerning the treatment, agalsidase alfa (Replagal®, Shire HGT Inc.) and agalsidase beta (Fabrazyme®, Genzyme Corp.), two recombinant α-GalA, bimonthly infused, have been shown to reduce Gb3 plasma levels (Schiffmann et al. 2001; Eng et al. 2001). Results on urinary Gb3 levels were less prominent (Schiffmann et al. 2001; Eng et al. 2001). Overall under these enzyme replacement therapies (ERT) and in the short term (from 20 to 25 weeks of treatment), sustained clinical benefits were observed such as reduction of left ventricle mass, stabilization of renal function and reduction of pain with in parallel a reduction of Gb3 contents in kidney, heart and skin (Schiffmann et al. 2001; Eng et al. 2001). Nevertheless, long-term effects (median follow-up of 6.0 years) appear more unobvious with the description of a persistent progression of cardiac fibrosis and renal failure and no significant benefit on cardiac death, renal death and stroke (Weidemann et al. 2013). Two recent reviews even reported no evidence for the use of ERT (El Dib et al. 2013) and only benefits in reducing left ventricular mass but limited renal effects (Rombach et al. 2014). Both reviews only considered six studies. One mechanism suspected in the incomplete long-term response to ERT is the development of immune responses against agalsidase. Actually, immune reactions are described in both treatment-naive and agalsidase-treated FD patients (Rozenfeld et al. 2009; De Francesco et al. 2013). Antibodies against agalsidase are notably well described, but their role remains unclear (Linthorst et al. 2004; Bénichou et al. 2009; Wilcox et al. 2012). In this review we present the spectrum of the immunological reactions, both innate and adaptive, observed in the natural history of FD and developed against agalsidase therapy, and their impact on outcome in FD.

Proinflammatory Pattern in Naive-Treatment FD Patients

1. Innate immunity response: the role of invariant natural killer T cells, CD1d/TLR4 pathway and Gb3 deposit.
   Fabry disease is characterized by a lack of α-GalA causing an accumulation of GSL in the late endocytic and lysosomal compartments. Self-GSL is recognized as an antigen by the invariant natural killer T cells (iNKTs). Indeed, iNKTs characterized by their Vα24-Jα T-cell receptor (TCR)-alpha chain rearrangement represent a subset of natural killer T cells. The iNKTs recognize as an antigen self-GSL presented by the major histocompatibility complex (MHC) class I-like molecule CD1d of antigen-presenting cells (APCs) (Spada et al. 1998). Human iNKTs are divided into three subsets according to the CD4 and CD8 expression

with a different inflammatory profile: positive only for CD4 (CD4 iNKTs), positive only for CD8 (CD8 iNKTs) and double negative for CD4 and CD8 (DN iNKTs) (Gumperz et al. 2002). Numerous quantitative and qualitative defects have been implicated in antimicrobial response defects, antitumor immunity and autoimmunity (Sugita et al. 2004; Chuang et al. 2012; Hunn and Hermans 2013). The recognition of GSL induces the release of various proinflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), interleukin 4 (IL-4), IL-5, IL-9, IL-10, IL-13 and IL-17 (Gumperz et al. 2002). It also upregulates CD40l inducing IL-10 and IL-12 secretion by dendritic cells (O'Reilly et al. 2011). A quantitative reduction of iNKTs has been reported in murine models of different LSDs including FD (Gadola et al. 2006; Macedo et al. 2012). In Fabry knockout (KO) mice, an age-associated increase in Gb3 storage was reported (Macedo et al. 2012). Concomitantly, a progressive decrease in iNKT rates was observed, which differs depending on the organs with a more significant reduction in the spleen (around 75%) and affecting primarily the CD4 iNKTs subset. ERT was shown to prevent the progression of this splenic iNKT deficiency in mouse (Macedo et al. 2012). In FD patients, under ERT for most of them, the quantitative reduction in the iNKT pool was not found in PBMCs (Balreira et al. 2008). This absence of disorder in iNKTs has been thought to result from the differences in intracellular trafficking of mouse and human CD1d molecules or the role of ERT. More recently, Pereira et al. reported a decrease in CD4 iNKTs, an increase of DN iNKTs and a reduction in the IL-4 production, without significant difference between ERT-treated and untreated patients (Pereira et al. 2013). Of note, only 4 patients were treated among the 15 included, limiting the impact of ERT. Interestingly, Rozenfeld and Balreira showed a low level of CD1d that could correspond to an increase of internalization and an increase in the expression of MHC class II in monocytes from Fabry patients (Balreira et al. 2008; Rozenfeld et al. 2009). Therefore it seems that the GSL accumulation in FD induces disturbances in iNKT distribution and plays a proinflammatory role via CD1d pathway.

The proinflammatory pattern in FD is well described with an increase of inflammatory cytokines (De Francesco et al. 2013). Urinary Gb3 levels were found to be highly correlated with increased IL-6 plasma levels ($r = 0.971$, $p < 0.01$) in 14 FD patients (Biancini et al. 2012). The supernatant of PBMC samples from 29 Fabry patients showed a significant increase in secreted IL-6 and IL-1β compared to 15 healthy controls, and dendritic cells (DCs) from the same Fabry patients revealed a trend towards higher basal levels of IL-1β ($p = 0.095$) and TNF-α ($p = 0.049$) secretion than controls. This production was abolished in vitro when using a toll-like receptor 4 (TLR4) blocking antibody. TLRs are innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs) of microorganisms. Via TLRs, DCs link innate and adaptive immunity (Iwasaki and Medzhitov 2004). TLR4 enhances DC maturation in a proinflammatory way inducing the secretion of IL-12 and IFN-γ but the inhibition of IL-4 (Caielli et al. 2010). It also triggers iNKT activation by inducing presentation of endogenous GSL by CD1d molecules. (Caielli et al. 2010). Pathological involvements of TLR4 pathway are reported through an endogenous ligand-mediated signal in an antibody-induced arthritis (Kim et al. 2012). TLRs also recognize endogenous ligands. Accumulated gangliosides in GM1 and GM2 gangliosidosis were reported to act as an endogenous ligand for TLR4 (Jou et al. 2006). Hence, Gb3 is questioned to act as an endogenous ligand to TLR4 (De Francesco et al. 2013). In fact, the Gb3 accumulation appears to be the starting point of an innate immunity reaction, involving CD1d and TLR4 pathways which would induce a dysregulation of one of the iNKT subsets and DCs, source of a pathological proinflammatory state with secretion of specific proinflammatory cytokines. We tried to summarize in Fig. 1 the interrelations between Gb3 accumulation, innate immunity cells and cytokines.

As previously mentioned, ERT could have a beneficial impact on innate immune response in FD in terms of iNKT cell counts (Macedo et al. 2012; Pereira et al. 2013). But no dedicated prospective and controlled study has been done to evaluate the impact of ERT on innate immune response as an endpoint in human. Besides, other therapeutic keys could arise from specific research on innate immunity. For example, lysosomal phospholipase A2 was reported to play a role in the generation of CD1d complexes and could therefore become a possible target like in coronary diseases (Paduraru et al. 2013; The STABILITY Investigators 2014).

But TLRs are not only expressed by immune cells. Endothelial cells, podocytes and kidney tubular epithelial cells also expressed TLR4 (Anders et al. 2004; Banas et al. 2008). Interestingly, Ma et al. recently reported the role of TLR4 activation in diabetic nephropathy (Ma et al. 2014). $TLR4^{-/-}$ diabetic mice had significantly attenuated albuminuria, reduced kidney hypertrophy and glomerular injury. They were also protected from fibrosis and tubular injury. First observed in hepatic fibrosis, TLR4 seems to drive a fibrogenic response through the TGF-β signalling pathway in diabetic nephropathy (Seki et al. 2007; Qian

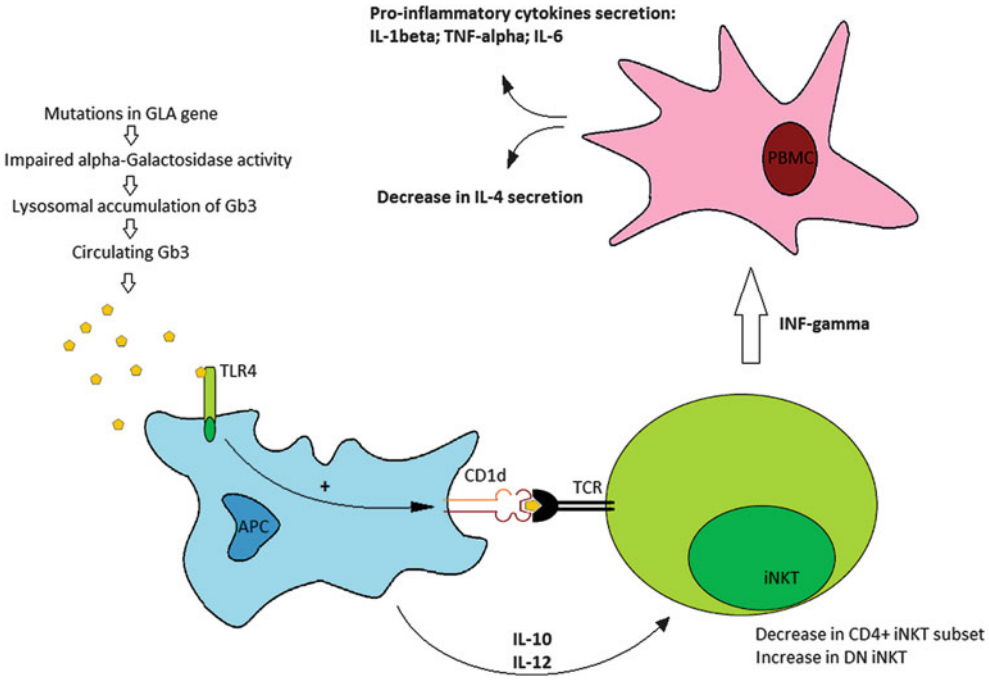


Fig. 1 Innate response observed in Fabry disease. *APCs* antigen-presenting cells, *Gb3* globotriaosylceramide, *iNKTs* invariant natural killer T cells (CD4+ or DN, double negative subsets), *PBMCs* peripheral blood mononuclear cells, *TCR* T-cell receptor, *TLR4* toll-like receptor 4

et al. 2008). Fabry nephropathy has similar aspects with diabetic nephropathy: glomerular and vascular changes with proteinuria then glomerulosclerosis, interstitial fibrosis and tubulopathy (Barbey et al. 2008). In a human podocyte model, lyso(Gb3) increased TGF-β expression, and anti-active TGF-β1 antibodies decreased fibrosis component such as fibronectin and type IV collagen (Sanchez-Niño et al. 2011). In all, Gb3 seems to activate the TLR4 pathway in podocytes and immune cells, leading to local inflammation, cellular injuries and interstitial remodelling.

2. Gb3 deposits induce a pro-oxidative pattern and alterations of the apoptotic pathway.

   Accumulation of Gb3 induces a pathological pro-oxidative state in endothelial cells by decreasing eNOS, enhancing iNOS and COX2 expression and upregulating the expression of cellular adhesion molecules such as ICAM-1, VCAM-1 and E-selectin (Shen et al. 2008; Namdar et al. 2012). Recently, 3-nitrotyrosine (3-NT), a specific marker for reactive nitrogen species and established cardiovascular disease in humans, was even suggested to be a biomarker for the vasculopathy in GLA knockout mice and classical FD patients. Indeed, in 13 FD patients, a more than sixfold elevation in 3-NT concentration was observed in comparison with 11 matched controls (Shu et al. 2014). The pathogenesis of vasculopathy in FD remains controversial. Rombach et al. discussed the role of Gb3 deposits in media layer leading to smooth muscle cell proliferation with arterial remodelling and shear stress (Rombach et al. 2010b). Increases of ROS and NF-κB and decrease of NO would then be the results of the angiotensin system activation. Another hypothesis proposed by Shu et al. is based on the sufficient role of Gb3 deposits to dysregulate eNOS pathway (Shu et al. 2014). Anyway, Gb3 accumulation triggers a pro-oxidative and proapoptotic pattern in endothelial cells and an upregulation of adhesion molecules with pathological effects that lead to diffuse vasculopathy.

   Another inflammatory mechanism can affect the apoptotic pathway in Fabry cells (DeGraba et al. 2000; Shen et al. 2008). Misfolded proteins can trigger the endoplasmic reticulum (ER) stress apoptotic pathway. This pathway is common to numerous inborn metabolic diseases. For example, missense mutations in GBA gene induce production of misfolded enzyme that accumulates in lysosomes of type 1 Gaucher disease patients (Wei et al. 2008). This pathological mechanism would be accessible to chaperone therapy, in which "chaperone" molecules bind to the inactive or unstable misfolded enzymes switching them into a functional shape (Boyd et al. 2013). Surprisingly, a study comparing 32 Fabry patients with 30 healthy controls showed that Fabry PBMCs displayed rather a mitochondrial than ER apoptotic pathway (De Francesco et al. 2011). This phenomenon could be explained by the different types of mutations (nonsense and missense) in patients, which can lead to very different

tertiary structure of proteins and then involve radically different ER stress behaviours. Of note, the type of mutation was not mentioned in the study of De Francesco et al.

Finally, another mechanism affects apoptosis regulation as Gb3 accumulation was associated with an increase of autophagosomes and a decrease of mammalian target of rapamycin (mTOR) and AKT signalling cascades in a human FD podocyte model. The latter are two well-known apoptosis inhibitors (Li et al. 2002; Liebau et al. 2013). Whether only Gb3 accumulation or both Gb3 and misfolded α-GalA are implicated is not fully clarified, but a proapoptotic state mediated by at least the intrinsic mitochondrial apoptotic pathway is described, with a pro-oxidative-associated pattern that could explain in part the systemic vasculopathy observed in FD. The benefits of ERT on these innate immune mechanisms have not been evaluated yet specifically. Although further studies are needed, we can hypothesize that the decrease of Gb3 could reduce inflammation and organ injury.

Immune Adaptive Responses Against Agalsidase in Fabry Disease

1. ERT in Fabry disease
   Two different recombinant α-GalA are available since 2001. Agalsidase alfa (Replagal®, Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) is produced in a human cell line by gene activation, with recommended infusion dosage of 0.2 mg/kg per 14 days (Schiffmann et al. 2000). Agalsidase beta (Fabrazyme®, Genzyme Corporation, Cambridge, MA, USA) is produced by classical cDNA transduction in Chinese hamster ovary (CHO) cells (Eng et al. 2001), with recommended infusion dosage of 1 mg/kg per 14 days. In the United States only agalsidase beta has been approved, whereas both molecules are available in Europe and many other countries. The Canadian Fabry disease initiative attempted a large head-to-head randomized trial (Sirrs et al. 2014). In this study, the primary endpoint was a composite clinical endpoint consisting of renal events, cardiovascular events, cerebrovascular events or death. No statistical difference in this endpoint was observed, but power was limited by the worldwide shortage of agalsidase beta. In the same way, no clear difference in clinical response and tolerance has appeared from the different long-term surveillance reports (Lidove et al. 2010). Of note, a significant correlation was reported between Gb3 clearance of the podocytes and cumulative agalsidase dose ($r = 0{,}804$; $p = 0{,}002$) using both preparations (Tondel et al. 2013). Literature supports benefits of an early initiation of ERT, permitting to stabilize kidney function and improve pain, quality of life and the Mainz Severity Score Index (MSSI) developed to quantify the overall severity of FD (Schiffmann et al. 2001, 2003; Eto et al. 2005; Germain et al. 2007; West et al. 2009; Lidove et al. 2010; Engelen et al. 2012). Nevertheless, a prospective follow-up of 66 male patients revealed that despite long-term ERT, glomerular filtration rate (GFR) declined ($p < 0.001$) and cardiac mass estimated by echocardiograms increased ($p < 0.001$), but the risk of developing a first or second major complication (defined by cardiac events such as rhythm trouble, infarction or congestion, cerebral strokes, end-stage renal disease (ESRD) or death) decreased per year of ERT (OR = 0.81 and OR = 0.52 respectively) (Rombach et al. 2013). Progression of the disease under ERT, estimated with GFR and cardiac mass, appears more important in males than in females (Rombach et al. 2013). ERT seems to have less benefit in advanced renal disease such as proteinuria superior than 1 g/day that is associated with continued loss of GFR (West et al. 2009). In advanced FD, the initiation of ERT seems of doubtful clinical benefits (Rombach et al. 2013; Weidemann et al. 2013). Irreversible organ damages such as fibrosis could explain this phenomenon. Furthermore, infusion-associated reactions (IARs) frequently occur with ERT in FD. Fortunately IARs are usually limited to fever and chills (Eng et al. 2001; Linthorst et al. 2004). These were more frequently experienced in patients with anti-agalsidase antibody-positive status (Smid et al. 2013).
2. Humoral responses against ERT: the lack of standardized assays
   As mentioned, the main issues in understanding the impact of antibody formation are both the safety and the long-term efficacy of ERT (Smid et al. 2013). Since ERT consists in infused recombinant proteins, the development of a humoral response is expected as previously demonstrated, for example, with recombinant factor VIII in the treatment of haemophilia (Lusher et al. 1993). This problem is also well known in other LSDs, such as Pompe disease. It has been demonstrated that in the infantile-onset form of Pompe disease, a significant proportion of patients (particularly the young cross-reactive immunologic material (CRIM)-negative status patients) develop a robust humoral immune response against the recombinant enzyme, attenuating treatment efficiency (de Vries et al. 2010). To date, in FD no standardized assay exists to detect the presence of anti-agalsidase antibodies (Schellekens 2008;

Lidove et al. 2010). In most of the trials and reports studying the IgG response against ERT, except those of the Dutch team of Aerts, Linthorst et al., antibody analyses were performed by the manufacturer of the infused agalsidase preparation (Linthorst et al. 2004; Ohashi et al. 2008; Vedder et al. 2008; Bénichou et al. 2009; Rombach et al. 2010a, 2012; Lidove et al. 2010; Wilcox et al. 2012). Linthorst et al. then Smid et al. showed that all the antibody-positive patients (assessed by the gold standard ELISA) demonstrated a neutralizing activity (Linthorst et al. 2004; Smid et al. 2013). Smid et al. studied the occurrence of IARs from 1999 to 2011 compared to the antibody status. As all antibody-positive patients first demonstrated neutralizing activity, they admitted the reciprocal proposition defining antibody-positive status with neutralizing tests in the course of the study. To our knowledge this reciprocal proposition has not been proven. Neutralizing technique does not take into account all the possible antibody targets, notably the molecules involved in the intracellular uptake, as evoked in an in vivo study in Fabry mice (Ohashi et al. 2008; Rombach et al. 2012). Therefore, comparing the immunogenicity of both treatments appears difficult. Nevertheless, agalsidase alfa seems less immunogenic than agalsidase beta (Lidove et al. 2010). To date, IgE antibodies have only been reported with agalsidase beta, only in males, sporadically and associated with severe IARs (Tanaka et al. 2010). Among the 51 patients receiving agalsidase beta in a double-blind placebo-controlled trial, one experienced anaphylaxis and had positive serum IgE test result and one experienced urticaria with throat congestion and had positive skin test results (Banikazemi et al. 2007). It could be the result of the production process using CHO, with a higher percentage of fully sialylated oligosaccharides and a higher level of phosphorylation (Lee et al. 2003; Bekri 2006; Ohashi et al. 2008; Deegan 2012; Ramaswami et al. 2012). In the latest report of postmarketing surveillance database on agalsidase beta, 73% of the patients developed IgG antibodies towards the exogenous enzyme (Wilcox et al. 2012). Concerning agalsidase alfa, 24% of the patients developed IgG antibodies (Lidove et al. 2010; Keating 2012). The different dosages of the two preparations could explain in part these results as Rombach reported no significant difference in antibody formation between the two preparations when using the same dosage of 0.2 mg/kg for both groups (Rombach et al. 2012). Increasing agalsidase dosage could therefore be associated with the development of antibodies limiting the expected dose-related benefits. All the data mention that females develop fewer antibodies, probably because of the residual enzyme activity due to their heterozygous status (Linthorst et al. 2004; Bénichou et al. 2009; Rombach et al. 2012). During a 1-year follow-up of 59 treated patients, 0/30 females but 17/29 males developed antibodies (Rombach et al. 2012). Antibodies towards agalsidase alfa and beta seem to show a complete cross-reactivity (Linthorst et al. 2004). It seems that men with nonsense mutations develop more antibodies than those with missense mutations, although certain missense mutations such as R342Q are associated with a high rate of seroconversion (Wilcox et al. 2012). Antibodies towards agalsidase seem to develop within 6 months of ERT with a median delay of around 6 weeks (Wilcox et al. 2004; Smid et al. 2013). They can become undetectable after previous positivity, patients then "tolerized" (Rombach et al. 2012; Wilcox et al. 2012). The effectiveness of immunosuppressive therapy in LSDs has already been described. Banugaria et al. reported that immune tolerance induction with rituximab, methotrexate and intravenous immunoglobulins increased the effectiveness of ERT in CRIM-negative infantile Pompe disease patients (Banugaria et al. 2013). Dickson et al. reported another example of such benefits in canine mucopolysaccharidosis I, when combining azathioprine and cyclosporine to ERT (Dickson et al. 2008). Interestingly, while the global impact of antibodies remains unclear, Garman et al. observed in 2004 a reduction of antibody response against agalsidase beta with methotrexate in Fabry mice, at the quite high dose of 10 mg/kg (Garman et al. 2004).

3. Impact of anti-agalsidase antibodies on enzymatic activity in vitro and FD biomarkers

As previously mentioned, Linthorst et al. first reported that all IgG-positive sera by ELISA assay showed a marked in vitro inhibition of enzyme activity (alfa and beta), ranging from 65 to 95% (Linthorst et al. 2004). Only scarce information is available concerning the effects of antibodies on intracellular enzymatic activity. Anti-agalsidase beta antibodies were found to be associated with an inhibition of enzyme activity in cultured fibroblasts of FD patients (Ohashi et al. 2008). A statistically significant association was also reported between high anti-agalsidase beta IgG titres and Gb3 deposition in dermal capillary endothelial cells in 54 males (Bénichou et al. 2009).

On the other hand, the impact of anti-agalsidase on biomarkers in FD is the source of a growing literature, being associated with an increase of urinary Gb3 levels without clear effect on Gb3 plasma levels (Schiffmann et al. 2006; Banikazemi et al. 2007; Vedder et al. 2008). The interpretation of these results is limited because much of the urinary Gb3 derives from podocytes and

tubular cells heterogeneously accessible to ERT. Indeed, agalsidase delivery to podocyte lysosome depends on the high-efficiency uptake by mannose-6-phosphate (M6P) receptor targeted by the M6P pattern of the recombinant enzyme but also on the megalin and sortilin uptakes, independently from M6P (Prabakaran et al. 2011). These secondary receptors could, however, be limited by specific neutralizing antibodies. As previously mentioned, lyso(Gb3) now appears a better biomarker of FD activity. A recent work conducted with 59 adult "classical" Fabry patients, from the Dutch cohort, treated by either agalsidase alfa or beta, reported that plasma lyso(Gb3) was significantly higher all along the 6-year follow-up in seropositive patients for anti-agalsidase antibodies determined by neutralizing tests (seronegative status was assessed when 5 μL of plasma neutralized less than 50% of the enzyme activity in vitro) (Rombach et al. 2012). In the same report, urinary Gb3 decreased only in seronegative patients. Interestingly, in seropositive males switched from an agalsidase beta dose of 0.2 mg/kg/per 2 weeks to 1.0 mg/kg per 2 weeks, plasma lyso(Gb3) and Gb3 additionally declined after 1 year ($p = 0.028$). Nevertheless, urinary Gb3 levels did not change, and biomarker levels remained higher than in seronegative patients ($p < 0.02$) (Rombach et al. 2012). All data are therefore concordant with a negative effect of anti-agalsidase antibodies on biomarkers of FD activity.

4. Impact of anti-agalsidase antibodies on clinical outcomes
   As previously mentioned, evidence of a pathogenic role of Gb3 accumulation is growing. The differences between the decreases of Gb3 and lyso(Gb3) depending on the presence of antibodies lead to believe that antibodies have a negative impact on clinical outcomes. Nevertheless, anti-agalsidase antibodies (alfa and beta) seem to have no influence on GFR slopes and cardiac mass nor the apparition of new white-matter lesions on cerebral magnetic resonance imaging (Schiffmann et al. 2006; Bénichou et al. 2009; Rombach et al. 2012). Some IARs were associated with high titre of antibodies, required hospitalization and caused significant disabilities or death (Smid et al. 2013). In this study, seropositive status was assessed by a neutralizing test that may be unsuitable for such safety studies. However further studies are needed with larger cohorts and standardized assays to determine the entire impact of these antibodies on clinical outcomes.
5. Cellular immune response against agalsidase
   To our knowledge, nothing is known about the possible cellular immune response triggered by agalsidase and its effects.

Conclusion

As Fabry disease is nowadays increasingly diagnosed, the description of the mechanisms involved in its pathogenesis, notably the innate immune reactions, appears essential. Numerous observations support the evidence of a proinflammatory state in Fabry cells, with a probable direct role of Gb3 which would activate TLR4 and CD1d pathways and enhance inflammatory cascades via iNKTs. Notably described in endothelial cells, this may in part explain the observed vasculopathy. TLR4 also enhances TGF-β expression in podocytes leading to the remodelling of the extracellular matrix and secondary irreversible damages such as fibrosis and could explain in part the Fabry nephropathy. Surprisingly, the description of the effects of ERT on these reactions is lacking.

Long-term and early-initiated ERT with both agalsidase alfa and beta appear to improve clinical outcomes when compared to placebo controls. Nevertheless, it prevents neither critical events nor irremediable progression of the disease. Anti-agalsidase antibodies are associated with a negative influence on biomarkers, enzymatic activity and intracellular Gb3 deposits, but the consequences on clinical outcomes are not perfectly clear, partly due to the lack of data and standardized assays.

Understanding the immune response in FD becomes a priority issue in the light of ERT limits. The identification of immunological pathways in FD physiopathology can lead to the development of targeted therapeutics. A better understanding of the immune response developed towards ERT could allow a better management of its effect and the development of new complementary strategies such as antioxidant, chaperones, gene therapies and/or immunosuppressive strategies.

Synopsis

Gb3, TLR4, invariant natural killer T cells and anti-agalsidase antibodies are key actors in immune responses observed in Fabry disease.

Compliance with Ethics Guidelines

Conflict of Interest

Wladimir Mauhin has received travel fees from Shire.

Olivier Lidove has received travel fees and speaker honoraria from Sanofi-Genzyme and Shire HGT and speaker honorarium from GSK.

Elisa Masat declares that she has no conflict of interest.

Federico Mingozzi declares that he has no conflict of interest.

Kuberaka Mariampillai declares that she has no conflict of interest.

Jean-Marc Ziza declares that he has no conflict of interest.

Olivier Benveniste declares that he has no conflict of interest.

Informed Consent

Not concerned.

Animal Rights

Not concerned.

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

Details of the Contributions of Individual Authors

WM, OL, EM, FM and OB: conception and design, analysis, interpretation of data and drafting the article

WM, OL and OB: revision

KM and JMZ: design, analysis and interpretation of data

Guarantor: OB

All the authors contributed to this manuscript and approved its revision and its submission.

This article has not been previously proposed for submission or publication.

References

Aerts JM, Groener JE, Kuiper S et al (2008) Elevated globotriaosylsphingosine is a hallmark of Fabry disease. Proc Natl Acad Sci U S A 105:2812–2817. doi:10.1073/pnas.0712309105

Anders H-J, Banas B, Schlöndorff D (2004) Signaling danger: toll-like receptors and their potential roles in kidney disease. J Am Soc Nephrol 15:854–867

Balreira A, Macedo MF, Girão C et al (2008) Anomalies in conventional T and invariant natural killer T-cell populations in Fabry mice but not in Fabry patients. Br J Haematol 143:601–604. doi:10.1111/j.1365-2141.2008.07380.x

Banas MC, Banas B, Hudkins KL et al (2008) TLR4 links podocytes with the innate immune system to mediate glomerular injury. J Am Soc Nephrol 19:704–713. doi:10.1681/ASN.2007040395

Banikazemi M, Bultas J, Waldek S et al (2007) Agalsidase-beta therapy for advanced Fabry disease: a randomized trial. Ann Intern Med 146:77–86

Banugaria SG, Prater SN, Patel TT et al (2013) Algorithm for the early diagnosis and treatment of patients with cross reactive immunologic material-negative classic infantile Pompe disease: a step towards improving the efficacy of ERT. PLoS One 8(6):e67052

Barbey F, Lidove O, Schwarting A (2008) Fabry nephropathy: 5 years of enzyme replacement therapy—a short review. NDT Plus 1 (1):11–19

Beck M (2006) Demographics of FOS – the Fabry outcome survey. In: Mehta A, Beck M, Sunder-Plassmann G (eds) Fabry disease: perspectives from 5 years of FOS. Oxford PharmaGenesis, Oxford

Bekri S (2006) Importance of glycosylation in enzyme replacement therapy. In: Mehta A, Beck M, Sunder-Plassmann G (eds) Source Fabry disease: perspectives from 5 years of FOS. Oxford PharmaGenesis, Oxford

Bénichou B, Goyal S, Sung C et al (2009) A retrospective analysis of the potential impact of IgG antibodies to agalsidase beta on efficacy during enzyme replacement therapy for Fabry disease. Mol Genet Metab 96:4–12. doi:10.1016/j.ymgme.2008.10.004

Biancini GB, Vanzin CS, Rodrigues DB et al (2012) Globotriaosylceramide is correlated with oxidative stress and inflammation in Fabry patients treated with enzyme replacement therapy. Biochim Biophys Acta 1822:226–232. doi:10.1016/j.bbadis.2011.11.001

Boyd RE, Lee G, Rybczynski P et al (2013) Pharmacological chaperones as therapeutics for lysosomal storage diseases. J Med Chem 56:2705–2725. doi:10.1021/jm301557k

Caielli S, Conforti-Andreoni C, Di Pietro C et al (2010) On/off TLR signaling decides proinflammatory or tolerogenic dendritic cell maturation upon CD1d-mediated interaction with invariant NKT cells. J Immunol 185:7317–7329. doi:10.4049/jimmunol.1000400

Chimenti C, Pieroni M, Morgante E et al (2004) Prevalence of Fabry disease in female patients with late-onset hypertrophic cardiomyopathy. Circulation 110:1047–1053. doi:10.1161/01.CIR.0000139847.74101.03

Chuang Y-P, Wang C-H, Wang N-C et al (2012) Modulatory function of invariant natural killer T cells in systemic lupus erythematosus. Clin Dev Immunol 2012:478429. doi:10.1155/2012/478429

De Francesco PN, Mucci JM, Ceci R et al (2011) Higher apoptotic state in Fabry disease peripheral blood mononuclear cells.: effect of globotriaosylceramide. Mol Genet Metab 104:319–324. doi:10.1016/j.ymgme.2011.06.007

De Francesco PN, Mucci JM, Ceci R et al (2013) Fabry disease peripheral blood immune cells release inflammatory cytokines: role of globotriaosylceramide. Mol Genet Metab 109:93–99. doi:10.1016/j.ymgme.2013.02.003

De Vries JM, van der Beek NAME, Kroos MA et al (2010) High antibody titer in an adult with Pompe disease affects treatment with alglucosidase alfa. Mol Genet Metab 101:338–345. doi:10.1016/j.ymgme.2010.08.009

Deegan PB (2012) Fabry disease, enzyme replacement therapy and the significance of antibody responses. J Inherit Metab Dis 35:227–243. doi:10.1007/s10545-011-9400-y

DeGraba T, Azhar S, Dignat-George F et al (2000) Profile of endothelial and leukocyte activation in Fabry patients. Ann Neurol 47:229–233

Dickson P, Peinovich M, McEntee M et al (2008) Immune tolerance improves the efficacy of enzyme replacement therapy in canine mucopolysaccharidosis I. J Clin Invest 118(8):2868–2876

El Dib RP, Nascimento P, Pastores GM (2013) Enzyme replacement therapy for Anderson-Fabry disease. Cochrane Database Syst Rev 2, CD006663. doi:10.1002/14651858.CD006663.pub3

Eng CM, Guffon N, Wilcox WR et al (2001) Safety and efficacy of recombinant human alpha-galactosidase A–replacement therapy in Fabry's disease. N Engl J Med 345:9–16. doi:10.1056/NEJM200107053450102

Engelen MA, Brand E, Baumeister TB et al (2012) Effects of enzyme replacement therapy in adult patients with Fabry disease on cardiac structure and function: a retrospective cohort study of the Fabry Munster Study (FaMuS) data. BMJ Open. doi:10.1136/bmjopen-2012-000879

Eto Y, Ohashi T, Utsunomiya Y et al (2005) Enzyme replacement therapy in Japanese Fabry disease patients: the results of a phase 2 bridging study. J Inherit Metab Dis 28:575–583. doi:10.1007/s10545-005-0575-y

Fuller M, Meikle PJ, Hopwood JJ (2006) Epidemiology of lysosomal storage diseases: an overview. In: Mehta A, Beck M, Sunder-Plassmann G (eds) Source Fabry disease: perspectives from 5 years of FOS. Oxford PharmaGenesis, Oxford

Gadola SD, Silk JD, Jeans A et al (2006) Impaired selection of invariant natural killer T cells in diverse mouse models of glycosphingolipid lysosomal storage diseases. J Exp Med 203:2293–2303. doi:10.1084/jem.20060921

Garman RD, Munroe K, Richards SM (2004) Methotrexate reduces antibody responses to recombinant human alpha-galactosidase A therapy in a mouse model of Fabry disease. Clin Exp Immunol 137:496–502. doi:10.1111/j.1365-2249.2004.02567.x

Germain DP, Waldek S, Banikazemi M et al (2007) Sustained, long-term renal stabilization after 54 months of agalsidase beta therapy in patients with Fabry disease. J Am Soc Nephrol 18:1547–1557. doi:10.1681/ASN.2006080816

Gumperz JE, Miyake S, Yamamura T, Brenner MB (2002) Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J Exp Med 195:625–636

Hunn MK, Hermans IF (2013) Exploiting invariant NKT cells to promote T-cell responses to cancer vaccines. Oncoimmunology 2:e23789. doi:10.4161/onci.23789

Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol 5:987–995. doi:10.1038/ni1112

Jou I, Lee JH, Park SY et al (2006) Gangliosides trigger inflammatory responses via TLR4 in brain glia. Am J Pathol 168:1619–1630. doi:10.2353/ajpath.2006.050924

Keating GM (2012) Agalsidase alfa: a review of its use in the management of Fabry disease. BioDrugs 26:335–354. doi:10.2165/11209690-000000000-00000

Kim JH, Kim HS, Kim HY et al (2012) Direct engagement of TLR4 in invariant NKT cells regulates immune diseases by differential IL-4 and IFN-γ production in mice. PLoS One 7:e45348. doi:10.1371/journal.pone.0045348

Lee K, Jin X, Zhang K et al (2003) A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. Glycobiology 13:305–313. doi:10.1093/glycob/cwg034

Li B, Desai SA, MacCorkle-Chosnek RA et al (2002) A novel conditional Akt "survival switch" reversibly protects cells from apoptosis. Gene Ther 9:233–244. doi:10.1038/sj.gt.3301641

Lidove O, West ML, Pintos-Morell G et al (2010) Effects of enzyme replacement therapy in Fabry disease–a comprehensive review of the medical literature. Genet Med 12:668–679. doi:10.1097/GIM.0b013e3181f13b75

Liebau MC, Braun F, Höpker K et al (2013) Dysregulated autophagy contributes to podocyte damage in Fabry's disease. PLoS One 8:e63506. doi:10.1371/journal.pone.0063506

Linthorst GE, Hollak CEM, Donker-Koopman WE et al (2004) Enzyme therapy for Fabry disease: neutralizing antibodies toward agalsidase alpha and beta. Kidney Int 66:1589–1595. doi:10.1111/j.1523-1755.2004.00924.x

Linthorst GE, Bouwman MG, Wijburg FA et al (2010) Screening for Fabry disease in high-risk populations: a systematic review. J Med Genet 47:217–222. doi:10.1136/jmg.2009.072116

Lukas J, Giese A-K, Markoff A et al (2013) Functional characterisation of alpha-galactosidase a mutations as a basis for a new classification system in fabry disease. PLoS Genet 9:e1003632. doi:10.1371/journal.pgen.1003632

Lusher JM, Arkin S, Abildgaard CF, Schwartz RS (1993) Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. N Engl J Med 328:453–459. doi:10.1056/NEJM199302183280701

Ma J, Chadban SJ, Zhao CY et al (2014) TLR4 activation promotes podocyte injury and interstitial fibrosis in diabetic nephropathy. PLoS One 9:e97985. doi:10.1371/journal.pone.0097985

MacDermot KD, Holmes A, Miners AH (2001a) Anderson-Fabry disease: clinical manifestations and impact of disease in a cohort of 98 hemizygous males. J Med Genet 38:750–760

MacDermot KD, Holmes A, Miners AH (2001b) Anderson-Fabry disease: clinical manifestations and impact of disease in a cohort of 60 obligate carrier females. J Med Genet 38:769–775

Macedo MF, Quinta R, Pereira CS, Sa Miranda MC (2012) Enzyme replacement therapy partially prevents invariant Natural Killer T cell deficiency in the Fabry disease mouse model. Mol Genet Metab 106:83–91. doi:10.1016/j.ymgme.2012.02.014

Mehta A, Beck M, Eyskens F et al (2010) Fabry disease: a review of current management strategies. QJM 103:641–659. doi:10.1093/qjmed/hcq117

Meikle PJ, Hopwood JJ, Clague AE, Carey WF (1999) Prevalence of lysosomal storage disorders. J Am Med Assoc 281:249–254

Nakao S, Kodama C, Takenaka T et al (2003) Fabry disease: detection of undiagnosed hemodialysis patients and identification of a "renal variant" phenotype. Kidney Int 64:801–807. doi:10.1046/j.1523-1755.2003.00160.x

Namdar M, Gebhard C, Studiger R et al (2012) Globotriaosylsphingosine accumulation and not alpha-galactosidase-A deficiency causes endothelial dysfunction in Fabry disease. PLoS One 7:e36373. doi:10.1371/journal.pone.0036373

O'Reilly V, Zeng SG, Bricard G et al (2011) Distinct and overlapping effector functions of expanded human CD4+, CD8α + and CD4-CD8α- invariant natural killer T cells. PLoS One 6:e28648. doi:10.1371/journal.pone.0028648

Ohashi T, Iizuka S, Ida H, Eto Y (2008) Reduced alpha-Gal A enzyme activity in Fabry fibroblast cells and Fabry mice tissues induced by serum from antibody positive patients with Fabry disease. Mol Genet Metab 94:313–318. doi:10.1016/j.ymgme.2008.03.008

Paduraru C, Bezbradica JS, Kunte A et al (2013) Role for lysosomal phospholipase A2 in iNKT cell-mediated CD1d recognition. Proc Natl Acad Sci U S A 110:5097–5102. doi:10.1073/pnas.1302923110

Pereira CS, Azevedo O, Maia ML et al (2013) Invariant natural killer T cells are phenotypically and functionally altered in Fabry disease. Mol Genet Metab 108:241–248. doi:10.1016/j.ymgme.2013.01.018

Prabakaran T, Nielsen R, Larsen JV et al (2011) Receptor-mediated endocytosis of alpha-galactosidase A in human podocytes in Fabry disease. PLoS One. doi:10.1371/journal.pone.0025065

Qian Y, Feldman E, Pennathur S et al (2008) From fibrosis to sclerosis: mechanisms of glomerulosclerosis in diabetic nephropathy. Diabetes 57:1439–1445. doi:10.2337/db08-0061

Ramaswami U, Parini R, Pintos-Morell G et al (2012) Fabry disease in children and response to enzyme replacement therapy: results from the Fabry Outcome Survey. Clin Genet 81:485–490. doi:10.1111/j.1399-0004.2011.01671.x

Rolfs A, Böttcher T, Zschiesche M et al (2005) Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study. Lancet 366:1794–1796. doi:10.1016/S0140-6736(05)67635-0

Rombach SM, Dekker N, Bouwman MG et al (2010a) Plasma globotriaosylsphingosine: diagnostic value and relation to clinical manifestations of Fabry disease. Biochim Biophys Acta 1802:741–748. doi:10.1016/j.bbadis.2010.05.003

Rombach SM, Twickler TB, Aerts JMFG et al (2010b) Vasculopathy in patients with Fabry disease: current controversies and research directions. Mol Genet Metab 99:99–108. doi:10.1016/j.ymgme.2009.10.004

Rombach SM, Aerts JMFG, Poorthuis BJHM et al (2012) Long-term effect of antibodies against infused alpha-galactosidase A in Fabry disease on plasma and urinary (lyso)Gb3 reduction and treatment outcome. PLoS One 7:e47805. doi:10.1371/journal.pone.0047805

Rombach SM, Smid BE, Bouwman MG et al (2013) Long term enzyme replacement therapy for Fabry disease: effectiveness on kidney, heart and brain. Orphanet J Rare Dis 8:47. doi:10.1186/1750-1172-8-47

Rombach SM, Smid BE, Linthorst GE et al (2014) Natural course of Fabry disease and the effectiveness of enzyme replacement therapy: a systematic review and meta-analysis : effectiveness of ERT in different disease stages. J Inherit Metab Dis. doi:10.1007/s10545-014-9677-8

Rozenfeld P, Agriello E, De Francesco N et al (2009) Leukocyte perturbation associated with Fabry disease. J Inherit Metab Dis 32(Suppl 1):S67–S77. doi:10.1007/s10545-009-1060-9

Sanchez-Niño MD, Sanz AB, Carrasco S et al (2011) Globotriaosylsphingosine actions on human glomerular podocytes: implications for Fabry nephropathy. Nephrol Dial Transplant 26:1797–1802. doi:10.1093/ndt/gfq306

Schellekens H (2008) The immunogenicity of therapeutic proteins and the Fabry antibody standardization initiative. Clin Ther 30(Suppl B):S50–S51

Schiffmann R, Murray GJ, Treco D et al (2000) Infusion of alpha-galactosidase A reduces tissue globotriaosylceramide storage in patients with Fabry disease. Proc Natl Acad Sci U S A 97:365–370

Schiffmann R, Kopp JB, Austin HA 3rd et al (2001) Enzyme replacement therapy in Fabry disease: a randomized controlled trial. J Am Med Assoc 285:2743–2749

Schiffmann R, Floeter MK, Dambrosia JM et al (2003) Enzyme replacement therapy improves peripheral nerve and sweat function in Fabry disease. Muscle Nerve 28:703–710. doi:10.1002/mus.10497

Schiffmann R, Ries M, Timmons M et al (2006) Long-term therapy with agalsidase alfa for Fabry disease: safety and effects on renal function in a home infusion setting. Nephrol Dial Transplant 21:345–354. doi:10.1093/ndt/gfi152

Schiffmann R, Waldek S, Benigni A, Auray-Blais C (2010) Biomarkers of Fabry disease nephropathy. Clin J Am Soc Nephrol 5:360–364. doi:10.2215/CJN.06090809

Seki E, De Minicis S, Osterreicher CH et al (2007) TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 13:1324–1332. doi:10.1038/nm1663

Shen J-S, Meng X-L, Moore DF et al (2008) Globotriaosylceramide induces oxidative stress and up-regulates cell adhesion molecule expression in Fabry disease endothelial cells. Mol Genet Metab 95:163–168. doi:10.1016/j.ymgme.2008.06.016

Shu L, Vivekanandan-Giri A, Pennathur S et al (2014) Establishing 3-nitrotyrosine as a biomarker for the vasculopathy of Fabry disease. Kidney Int. doi:10.1038/ki.2013.520

Sirrs SM, Bichet DG, Casey R et al (2014) Outcomes of patients treated through the Canadian Fabry disease initiative. Mol Genet Metab 111:499–506. doi:10.1016/j.ymgme.2014.01.014

Smid BE, Hoogendijk SL, Wijburg FA et al (2013) A revised home treatment algorithm for Fabry disease: influence of antibody formation. Mol Genet Metab 108:132–137. doi:10.1016/j.ymgme.2012.12.005

Spada FM, Koezuka Y, Porcelli SA (1998) CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J Exp Med 188:1529–1534

Spada M, Pagliardini S, Yasuda M et al (2006) High incidence of later-onset fabry disease revealed by newborn screening. Am J Hum Genet 79:31–40. doi:10.1086/504601

Sugita M, Cernadas M, Brenner MB (2004) New insights into pathways for CD1-mediated antigen presentation. Curr Opin Immunol 16:90–95

Tanaka A, Takeda T, Hoshina T et al (2010) Enzyme replacement therapy in a patient with Fabry disease and the development of IgE antibodies against agalsidase beta but not agalsidase alpha. J Inherit Metab Dis 33(Suppl 3):S249–S252. doi:10.1007/s10545-010-9136-0

The STABILITY Investigators (2014) Darapladib for preventing ischemic events in stable coronary heart disease. N Engl J Med. doi:10.1056/NEJMoa1315878

Thomaidis T, Relle M, Golbas M et al (2009) Downregulation of alpha-galactosidase A upregulates CD77: functional impact for Fabry nephropathy. Kidney Int 75:399–407. doi:10.1038/ki.2008.576

Thomas AS, Mehta AB (2013) Difficulties and barriers in diagnosing Fabry disease: what can be learnt from the literature? Expert Opin Med Diagn 7:589–599. doi:10.1517/17530059.2013.846322

Tondel C, Bostad L, Larsen KK (2013) Agalsidase benefits renal histology in young patients with Fabry disease. J Am Soc Nephrol 24(1):137–148. doi:10.1681/ASN.2012030316

Vedder AC, Linthorst GE, van Breemen MJ et al (2007) The Dutch Fabry cohort: diversity of clinical manifestations and Gb3 levels. J Inherit Metab Dis 30:68–78. doi:10.1007/s10545-006-0484-8

Vedder AC, Breunig F, Donker-Koopman WE et al (2008) Treatment of Fabry disease with different dosing regimens of agalsidase: effects on antibody formation and GL-3. Mol Genet Metab 94:319–325. doi:10.1016/j.ymgme.2008.03.003

Waldek S, Patel MR, Banikazemi M et al (2009) Life expectancy and cause of death in males and females with Fabry disease: findings from the Fabry Registry. Genet Med 11:790–796. doi:10.1097/GIM.0b013e3181bb05bb

Wang RY, Lelis A, Mirocha J, Wilcox WR (2007) Heterozygous Fabry women are not just carriers, but have a significant burden of disease and impaired quality of life. Genet Med 9:34–45

Wei H, Kim S-J, Zhang Z et al (2008) ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders and are alleviated by chemical chaperones. Hum Mol Genet 17:469–477. doi:10.1093/hmg/ddm324

Weidemann F, Niemann M, Störk S et al (2013) Long-term outcome of enzyme-replacement therapy in advanced Fabry disease: evidence for disease progression towards serious complications. J Intern Med 274:331–341. doi:10.1111/joim.12077

West M, Nicholls K, Mehta A et al (2009) Agalsidase alfa and kidney dysfunction in Fabry disease. J Am Soc Nephrol 20:1132–1139. doi:10.1681/ASN.2008080870

Wilcox WR, Banikazemi M, Guffon N et al (2004) Long-term safety and efficacy of enzyme replacement therapy for Fabry disease. Am J Hum Genet 75:65–74

Wilcox WR, Linthorst GE, Germain DP et al (2012) Anti-α-galactosidase A antibody response to agalsidase beta treatment: data from the Fabry Registry. Mol Genet Metab 105:443–449. doi:10.1016/j.ymgme.2011.12.006

Winchester B, Young E (2006) Biochemical and genetic diagnosis of Fabry disease. In: Mehta A, Beck M, Sunder-Plassmann G (eds) Source Fabry disease: perspectives from 5 years of FOS. Oxford PharmaGenesis, Oxford

JIMD Reports
DOI 10.1007/8904_2014_405

RESEARCH REPORT

Asparagine Synthetase Deficiency: New Inborn Errors of Metabolism

Majid Alfadhel • Muhammad Talal Alrifai • Daniel Trujillano • Hesham Alshaalan • Ali Al Othaim • Shatha Al Rasheed • Hussam Assiri • Abdulrhman A. Alqahtani • Manal Alaamery • Arndt Rolfs • Wafaa Eyaid

Received: 23 September 2014 / Revised: 12 December 2014 / Accepted: 22 December 2014 / Published online: 8 February 2015

Abstract *Background*: Asparagine synthetase deficiency (ASD) is a newly identified neurometabolic disorder characterized by severe congenital microcephaly, severe global developmental delay, intractable seizure disorder, and spastic quadriplegia. Brain MRI showed brain atrophy, delayed myelination, and simplified gyriform pattern.

Methods: We report ASD deficiency in a 2- and 4-year-old sibling. On them, we described clinical, biochemical, and molecular findings, and we compared our results with previously reported cases.

Results: We identified a homozygous novel missense mutation in *ASNS* gene in both probands and we demonstrated low CSF and plasma asparagine in both patients.

Conclusions: Clinicians should suspect ASD deficiency in any newborn presented with severe congenital microcephaly followed by severe epileptic encephalopathy and global developmental delay. CSF asparagine level is low in this disorder while plasma may be low.

Communicated by: Jutta Gaertner

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2014_405) contains supplementary material, which is available to authorized users.

M. Alfadhel (✉) · S. Al Rasheed · W. Eyaid
Division of Genetics, Department of Pediatrics, King Abdulaziz Medical City, Riyadh, Saudi Arabia
e-mail: dralfadhelm@gmail.com

M. Alfadhel · M.T. Alrifai · H. Alshaalan · A. Al Othaim · S. Al Rasheed · W. Eyaid
King Saud bin Abdulaziz University for Health Sciences, King Abdulaziz Medical City, Riyadh, Saudi Arabia

M.T. Alrifai
Division of Neurology, Department of Pediatrics, King Abdulaziz Medical City, Riyadh, Saudi Arabia

D. Trujillano · A. Rolfs
Centogene AG, Schillingallee 68, 18057, Rostock, Germany

H. Alshaalan
Department of Radiology, King Abdulaziz Medical City, Riyadh, Saudi Arabia

A. Al Othaim · H. Assiri
Biochemical Genetics Lab, Department of Pathology, King Abdulaziz Medical City, Riyadh, Saudi Arabia

A.A. Alqahtani · M. Alaamery
King Abdullah International Medical Research Center (KAIMRC), Riyadh, Saudi Arabia

M. Alaamery
The Brain Genome Laboratory (BGL), King Abdulaziz Medical City, Riyadh, Saudi Arabia

Introduction

Asparagine synthetase deficiency (ASD) is a new rare autosomal recessive neurometabolic disease which is caused by homozygous or compound heterozygous mutation in the *ASNS* gene on chromosome 7q21 (Ruzzo et al. 2013). It is described recently by Ruzzo et al. (2013) who reported nine cases from four unrelated families; they have a distinct form of severe encephalopathy associated with congenital microcephaly, progressive brain atrophy, intractable seizure, and profound developmental delay (Ruzzo et al. 2013). They had axial hypotonia with severe appendicular spasticity (Bourgeron et al. 1994; Ruzzo et al. 2013). As this is a newly described disease, there is not much data about clinical and biochemical phenotype. In this case report, we elaborate more on the clinical, biochemical, and molecular findings of 2 siblings with asparagine synthetase deficiency and we compared our

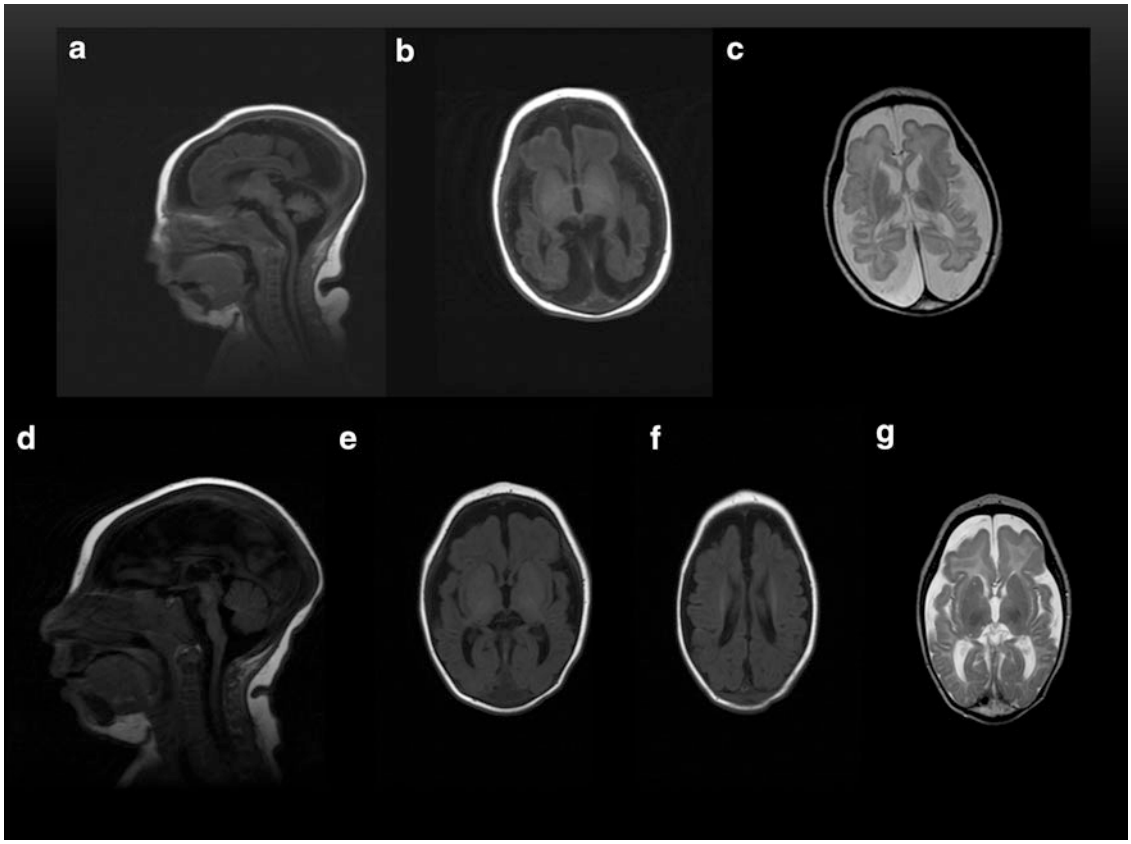


Fig. 1 Brain MRI of a 14-day-old girl (**a**–**c**). (**a**) Sagittal T1 shows severe microcephaly and cerebral atrophy. (**b**) Axial T1 shows simplified gyriform pattern of the frontal lobes with delayed myelination. (**c**) Axial T2 shows normal basal ganglia. Brain MRI of a 4-month-old boy (**d**–**g**). (**d**) Sagittal T1 shows severe microcephaly and cerebral atrophy. (**e**, **f**) Axial T1 shows simplified gyriform pattern of the frontal lobes with delayed myelination. (**g**) Axial T2 shows normal basal ganglia, frontal lobes simplified gyriform pattern, and delayed myelination

results to the previously reported cases. To the best of our knowledge, this is the first time in the literature that confirmed low CSF asparagine in this disorder and the third report in the literature regarding this new inborn error of neurometabolic disorder.

Case Report

Patient 1 was a full-term baby boy born by cesarean section due to fetal distress and failure to progress to the first-cousin Saudi parents. His birth weight was 3.1 kg (10–25th percentile), length 45 cm (<5th percentile), head circumference 30 cm (<5th percentile), and Apgar score 8 and 9 at 5 and 10 min, respectively. Few hours after birth, he started to have intractable seizure. He stayed in the nursery for 6 days with myoclonic seizures until controlled with multiple antiepileptic medications. Then he was discharged on these medications and continued to have an on-and-off attack of myoclonic seizure. On examination, his growth parameters continued to be less than 5th percentile and growth arrested. Electroencephalogram (EEG) showed multifocal epileptiform discharges, diffuse slowing, and paroxysmal attenuation in sleep. Brain MRI showed severe microcephaly, brain atrophy, evidence of simplified gyral pattern, and delayed myelination (Fig. 1). Modified barium swallow showed minimal laryngeal penetration without aspiration. Upper GI study showed moderate to severe reflux with no malrotation.

Patient 2 was the sister born by normal spontaneous vaginal delivery. Her birth weight was 2.65 kg (below 10th percentile) and the head circumference was 26.5 cm (far below 5th percentile) and length was 42 cm (below 10th percentile). Apgar scores were 7 and at 1 min and 8 at 5 min. She was admitted to NICU after 5 min of age with severe microcephaly and seizure and she has the similar course and clinical phenotype as her brother.

On examination of both siblings at 2 and 4 years of age, both have severe microcephaly and all growth parameters <3rd percentile. They have subtle dysmorphic features (microcephaly, brachycephaly, pear-like head shape, micrognathia). Neurological examination showed axial hypotonia

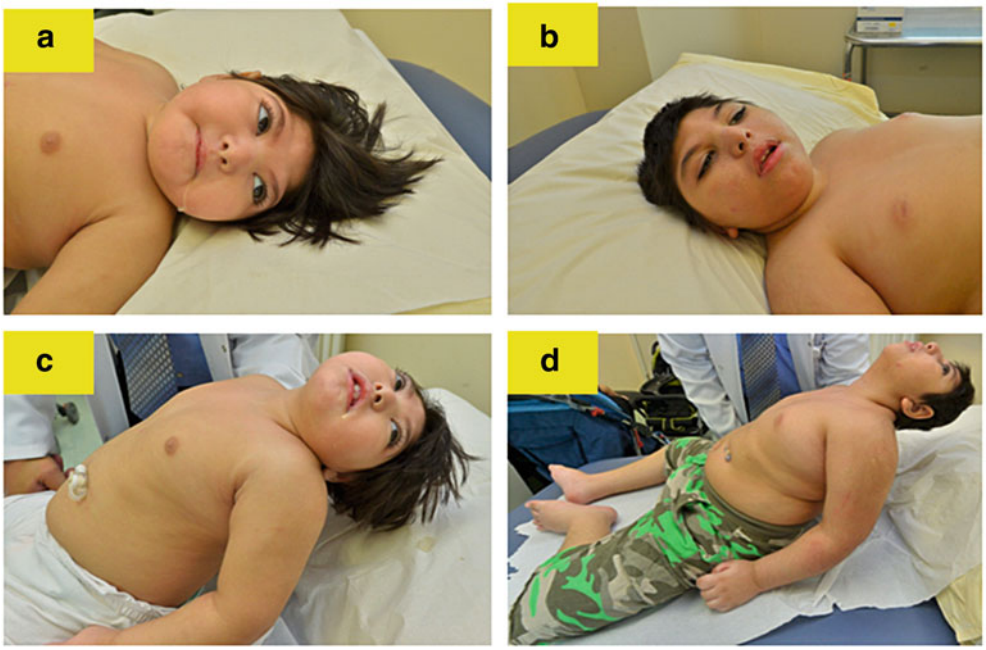


Fig. 2 Subtle dysmorphic features: microcephaly, brachycephaly, pear-like head shape, micrognathia, and axial hypotonia (**a**–**d**)

and appendicular hypertonia with hyperreflexia (Fig. 2). They continued to have severe developmental delay and not acquiring any milestones. Other system examinations were unremarkable. Both continued to have intractable seizure refractory to antiepileptic medications. Milk scan showed recurrent gastroesophageal reflux.

All biochemical and molecular investigations including acylcarnitine profile and urine organic acids, creatine kinase (CK) level, total homocysteine, lactic acid, ammonia level, chromosomal analysis, and CGH microarray were unremarkable. The concentration of CSF neurotransmitters homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) was moderately reduced. Molecular genetic testing of *MC2R*, *PNKP*, and *ASPM* was negative.

Further analysis by whole exome sequencing (WES) performed on both siblings and parents identified a previously unreported homozygous mutation in exon 10 of the asparagine synthetase (*ASNS*) gene (c.1160A>G[p.Tyr377Cys]) in both siblings. The parents confirmed to be carriers for this mutation and the discovered mutation validated by Sanger sequencing methodology (for detailed WES methodology, filtering strategy, and average coverage, please see supplementary materials).

Discussion

Our report showed the similar findings delineated by previous reports (Ruzzo et al. 2013; Ben-Salem et al. 2014). Table 1 compared the clinical findings in this report with the former ones. In 12 children described so far, all has the following cardinal features which are severe congenital microcephaly (100%), ranging between 26.5 and 31.5 cm; severe developmental delay and the patients just lying in the bed, not fixing or following and not acquiring any milestones; appendicular hypertonia; and hyperreflexia (100%). Majority of patients has additional clinical features including intractable seizure (9/12, 75%), axial hypotonia (8/12, 67%), and hyperekplexia (3/12, 25%). The type of seizure is not specific including myoclonic, tonic, spasm, and generalized tonic-clonic seizure which are refractory to antiepileptic medications. EEG pattern is reported differently but with main features such as multiple independent spike foci (8/12, 67%); other EEG findings were hypsarrhythmia, burst suppression, disorganized background. Radiologically, all patients have severe microcephaly, brain atrophy, and delayed myelination (100%). Other features include simplified gyriform pattern (9/12, 75%) and decreased size pons (7/12, 58%) (Fig. 2). The consanguinity found in 4/6 families (67%) suggests that this disorder is inherited as autosomal recessive pattern. Furthermore, the ethnicities are diverse including Iranian Jews, French Canadian, Bangladeshi, and Saudi Arabian in this report confirming that this disorder is pan-ethnic. In this report, we presented a novel missense mutation that was not described by previous reports in exon 10 of the *ASNS* gene. The pathogenicity and functional impact of this novel variant is supported by SIFT and MutationTaster software

Table 1 Demographic and clinical data of the presented cases compared to previously reported cases

	Our report	Ruzzo et al. (2013)	Ben-Salem et al. (2014)
Number of patients	2	9	1
Number of families	1	4	1
Age	(2 years, 4 years)	9 months to 14 years	5 years
Sex (M:F)	1:1	8:1	1:0
Ethnicity	Saudi Arabian	Iranian Jews, French Canadian, Bangladeshi	Emirati
Consanguinity	Yes	Yes in 2 families	Yes
Mutation in *ASNS* gene	c. 1160 A>G(p.Tyr377Cys) Saudi siblings	c.1084T>G(p. phe362Val) c.1648C>T (p.Arg550Cys c.1648C>T(p. Arg550Cys)/ c.17C>A(p. Ala6Glu	c.1193A>C(p. Tyr398Cys)
Types of mutation	Missense and homozygous	Missense, homozygous, and compound heterozygous	Missense and homozygous
Developmental delay	Severe	Severe	Severe
Progressive microcephaly	Both	Eight out of nine	Yes
Axial hypotonia	Both	Five out of nine	
Spastic quadriplegia	Both	All	Yes
Seizure	Both	Six out of nine	Yes
Type of seizure	Both have GTC and myoclonic[a]	Three of them have spasm, tonic, myoclonic, and GTC Two of them have partial complex One of them has tonic and orobuccal	Myoclonic
EEG pattern	MISF[b]	Two of them have bursts and MISF Three of them have hypsarrhythmia and MISF	MISF
MRI	Both have severe microcephaly, brain atrophy and delayed myelination, and simplified gyriform pattern	All have the severe microcephaly, brain atrophy, and delayed myelination. 6/9 have decreased size pons and simplified gyriform pattern	Severe microcephaly, thin corpus callosum, ventriculomegaly, brain atrophy, decreased size pons, and simplified gyriform pattern

M male, *F* female
[a] Generalized tonic-clonic seizure
[b] Multiple independent spike foci

analyses. Furthermore, it is located in a moderately conserved nucleotide and highly conserved amino acid position, with large physiochemical differences between the amino acids tyrosine and cysteine. In addition, mutation-specific testing in both parents confirmed carrier status.

Biochemically, asparagine synthetase also known as aspartate-ammonia ligase, symbolic as ASNS, is an enzyme involved in the biosynthesis of asparagine from aspartate through an ATP-dependent transaminated reaction (Zhang et al. 1989). This conversion takes place in the presence of glutamine which acts as amino group donor in reaction (Fig. 3) (Zhang et al. 1989; Ruzzo et al. 2013). This suggested that asparagine would be low in body fluids if this enzyme is deficient and more specifically in the brain as *ASNS* is highly expressed in adult brain (Hongo et al. 1996; Ruzzo et al. 2013) Although this was not demonstrated by the Ruzzo et al. (2013) report because the CSF

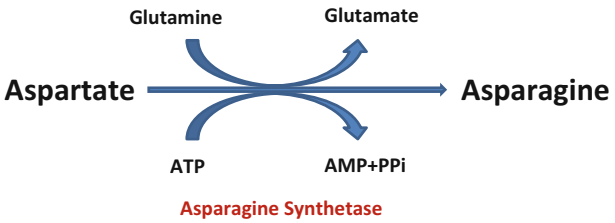


Fig. 3 Asparagine synthetase enzyme converts aspartate to asparagine in the presence of glutamine which is the amino group donor in this reaction

Table 2 Biochemical findings

Amino acid levels[a]	Patient 1	Patient 2	Ruzzo et al. (2013) and Ben-Salem et al. (2014)	%
Asparagine level (plasma) (33–68.4 μmol/L)	10 μmol/L (low)	6 μmol/L (low)	(11–57 μmol/l), 2/5 low, others: normal	57
Asparagine level (CSF) (1.1–6.9 μmol/L)	Not detected	1 μmol/L	NA	100
Glutamine level (plasma) (254–823 μmol/L)	339 μmol/L, normal	328 μmol/L, normal	(439–1.250 μmol/l), 2/4 high, others: normal	33
Glutamine level (CSF) (356–680 μmol/L)	922 μmol/L, high	574 μmol/L, normal	NA	50

NA not available
[a] Reference ranges have been validated locally by our own population age related

asparagine was not measured in their patients, our findings support this theory as CSF asparagine levels were low in both siblings. Plasma asparagine levels were low in the presented cases but were only low in 2/5 of previous patients (Table 2).

ASD is one of aminoacidopathies that will be an another example which illustrate the principle of mechanism of occurring the inborn errors of metabolism which are either due to accumulation of substrate or deficiency of product or transport defect (Scriver 2001). Therefore, it will be added to other synthesis defects like creatine deficiency syndromes (Stockler et al 1994), glutamine synthetase deficiency (Haberle et al 2005), and the serine synthetic defects (van der Crabben et al. 2013). In all aforementioned disorders, the treatment is the administration of the deficient product to the affected individuals and the response was variable. It will be exciting to see the response of the patients with ASD to asparagine replacement; however, given that the phenotype is present since birth, it makes difficult to predict that such treatment will be curative (Ruzzo et al. 2013).

In conclusion, we alert the clinicians to consider ASD deficiency in any child presented with congenital microcephaly, epileptic encephalopathy and severe developmental delay. For the first time in the literature, our report confirms low CSF asparagine level in patients with ASD deficiency.

Take-Home Message

Asparagine synthetase deficiency is characterized by severe congenital microcephaly followed by childhood global developmental delay, central hypotonia, spastic quadriplegia and intractable seizure disorder. CSF asparagine level is low in this disorder while plasma may be low. Brain MRI showed microcephaly, brain atrophy, and delayed myelination and simplified gyriform pattern.

Conflict of Interest

Majid Alfadhel, Muhammad Talal Alrifai, Daniel Trujillano, Hesham Alshaalan, Ali Al Othaim, Shatha Al Rasheed, Hussam Assiri, Abdulrhman A Alqahtani, Manal Alaamery, Arndt Rolfs, and Wafaa Eyaid declare that they have no conflict of interest.

Informed Consent

Informed consent was obtained from parents of the patients included in the study. Proof that informed consent was obtained is available upon request.

Authors' Contributions

MAF performed the majority of work associated with preparing, writing, and submitting the manuscript and contributed to the clinical diagnosis and management of the patients. MTR edited the manuscript and contributed to the clinical diagnosis and management of the patients. DT performed the molecular testing and contributed to the diagnosis of the patients and editing the manuscript. HA assessed and described the radiological findings obtained from the patients. AA edited the manuscript and contributed to biochemical investigations of the patients. SR edited the manuscript and contributed to clinical management of the patients. HAS summarized the clinical findings and contributed to the writing of the first draft. AQ summarized the clinical findings and contributed to the writing of the first draft. AR edited the manuscript, performed the molecular testing, and contributed to the diagnosis of the patients. WE edited the manuscript and contributed to the clinical diagnosis and management of the patients.

References

Ben-Salem S, Gleeson JG, Al-Shamsi AM et al (2014) Asparagine synthetase deficiency detected by whole exome sequencing causes congenital microcephaly, epileptic encephalopathy and psychomotor delay. Metab Brain Dis. doi:10.1007/s1011-014-9618-0

Bourgeron T, Chretien D, Poggi-Bach J et al (1994) Mutation of the fumarase gene in two siblings with progressive encephalopathy and fumarase deficiency. J Clin Invest 93:2514

Haberle J, Gorg B, Rutsch F et al (2005) Congenital glutamine deficiency with glutamine synthetase mutations. N Engl J Med 353:1926–1933

Hongo S, Chiyo T, Takeda M (1996) Cloning of cDNA for asparagine synthetase from rat Sertoli cell. Biochem Mol Biol Int 38:189–196

Ruzzo EK, Capo-Chichi J-M, Ben-Zeev B et al (2013) Deficiency of asparagine synthetase causes congenital microcephaly and a progressive form of encephalopathy. Neuron 80:429–441

Scriver CR (2001) The metabolic & molecular bases of inherited disease. McGraw-Hill, New York/London

Stockler S, Holzbach U, Hanefeld F et al (1994) Creatine deficiency in the brain: a new, treatable inborn error of metabolism. Pediatr Res 36:409–413

van der Crabben SN, Verhoeven-Duif NM, Brilstra EH et al (2013) An update on serine deficiency disorders. J Inherit Metab Dis 36:613–619

Zhang YP, Lambert MA, Cairney AE, Wills D, Ray PN, Andrulis IL (1989) Molecular structure of the human asparagine synthetase gene. Genomics 4:259–265

JIMD Reports
DOI 10.1007/8904_2015_406

RESEARCH REPORT

Occurrence of Malignant Tumours in the Acute Hepatic Porphyrias

Estefanía Lang · Martin Schäfer · Holger Schwender · Norbert J. Neumann · Jorge Frank

Received: 25 September 2014 / Revised: 05 January 2015 / Accepted: 07 January 2015 / Published online: 22 February 2015

Abstract The porphyrias are a group of inherited metabolic diseases resulting from enzymatic deficiencies of specific haem biosynthetic enzymes. They can be classified as primarily acute and non-acute types. Clinically, the acute hepatic porphyrias (AHPs) are characterised by acute neurovisceral attacks. Patients with AHP may be at increased risk for development of hepatocellular carcinoma (HCC). However, systematic studies on the occurrence of other malignancies in patients with the AHPs have not been performed to date. Here, we studied the development of HCC and distinct malignant tumours in patients with the AHPs registered in a single European porphyria specialist centre. A questionnaire was designed and sent to all individuals ($n = 122$) diagnosed between 1970 and 2012 of whom a valid address was available ($n = 82$), requesting information on their personal and family history of cancer. Statistical analysis was performed to calculate incidence, prevalence and relative risk of HCC. To calculate confidence intervals, a Poisson distribution was assumed. Forty-nine patients (59.8%) returned a completed questionnaire. Overall, HCC was diagnosed in one female (2.1%), and the remaining patients reported on six distinct malignancies. We were able to confirm that HCC is an important complication in AHP. The patients in our cohort had an approximately 35-fold increased risk of developing HCC, similar to observations in other European countries. In addition, we detected colon, breast, uterine and thyroid cancer as well as lymphoma and a liver metastasis in patients with AHP. However, considering the small number of tumours and patients studied here, the data should be interpreted with caution, and further studies on cancer occurrence in AHP patients will require a multicentre setting.

Abbreviations

ADDP	δ-Aminolaevulinic acid dehydratase-deficient porphyria
AHP	Acute hepatic porphyria
AHPs	Acute hepatic porphyrias
AIP	Acute intermittent porphyria
HCC	Hepatocellular carcinoma
HCP	Hereditary coproporphyria
VP	Variegate porphyria

Introduction

The porphyrias are a group of rare inherited metabolic diseases that result from a catalytic deficiency of one of the eight enzymes along the haem biosynthetic pathway. They can be classified as acute hepatic, hepatic cutaneous and erythroid cutaneous forms. The acute hepatic porphyrias (AHPs) are characterised by life-threatening acute neurovisceral attacks. The AHPs include autosomal dominant acute intermittent porphyria (AIP), variegate porphyria (VP), hereditary coproporphyria (HCP) and autosomal recessive δ-aminolaevulinic acid dehydratase-deficient porphyria (ADDP). Clinically, the AHPs are characterised by the sudden onset of unspecific neurovisceral symptoms,

Communicated by: John Christodoulou, MB BS PhD FRACP FRCPA

Competing interests: None declared

E. Lang · N.J. Neumann · J. Frank (✉)
Department of Dermatology and European Porphyria Specialist Centre Düsseldorf, Heinrich Heine University Düsseldorf, Moorenstr. 5, 40225, Düsseldorf, Germany
e-mail: jorge.frank@med.uni-duesseldorf.de

M. Schäfer · H. Schwender
Mathematical Institute, Heinrich Heine University Düsseldorf, 40225, Düsseldorf, Germany

including colicky abdominal pain, nausea and vomiting, constipation, tachycardia, hypertension, paraesthesia, muscle and back pain, para- and tetraplegia, encephalopathy, paralysis, anxiety and acute psychosis. VP and HCP are sometimes referred to as neurocutaneous porphyrias because they can also manifest with blistering photosensitivity on sun-exposed areas of the body.

A serious complication in the AHPs is the development of hepatocellular carcinoma (HCC). HCC is the most common primary malignant liver tumour and one of the most common malignant tumours worldwide (Mendy and Walton 2009). More than 500,000 new cases are diagnosed each year, with the highest incidence being observed in Asia and Africa (Mendy and Walton 2009; Nordenstedt et al. 2010). However, the incidence has also increased in the United States and Europe during the past decade (Llovet et al. 2003). Well-known risk factors for the development of HCC include alcohol ingestion, hepatitis B and C virus infection and liver cirrhosis (Hardell et al. 1984; Fattovich et al. 2004). Several mortality and case–control studies have demonstrated an association between this type of liver cancer and the AHPs in Europe (Lithner and Wetterberg 1984; Bengtsson and Hardell 1986; Kauppinen and Mustajoki 1988; Andersson et al. 1996; Linet et al. 1999; Andant et al. 2000; Schneider-Yin et al. 2009; Innala and Andersson 2011; Elder et al. 2013; Sardh et al. 2013). Concerning other malignant tumour entities, however, systematic studies on their occurrence in AHP patients have not yet been conducted. To date, only few single cases have been reported (Scarlett et al. 1995; Schaffer et al. 2001; Forget et al. 2001; Davies and Whitaker 2002; Kristiansen and Langkjer 2006; Hanneken et al. 2009; Cunningham et al. 2010; Mañas Gómez et al. 2011).

Here, we sought to assess the frequency of HCC and other malignancies in patients with AHP and their first-degree relatives in a single European porphyria specialist centre and, in particular, whether the frequency of HCC differs from that encountered in other European countries.

Material and Methods

Patients and Questionnaire

In order to gain insight into individual medical histories, we developed a questionnaire (Fig. 1) that we sent to all patients with AHP ($n = 122$) from our porphyria registry, comprising 97 patients with AIP, 20 with VP, 4 with HCP and one patient with ADDP. Most of these patients resided in North Rhine-Westphalia (NRW), which currently has 17.84 million inhabitants and is the most populated state of Germany. We requested personal and family histories of malignant tumours and symptoms of the disease as well as health behaviour. Additionally, patients with the neurocutaneous porphyrias (VP and HCP) received a second questionnaire regarding sun exposure, ingestion of phototoxic and photoprotective drugs and cutaneous symptoms (data not shown). All participants in this study provided informed consent, in accordance with guidelines set forth by the Ethics Committee of the Heinrich Heine University Düsseldorf and the Declaration of Helsinki principles.

Incidence and Prevalence of AHP and Relative Risk of HCC

The incidence of AHP in our cohort was calculated as the ratio of the number of symptomatic patients who were first diagnosed between January 2007 and December 2009 to the number of patient-years corresponding to the reference population in that period, according to Elder et al. (2013). The prevalence was calculated as the product of the incidence rate and the mean duration of disease. As mean duration of disease, 45 years was used for AIP and 40 years for VP, in accordance with Elder et al. (2013)

To calculate confidence intervals, a Poisson distribution was assumed for the observed cases (Ulm 1990). The relative risk for developing HCC was measured by the ratio of the observed number of HCC cases among porphyric patients to the number of expected HCC cases to be among them (Kauppinen and Mustajoki 1988; Andant et al. 2000). The number of expected cases was calculated by multiplying, within each 5-year age group, the corresponding mean incidence of HCC in NRW for the years 2010–2011, according to data derived from the cancer registry NRW database (http://www.krebsregister.nrw.de), by the number of patient-years in our AHP cohort. To standardise for gender, the incidence rates in the age groups were calculated as a weighted mean of the respective gender-specific incidence rates, where the weights correspond to the age group-specific gender distribution in the AHP cohort.

The population of NRW was obtained from the State Office for Data Processing and Statistics NRW website (http://www.it.nrw.de) and averaged across year values. The populations of the European countries were obtained from the Eurostat website (http://epp.eurostat.ec.europa.eu) and averaged across year values between January 2007 and January 2010 (reference date January 1st). HCC cases in 5-year age groups for HCC in NRW were obtained from the cancer registry NRW website that was first established in

Questionnaire (part 1)

1. When was the diagnosis of porphyria made?

2. What form of porphyria do you have (if known)?

3. Have you ever suffered from an acute porphyria attack? yes☐ no☐
If yes, when?

3a. Do you regularly suffer from acute porphyria attacks? yes☐ no☐

4. Are other family members affected by porphyria? yes☐ no☐
If yes, who?

5. Do you have children? yes☐ no☐

6. Do you have any concomitant diseases? yes☐ no☐

7. Do you regularly visit a physician? yes☐ no ☐

8. Do you take any medication? yes☐ no☐
If yes, what medication?

9. Do you take sedatives? yes☐ no☐
If yes, which one(s)?

10. If you are a woman: Do you take regular hormonal contraceptives?
yes☐ no☐
If yes, which one? Birth controll pill☐ IUD☐ Ring☐ other:

11. Do you attend to cancer screening? (colon, skin, prostata, cervix, breast)
yes☐ no☐

12. Do you have / did you have any tumours? yes☐ no☐
If yes, what kind of tumour? (if known)

13. Does anyone in your family suffer from a tumour? yes☐ no☐
If yes, who? What tumour (if known)?

14. Have you ever had moles or tumours of the skin removed? yes☐ no☐

15. Have you ever had a diagnosis of non-melanoma skin cancer precursor (actinic keratosis, Bowen's disease), or-non melanoma skin cancer (so-called basal cell carcinoma or squamous cell carcinoma)?
yes☐ no☐

16. Have you ever had a diagnosis of melanoma precursor (lentigo maligna), or malignant melanoma?
yes☐ no☐

17. Do you suffer from stomach, intestine, kidney, liver or skin disease or are there any other organ diseases? yes☐ no☐

18. Have you ever had a diagnosis of liver infection (hepatitis) or other liver diseases?
yes☐ no☐

19. Have you ever had surgery of the stomach, intestine or other organs?
If yes, why? yes☐ no☐

Fig. 1 Questionnaire sent to every participant in this study. We requested information on the individual medical history, including personal and family history of tumours and symptoms of the disease as well as health behaviour

2005. Raw incidence rates were calculated based on the HCC cases using the NRW population in 5-year age groups as available from the State Office for Data Processing and Statistics NRW website. Data were analysed using the software R, version 3.0.2 (R Core Team, 2013).

Results

Questionnaire

Only 82 of the 122 AHP patients registered in our centre could be reached by mail. 49 of these 82 patients (59.8%) returned a completed questionnaire, including 38 with AIP, 10 with VP and 1 patient with ADDP. None of the patients with HCP contributed to our study. Patients reported on a total of seven malignant tumours (Table 1).

Malignant Tumours

The group of malignant tumours contained one HCC, one colon carcinoma, one liver metastasis of a colon carcinoma, one breast carcinoma, one malignant uterus tumour, one thyroid carcinoma and one non-Hodgkin lymphoma (Table 1).

Incidence of AHP

The incidence rate of AHP in NRW in the years 2007–2009 was 0.26 per million per year (95% CI: 0.14–0.44). Considering the two most frequent types of AHP separately, the incidence rate of AIP was 0.20 per million per year (95% CI: 0.10–0.37), and the incidence rate of VP was 0.06 per million per year (95% CI: 0.01–0.16).

Prevalence of AHP

The prevalence of AIP for NRW in the years 2007–2009 was 9.19 per million (95% CI: 4.59–16.44) and the prevalence of VP was 2.23 per million (95% CI: 0.46–6.51).

Relative Risk of Developing HCC

AHP patients in NRW had an approximately 35-fold increased risk of developing HCC (relative risk: 34.64, 95% CI: 0.88–193.01).

Table 1 Malignant tumours in patients with acute hepatic porphyria in this study

	Liver	Colon	Breast	Uterus	Thyroid	Blood
Malignant tumours	2	1	1	1	1	1

Discussion

Previously, systematic studies on the spectrum of malignant tumours in patients with AHP have not yet been conducted in any porphyria specialist centre. In this respect, it is important to state that our data showed an incidence of AHP in our cohort that is similar to that encountered in other European countries (Elder et al. 2013). In contrast to most epidemiological studies on the incidence of tumours, which are usually carried out on large cohorts from the general population, this work focuses on a small group of rare hereditary metabolic diseases, which affect only a few individuals. However, the increased incidence of certain tumour entities within a group of rare diseases could provide clues as to whether any of these tumours might have an immediate association with a specific acute porphyria variant. Thus, the work presented here aims at contributing to a better understanding of the occurrence of cancer in patients with AHP.

One of the most severe complications in the AHPs is the development of HCC, a tumour with poor prognosis. Since the first report on the concomitant occurrence of HCC and AIP in 1984, several studies have shown an association between this type of primary liver cancer and the AHPs in Europe (Lithner and Wetterberg 1984; Hardell et al. 1984; Bengtsson and Hardell 1986; Tidman et al. 1989; Grabczynska et al. 1996; Andersson et al. 1996; Linet et al. 1999; Andant et al. 2000; Schneider-Yin et al. 2009; Innala and Andersson 2011; Elder et al. 2013; Sardh et al. 2013). However, some of the aforementioned studies linking HCC to AHP certainly include cases in which there were further risk factors. Whenever case numbers are small, even one or two of these factors can introduce bias. Although we tried to address such a putative bias by sending a questionnaire to our patients, we are aware that this questionnaire may not be exhaustive regarding individual risk profiles since we did not ask for, e.g. alcohol ingestion and cigarette smoking. Further, questionnaire-based assessments may not be entirely reliable.

In our study, we detected a female with AIP who developed a HCC. Based on these data, patients with AHPs in NRW have a 35-fold increased risk to develop a HCC (relative risk: 34.64, 95% CI: 0.88–193.01). This result is consistent with the data of other European porphyria centres, in particular France (Andant et al. 2000). In these studies, AHP patients in France had a 36-fold relative risk and those from Finland a 61-fold relative risk to develop HCC (Kauppinen and Mustajoki 1988; Andant et al. 2000). The considerable amount of uncertainty in our estimate is due to the small number of AHP patients developing a HCC in our cohort.

Table 2 Chronologic overview on the major studies regarding the occurrence of hepatocellular carcinoma in the acute hepatic porphyrias

Study	Sample size	Number of hepatocellular carcinomas
Lithner and Wetterberg (1984)	Unknown	11
Hardell et al. (1984)	78	3
Bengtsson and Hardell (1986)	83	5
Kauppinen and Mustajoki (1988)	245	7
Andersson et al. (1996)	33	9
Andant et al. (2000)	650	7
Schneider-Yin et al. (2009)	145	4
Innala and Andersson (2011)	62	22
Elder et al. (2013)	204	14
Sardh et al. (2013)	179	19
Lang et al. (this study)	49	1

Studies on the association between AHPs and HCC were also conducted in Switzerland, Sweden and Great Britain. However, the results did not include relative risks for the development of this tumour. In Switzerland, two AIP patients and two VP patients with HCC were observed over a period of 15 years (Schneider-Yin et al. 2009). Sweden is the only European country from which data derived from different geographic regions and porphyria centres are available. In 1986, Bengtsson et al. reported on five patients with AIP who developed HCC (Bengtsson and Hardell 1986). In a retrospective study from 1996, Andersson and colleagues identified nine patients with AIP from Northern Sweden who had HCC (Andersson et al. 1996). Later, the same group reported on 22 patients with AIP and HCC (Innala and Andersson 2011). Recently, Sardh et al. detected 23 primary liver tumours in patients with AHP (Sardh et al. 2013). An overview of the major studies on the concomitant occurrence of HCC and AHP is given in Table 2. In 1989 and 1996, two patients with VP and HCC were reported from Great Britain (Tidman et al. 1989; Grabczynska et al. 1996).

To date, the pathological mechanisms underlying the development of HCC in patients with AHP are largely unknown (Andant et al. 2000). Although in the general population cirrhosis is the most important aetiological factor, it seems to be only of minor importance in patients with AHP (Fattovich et al. 2004; Mullhaupt et al. 2008; Deybach and Puy 2011; Zhou et al. 2014). In support of this notion, we did not find any patient with liver cirrhosis in our study. Furthermore, only one patient with AIP and concomitant hepatitis C virus infection was detected.

Regarding the occurrence of non-hepatic tumours in patients with AHP, there are reports on breast cancer, thyroid cancer and non-Hodgkin lymphoma (Scarlett et al. 1995; Schaffer et al. 2001; Forget et al. 2001; Davies and Whitaker 2002; Kristiansen and Langkjer 2006; Mañas Gómez et al. 2011). However, these were only few and mostly incidental cases and systematic studies from a porphyria specialist centre have not been performed yet. Interestingly, malignant tumours of the gastrointestinal tract have so far not been reported in association with AHP.

In our cohort, one patient with VP developed colorectal cancer with hepatic metastasis (Hanneken et al. 2009). Of note, three other patients with VP had a positive family history with regard to colon cancer. Even more interestingly, two patients suffered from Crohn's disease, a chronic inflammatory bowel disorder that is a known risk factor for the development of malignant intestinal tumours such as colon cancer (Cunningham et al. 2010). Considering these data, the question arises, if VP could confer some degree of susceptibility for the development of gastrointestinal disease and colorectal cancer. However, there are certain statistical limitations of observational studies suggesting a putative association or a causative link between two conditions of which one is very rare. Thus, we are well aware that the number of patients studied here is too small to draw such a conclusion. Furthermore, we cannot compare our data with that from other porphyria centres at this time.

With regard to other types of cancer, one individual with AIP was diagnosed with breast cancer, and three other patients with AIP had a positive family history regarding this malignancy. Additionally, one AIP patient in our cohort had cervical cancer, one patient with AIP had thyroid cancer, and another individual with VP suffered from non-Hodgkin lymphoma.

Certainly, our data should be interpreted with caution because the sample studied was relatively small and contained only one patient with HCC. Therefore, the likelihood of error cannot be completely ruled out, in particular since the sample may not have captured all the characteristics and variables of the target population, making it difficult to distinguish real differences from random variation. Notwithstanding, our data indicate that the patients with AHP in our cohort have an approximately 35-fold increased risk of developing HCC. This is in support of previous reports from other European countries, which may suggest that the AHP reflect an important aetiological risk factor for this tumour entity. For the first time, we systematically studied the occurrence of malignant tumours other than HCC within a cohort of AHP patients. In order to obtain results based on a larger number of cases than in our study focusing on the NRW region of Germany, our data on the frequency of non-hepatic malignancies in the AHPs

require confirmation from other porphyria specialist centres, preferably in a multicentre setting. Without such information, our findings regarding the occurrence of malignancies other than hepatic cancer are difficult to interpret.

Synopsis

We performed a systematic analysis of the occurrence of malignant tumours in patients with acute hepatic porphyrias, which indicates that German patients with this type of porphyria have an approximately 35-fold increased risk of developing hepatocellular carcinoma.

Author Contribution

E.L. and J.F. were involved in conception and design of the study, analysis and interpretation of data and drafting the article. M.S. and H.S. were involved in analysis and interpretation of data and critical revision of the manuscript. N.J.N. was involved in conception and design of the study and critical revision of the manuscript.

Compliance with Ethics Guidelines

Conflict of Interest

Estefanía Lang, Martin Schäfer, Holger Schwender, Norbert J. Neumann, and Jorge Frank declare that they have no conflict of interest.

References

Andant C, Puy H, Bogard C et al (2000) Hepatocellular carcinoma in patients with acute hepatic porphyria: frequency of occurrence and related factors. J Hepatol 32:933–939

Andersson C, Bjersing L, Lithner F (1996) The epidemiology of hepatocellular carcinoma in patients with acute intermittent porphyria. J Intern Med 240:195–201

Bengtsson NO, Hardell L (1986) Porphyrias, porphyrins and hepatocellular cancer. Br J Cancer 54:115–117

Cunningham D, Atkin W, Lenz H-J et al (2010) Colorectal cancer. Lancet 375:1030–1047. doi:10.1016/S0140-6736(10)60353-4

Davies JH, Whitaker SJ (2002) Chlorambucil and acute intermittent porphyria. Clin Oncol (R Coll Radiol) 14:491–493

Deybach J-C, Puy H (2011) Hepatocellular carcinoma without cirrhosis: think acute hepatic porphyrias and vice versa. J Intern Med 269:521–524. doi:10.1111/j.1365-2796.2011.02358.x

Elder G, Harper P, Badminton M et al (2013) The incidence of inherited porphyrias in Europe. J Inherit Metab Dis 36:849–857. doi:10.1007/s10545-012-9544-4

Fattovich G, Stroffolini T, Zagni I, Donato F (2004) Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 127:S35–S50

Forget F, Awada A, Klastersky J (2001) Anticancer chemotherapy in a patient with prior history of acute intermittent porphyria. A case report and review of the literature. Support Care Cancer 9:465–466

Grabczynska SA, McGregor JM, Hawk JL (1996) Late onset variegate porphyria. Clin Exp Dermatol 21:353–356

Hanneken S, Kuerten V, Hoernke M, Neumann NJ (2009) Metastatic colon cancer triggering an acute attack of variegate porphyria. Int J Colorectal Dis 24:127–128. doi:10.1007/s00384-008-0537-6

Hardell L, Bengtsson NO, Jonsson U et al (1984) Aetiological aspects on primary liver cancer with special regard to alcohol, organic solvents and acute intermittent porphyria–an epidemiological investigation. Br J Cancer 50:389–397

Innala E, Andersson C (2011) Screening for hepatocellular carcinoma in acute intermittent porphyria: a 15-year follow-up in northern Sweden. J Intern Med 269:538–545. doi:10.1111/j.1365-2796.2010.02335.x

Kauppinen R, Mustajoki P (1988) Acute hepatic porphyria and hepatocellular carcinoma. Br J Cancer 57:117–120

Kristiansen C, Langkjer ST (2006) Treatment and treatment considerations in a patient with advanced breast cancer and acute intermittent porphyria. Acta Oncol 45:337–339. doi:10.1080/02841860500434697

Linet MS, Gridley G, Nyrén O et al (1999) Primary liver cancer, other malignancies, and mortality risks following porphyria: a cohort study in Denmark and Sweden. Am J Epidemiol 149:1010–1015

Lithner F, Wetterberg L (1984) Hepatocellular carcinoma in patients with acute intermittent porphyria. Acta Med Scand 215:271–274

Llovet JM, Burroughs A, Bruix J (2003) Hepatocellular carcinoma. Lancet 362:1907–1917. doi:10.1016/S0140-6736(03)14964-1

Mañas Gómez MJ, GonzÄlez O, Vilallonga R, Armengol Carrasco M (2011) Severe hyponatremia in postoperatory of papillary thyroid carcinoma as a manifestation of acute intermittent porphyria. Med Clin (Barc) 136:556–557. doi:10.1016/j.medcli.2010.09.037

Mendy M, Walton R (2009) Molecular pathogenesis and early detection of hepatocellular carcinoma–perspectives from West Africa. Cancer Lett 286:44–51. doi:10.1016/j.canlet.2009.04.039

Mullhaupt B, Junker C, Wuest E, Renner EL (2008) Mortality from primary liver cancer in Switzerland from 1975 to 1994. Swiss Med Week 138:313–316

Nordenstedt H, White DL, El-Serag HB (2010) The changing pattern of epidemiology in hepatocellular carcinoma. Dig Liver Dis 42 (Suppl 3):S206–S214. doi:10.1016/S1590-8658(10)60507-5

Sardh E, Wahlin S, Björnstedt M et al (2013) High risk of primary liver cancer in a cohort of 179 patients with Acute Hepatic Porphyria. J Inherit Metab Dis 36:1063–1071. doi:10.1007/s10545-012-9576-9

Scarlett JD, Corry J, Jeal PN (1995) Cytotoxic chemotherapy and radiotherapy in a patient with breast cancer and variegate porphyria (VP). Aust N Z J Med 25:742–743

Schaffer M, Schaffer PM, Panzer M et al (2001) Porphyrias associated with malignant tumors: results of treatment with ionizing irradiation. Onkologie 24:170–172

Schneider-Yin X, Harms J, Minder EI (2009) Porphyria in Switzerland, 15 years experience. Swiss Med Week 139:198–206

Tidman MJ, Higgins EM, Elder GH, MacDonald DM (1989) Variegate porphyria associated with hepatocellular carcinoma. Br J Dermatol 121:503–505

Ulm K (1990) A simple method to calculate the confidence interval of a standardized mortality ratio (SMR). Am J Epidemiol 131:373–375

Zhou Y-M, Zhang X-F, Li B et al (2014) Prognosis after resection of hepatitis B virus-related hepatocellular carcinoma originating from non-cirrhotic liver. Ann Surg Oncol. doi:10.1245/s10434-014-3505-0

JIMD Reports
DOI 10.1007/8904_2015_407

CASE REPORT

Improvement in Bone Mineral Density and Architecture in a Patient with Gaucher Disease Using Teriparatide

Aneal Khan • David A. Hanley • Colleen McNeil • Steven Boyd

Received: 10 November 2014 / Revised: 04 January 2015 / Accepted: 07 January 2015 / Published online: 03 March 2015

Abstract Gaucher disease is an autosomal recessive lysosomal storage disorder caused by deficiency of the enzyme acid beta-glucosidase (glucocerebrosidase) due to mutations in the GBA gene. The most common form (type I) is associated with severe hematologic, visceral and bone disease. Disease-modifying treatments, such as enzyme replacement therapy and substrate reduction therapy, can improve the hematologic and visceral aspects of the disease but success with improving severe osteopenia, which can increase the risk of fractures, is limited. Our case involves a patient with complex disease affecting bone health including Gaucher disease (type I), Sjögren syndrome, rheumatoid arthritis and corticosteroid use who did not respond to long term use of bisphosphonates. We report an improvement in bone mineral density and bone architecture commensurate with a reduced incidence of fractures in whom we used teriparatide (human parathyroid hormone (PTH; 1-34) to treat severe osteopenia. We conclude that teriparatide should be considered for further studies as an agent to improve bone mineral density in patients with Gaucher disease.

Communicated by: Maurizio Scarpa, M.D, Ph.D

Competing interests: None declared

A. Khan (✉)
Assistant Professor of Pediatrics and Medical Genetics, The University of Calgary, Alberta Children's Hospital, 2888 Shaganappi Trail NW, Calgary, AB, Canada T3B 6A8
e-mail: khaa@ucalgary.ca

D.A. Hanley
Departments of Medicine, Community Health Sciences and Oncology, Richmond Road Diagnostic and Treatment Centre, University of Calgary, 1820 Richmond Road SW, Calgary, AB, Canada T2T 5 C7

C. McNeil
Alberta Health Services, Alberta Children's Hospital, 2888 Shaganappi Trail NW, Calgary, AB, Canada T3B 6A8

S. Boyd
Schulich School of Engineering, University of Calgary, 2500 University Drive, NW, Calgary, AB, Canada T2N 1N4

Introduction

Gaucher disease (OMIM 230800) is an autosomal recessive lysosomal disorder caused by deficiency of the enzyme acid beta-glucosidase (glucocerebrosidase) due to mutations in the *GBA* gene. Accumulation of glucosylceramide in mononuclear phagocytes leads to the characteristic appearance of "Gaucher" cells that can be identified in a number of tissues but most commonly in the bone marrow and liver (Thomas et al. 2014). The most common form of Gaucher disease results in hematologic, visceral, and bone abnormalities with sparing of the central nervous system (type I). Patients can present at any age with anemia, thrombocytopenia, hepatosplenomegaly, and bone disease. The bone disease can have protean manifestations (Wenstrup et al. 2002), but there are three general patterns of bone involvement that lead to adverse outcomes: (1) osteopenia, (2) infiltrative bone disease, and (3) ischemic bone disease. Osteopenia is the most common feature and along with bone pain together is more prevalent in patients that have had a splenectomy (Charrow et al. 2000). Osteopenia in patients with a lumbar spine dual X-ray absorptiometry (DXA) *z*-score less than or equal to – 1.0 can increase the risk of fracture at any skeletal site (Khan et al. 2012). Infiltrative disease, from Gaucher cells, can damage bone architecture, most commonly affects the spine and one of

the reasons why disease control should be achieved as soon as possible (Wenstrup et al. 2007; Charrow et al. 2007). Ischemic disease, for which avascular necrosis is the most debilitating disease, is poorly understood but has been related to anemia (Khan et al. 2012).

The only approved disease-modifying treatments for Gaucher disease in Canada are imiglucerase, velaglucerase, taliglucerase (enzyme replacement therapy), and miglustat (substrate reduction therapy). Although these treatments are primarily designed at reducing the burden of accumulated glucosylceramide and generally improve both the hematologic and visceral aspects of Gaucher disease, they are not specifically targeted to treating the bone disease which has been a greater challenge. Fracture prevention strategies have focused on standard osteoporosis therapy recommendations, including attention to calcium and vitamin D status, lifestyle recommendations, and the use of bisphosphonates such as alendronate – the most commonly chosen agent (Cox et al. 2008). Bisphosphonates have been the front-line therapy for postmenopausal osteoporosis for many years, because of strong fracture prevention data in clinical trials, and mechanism of action is thought to be related to suppression of bone resorption and a slowing of bone turnover. The only bone-anabolic agent currently available for clinical use is teriparatide (human parathyroid hormone (PTH 1–34)). Largely because of cost, teriparatide tends to be used in cases where standard osteoporosis therapy appears to be ineffective, such as recurring fractures and loss of bone density (Hanley et al. 2008). This is the first reported case of using teriparatide in a patient with Gaucher disease, and we show a dramatic improvement in both bone mineral density and architecture compared to previous standard treatments. We suggest that teriparatide may be a useful medication to consider for further studies to treat low bone mineral density in patients with Gaucher disease.

Case Report

The patient is a 65-year-old woman with Gaucher disease type I at 30 years of age and a concurrent history of osteopenia and multiple fractures. She also had a diagnosis of chronic obstructive pulmonary disease which was assessed to be the cause of her mild pulmonary hypertension. She had not had a splenectomy. While being monitored and treated for her Gaucher disease and osteopenia, she was also diagnosed 3 years later with rheumatoid arthritis and later Sjögren syndrome. The features of Sjögren syndrome were mild and involved mainly dryness of the mouth and eyes. Her Gaucher disease genotype showed compound heterozygosity with two mutations: (1) N370S=c.1226A>G (p.Asn409Ser) and (2) G377S=c.1246G>A (p.Gly416Ser). Her peripheral T-lymphocyte activity of beta-glucosidase was lymphocyte beta-glucosidase activity 1.7 nmol/h/mg protein. She was on standard clinical management of her Gaucher disease. Her average imiglucerase dose during the interval of teriparatide use 54 U/kg, and she was on a prescribed dose of 60 U/kg 3 years prior to the first observation point. From 8 years before the first observation point, she was on 30 U/kg of imiglucerase. Treatments for her rheumatoid arthritis have included Salazopyrin, methotrexate, etanercept, and Arthrotec. For her osteoporosis, she had 13 years of bisphosphonate therapy with (cyclical etidronate and then alendronate at standard doses). She had very low bone density by DXA and a high rate of fracture with no clinical or densitometry improvement noted during bisphosphonate therapy. She was therefore started on treatment with teriparatide at a standard dose (subcutaneous injections of 20 micrograms daily) from January 2009 to January 2011.

Baseline laboratory parameters and clinical data were monitored, as per her usual clinical care, using intake parameters as per the Gaucher Registry (https://www.registrynxt.com/Pages/Home.aspx). The Gaucher Registry is the largest international registry that records voluntary data on patients with Gaucher disease. Serum 25-hydroxy vitamin D levels were normal throughout this period (>80 nmol/L).

Areal bone densitometry (aBMD) of lumbar spine and hip was measured by dual X-ray absorptiometry (DXA) using a Hologic QDR 4500W® (Hologic Inc., Bedford, MA, USA). High-resolution peripheral computed tomography (HR-pQCT) of the peripheral limbs was performed using XtremeCT® (Scanco Medical, Brüttisellen, Switzerland) (Cheung et al. 2013). Baseline measurements were taken 4 months before the start of teriparatide. We used the following measurements from HR-pQCT reported in equivalent mg/cm^3 of hydroxyapatite for the entire scanned region: total density in a scanned region (BMD, mg/cm^3), cortical density (Ct.BMD, mg/cm^3), and trabecular density (Tb.BMD, mg/cm^3). Additionally, morphometric parameters included the trabecular thickness, trabecular number (TbTh., mm, TbN, mm^{-1}), and cortical thickness (CtTh, mm). The trabecular number is determined based on a direct analysis of the 3D data, and then trabecular thickness and separation are derived (MacNeil and Boyd 2007; Weinreb et al. 2007).

Results

The hematologic and visceral manifestations of Gaucher disease were considered well controlled in this patient for many years prior to the use of teriparatide. During this time, she maintained a normal and stable peripheral white blood cell and platelet count and hemoglobin level and platelets. Gaucher disease biomarkers ranged from: chitotriosidase

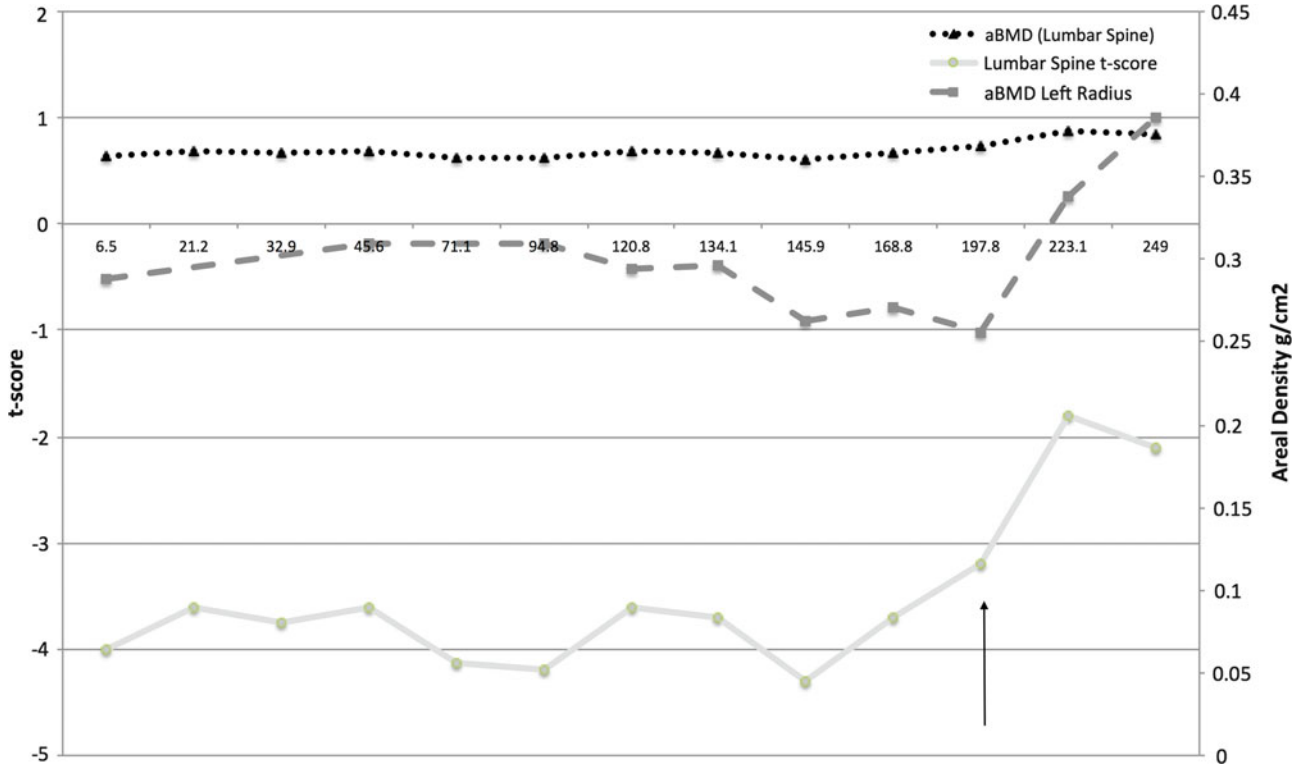


Fig. 1 Areal bone mineral density (aBMD) of the lumbar spine (*dotted*), left radius (*dashed*) in g/cm^2 along the right axis, and lumbar spine *t*-score (*solid*) along the left axis plotted against the number of months since the start of enzyme replacement therapy for Gaucher disease along the *x*-axis. *Arrow* marks the start of teriparatide

138–557 nmol/h/mL (reference 4–120 nmol/h/mL), angiotensin-converting enzyme (ACE) 30–62 IU/L (reference 25–106), and tartrate-resistant acid phosphatase (TRAP) 4.3–12.6 IU/L (reference 3–10 IU/L). Spleen and liver volumes were normal and stable.

Prior to teriparatide treatment, DXA measurements showed stable lumbar spine aBMD. Pre- and post-teriparatide treatment measured over 25 months showed a 19.7% increase in lumbar mineral density (0.732–0.876 g/m^2), and the *t*-score increased from −3.2 to −1.8 (measured over 25 months). Right radial aBMD increased by 32% (0.256–0.338 g/m^2) (Fig. 1). A follow-up scan 26 months later showed the lumbar mineral density was sustained at 0.848 g/m^2, the radial density continued to increase to 0.386 g/m^2, and the *t*-score showed a slight decrease to −2.1 standard deviations.

HR-pQCT data showed the left forearm BMD increased by 33% (127.0–137.7 mg HA/cm^3) which closely matches the result from DXA in the same region. The compact (cortical) bone mineral density (Ct.BMD) did not change (788.7–793.0 mg HA/cm^3; +0.5%), while trabecular density increased (Tb.BMD) by 170.4% (11.5–31.1 mg HA/cm^3), cortical thickness (CtTh) by 23.9% (0.46–0.57 mm), trabecular number (TbN) by 28.9% (0.45–0.58 /mm), and trabecular thickness (TbTh.) by 114.3% (0.021–0.045 mm) (Fig. 2). These numbers were sustained on an HR-pQCT scan 3 years later with a radial BMD of 200 mg HA/cm^3, Ct.BMD 869.6 mg HA/cm^3, Tb.BMD 35.0 mg HA/cm^3, CtTh 0.72 mm, TbN 0.66 mm^{-1}, and TbTh. 0.044 mm. The increases in left limb BMD appear to be related to initial increases in trabecular mineral density (Tb.BMD) and remodeling showing increased trabecular number and trabecular thickness. The initial increases in compact bone density were minor, but a larger increase was seen at the 3-year follow-up. Cortical thickness (CtTh) improved steadily throughout this period.

Between 21 January 1992 and 19 January 2009, she had 32 bone events (4.6 bone events/year) of which 9 were fractures. Since the teriparatide treatment, she has had five bone events over 5 years (1 bone event/year) of which one was a fracture. A bone event was any one of fracture, avascular necrosis, marrow infiltration, infarction, lytic lesion, or Erlenmeyer flask deformity. Over the past 3 years, she has not reported any fracture despite restarting her corticosteroids to treat her arthritis.

Discussion

Patients with Gaucher disease can have protean manifestations of bone disease that have a significant impact on

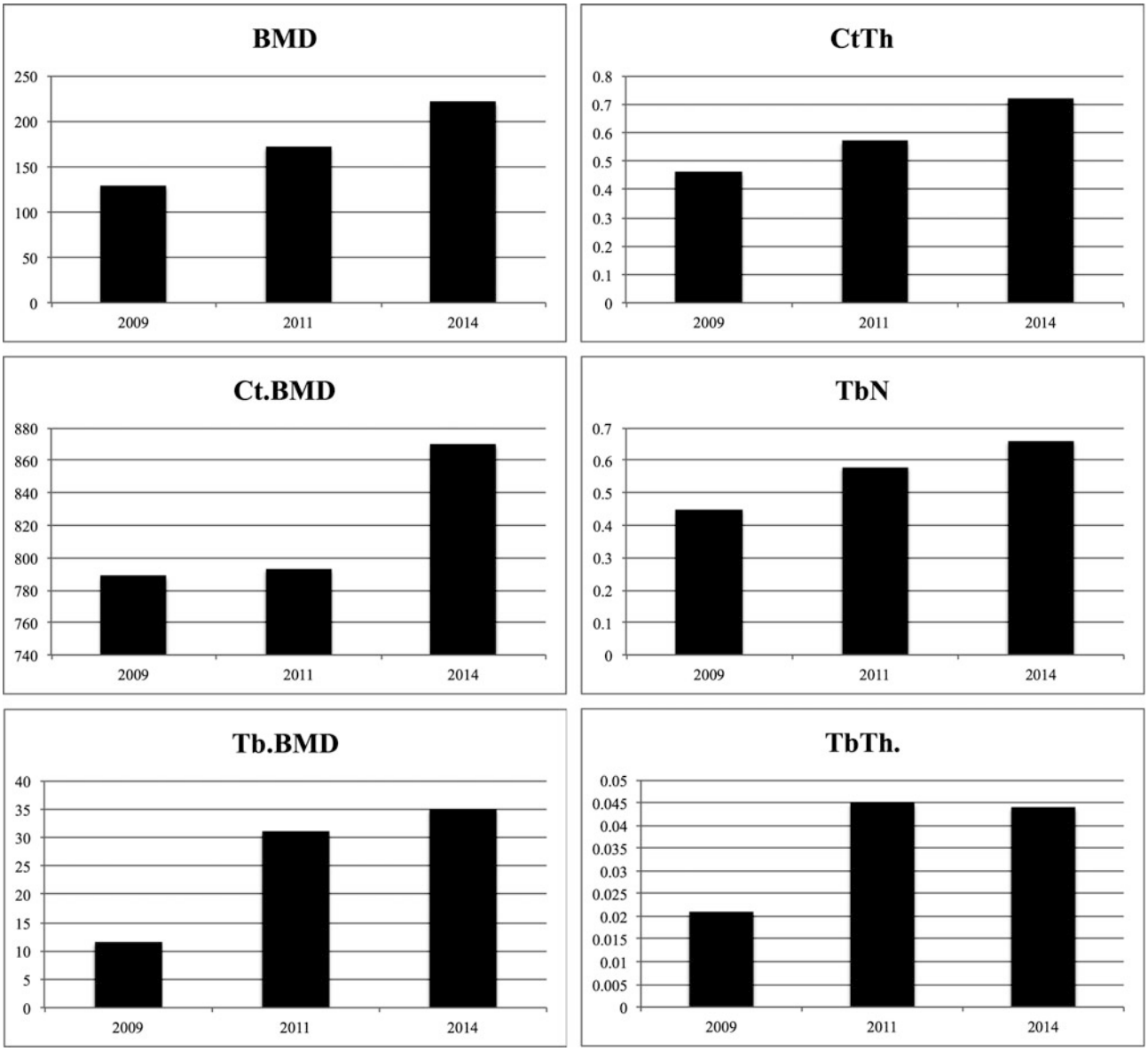


Fig. 2 High-resolution peripheral quantitative computed tomography (HR-pQCT) of the left radius before (2009) and after treatment with teriparatide (2011) and at follow-up (2014). Values on the *y*-axis correspond to the following units: BMD in mg/cm^3, Ct.BMD in mg/cm^3, Tb.BMD in mg/cm^3, TbTh. in mm, TbN in mm^{-1}, CtTh in mm

morbidity and quality of life (Weinreb et al. 2007). In the general population, fractures can increase morbidity and mortality although mortality from fractures has not been specifically studied in Gaucher disease. Compared to the hematologic parameters and visceral disease that have had traditionally been shown to have a fairly rapid response to enzyme replacement therapy, the responses in the bone have taken a longer time and have been more difficult to study. In this patient, there were a number of underlying factors, including rheumatoid arthritis and oral corticosteroid use that we expect would have impacted bone mineral density. However, very little improvement in bone density was observed after years of using standard doses of enzyme replacement therapy and bisphosphonates and an adequate intake of vitamin D and calcium.

Low bone mineral density of the lumbar spine has been shown to be a risk factor for fractures in patients with Gaucher disease (Khan et al. 2012). Wenstrup and colleagues showed that treatment with imiglucerase is associated with an increase in BMD *T*-scores in a dose-dependent manner, but the changes could take years to materialize (Wenstrup et al. 2007). In a recently published trial from our centre, Macdonald and colleagues showed teriparatide-improved bone mineral density and architecture using HR-pQCT in postmenopausal women with osteoporosis (Macdonald et al. 2011). We have also shown in

another lysosomal storage disease, Pompe disease, that bone architecture measured using HR-pQCT can provide valuable insight into the remodeling that takes place from different treatments (Khan et al. 2013).

There currently is no standard treatment for osteopenia in Gaucher disease. We feel this case is significant for a number of reasons. This is the first reported case of the use of teriparatide in a patient with Gaucher disease and severe bone disease, showing an improvement in bone mineral density and architecture. The patient's physicians chose to treat her with teriparatide because she was continuing to fracture despite standard osteoporosis management including bisphosphonate therapy. The treatment was well tolerated, and no adverse effects were noted.

The common target of treatment of low bone mass and fractures in Gaucher disease focuses on methods to increase bone mineral density, mass, and strength. DXA is commonly used as an assessment tool but provides an areal density – a two-dimensional surrogate – and does not measure true bone mineral density (a volumetric measurement). HR-pQCT can estimate a true three-dimensional density in both the cortical and trabecular compartments of the region of interest. In this patient, cortical density was normal at baseline, whereas all other architectural measurements were at least two standard deviations below the mean. This indicates that in our patient, cortical mineral density appears to have been defended the greatest prior to teriparatide treatment. This would appear to be a reasonable response since most of the strength in bone is in cortical bone. The most substantial improvements were in the trabecular compartment leading to increased overall density (D100) and trabecular remodeling, and at 3-year follow-up, the cortical thickness was also higher. These observations are entirely consistent with the known action of teriparatide therapy in osteoporosis, with marked increases in trabecular bone mass, especially in the spine, and increased cortical remodeling (Hanley et al. 2008). Increased total bone mineral content during teriparatide therapy is reflected by increased aBMD measurements by DXA as well. We therefore feel our case illustrates that teriparatide therapy strengthens trabecular bone, making more bone rather than simply increasing mineral density of cortical bone, which is likely to be a useful target in the treatment of Gaucher bone disease.

In this patient, there are a number of factors contributing to low bone density. Although the osteopenia and Gaucher disease were diagnosed prior to the diagnosis of rheumatoid arthritis and the use of corticosteroids, at the time of teriparatide use, we cannot delineate the contribution of each of these factors to the osteopenia. However, we note that the improvements after teriparatide use occurred even with the multitude to factors contributing to long-standing osteoporosis that did not respond to years of bisphosphonate therapy.

In conclusion, our case report illustrates improvement in bone mineral density and architecture with no adverse effects from using teriparatide in a patient with Gaucher disease and complex bone disease.

Synopsys

Teriparatide can increased bone mineral density in a patient with Gaucher disease.

Compliance with Ethics Guidelines

Conflict of Interest

Aneal Khan has received speaker honorarium, travel grants, and consultation fees from Genzyme Corporation® (a Sanofi® Company) and is a member of the International Collaborative Gaucher Group (ICGG).

David Hanley and Steven Boyd have declared no conflict of interest.

Colleen McNeil has received travel grants from Genzyme Corporation® (a Sanofi® Company).

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (Wenstrup et al. 2007). Teriparatide is labeled for the use for severe osteoporosis and approved for such use by Health Canada (Drug Identification Number 02254689). Therefore, this case reports an observation on a clinically approved use of the drug, is not a research trial, and does not require application to research ethics for research consent. Dual X-ray absorptiometry is used in standard clinical practice to monitor patients with osteoporosis and does not require research consent. High-resolution peripheral computed tomography is a research application, and we have obtained informed consent for its application in the subject of this case report.

Author Contributions

Aneal Khan helped design the observation plan from which the data was generated, analyzed the data, constructed, and edited the manuscript.

David Hanley helped design the observation plan, analyzed the data, and edited the manuscript.

Colleen McNeil helped analyze the data and review the manuscript.

Steven Boyd helped design the observation plan, analyzed the data, and edited the manuscript.

References

Charrow J, Andersson HC, Kaplan P, Kolodny EH, Mistry P, Pastores G et al (2000) The Gaucher registry: demographics and disease characteristics of 1698 patients with Gaucher disease. Arch Intern Med 160(18):2835–2843

Charrow J, Dulisse B, Grabowski GA, Weinreb NJ (2007) The effect of enzyme replacement therapy on bone crisis and bone pain in patients with type 1 Gaucher disease. Clin Genet 71(3):205–211

Cheung AM, Adachi JD, Hanley DA, Kendler DL, Davison KS, Josse R et al (2013) High-resolution peripheral quantitative computed tomography for the assessment of bone strength and structure: a review by the Canadian Bone Strength Working Group. Curr Osteoporos Rep 11(2):136–146

Cox TM, Aerts JM, Belmatoug N, Cappellini MD, vom Dahl S, Goldblatt J et al (2008) Management of non-neuronopathic Gaucher disease with special reference to pregnancy, splenectomy, bisphosphonate therapy, use of biomarkers and bone disease monitoring. J Inherit Metab Dis 31(3):319–336

Hanley D, Watson P, Hodsman A, Dempster DM (2008) Pharmacologic mechanisms of therapeutics: parathyroid hormone. In: Bilezikian JP, Raisz LG, Martin TJ (eds) Principles of bone biology, 3rd edn. Academic/Elsevier, San Diego, pp 1661–1695

Khan A, Hangartner T, Weinreb NJ, Taylor JS, Mistry PK (2012) Risk factors for fractures and avascular osteonecrosis in type 1 Gaucher disease: a study from the International Collaborative Gaucher Group (ICGG) Gaucher Registry. J Bone Miner Res 27(8):1839–1848

Khan A, Weinstein Z, Hanley DA, Casey R, McNeil C, Ramage B et al (2013) In vivo bone architecture in Pompe disease using high-resolution peripheral computed tomography. JIMD Rep 7:81–88

Macdonald HM, Nishiyama KK, Hanley DA, Boyd SK (2011) Changes in trabecular and cortical bone microarchitecture at peripheral sites associated with 18 months of teriparatide therapy in postmenopausal women with osteoporosis. Osteoporos Int 22(1):357–362

MacNeil JA, Boyd SK (2007) Accuracy of high-resolution peripheral quantitative computed tomography for measurement of bone quality. Med Eng Phys 29(10):1096–1105

Thomas AS, Mehta A, Hughes DA (2014) Gaucher disease: haematological presentations and complications. Br J Haematol 165(4):427–440

Weinreb N, Barranger J, Packman S, Prakash-Cheng A, Rosenbloom B, Sims K et al (2007) Imiglucerase (Cerezyme) improves quality of life in patients with skeletal manifestations of Gaucher disease. Clin Genet 71(6):576–588

Wenstrup RJ, Roca-Espiau M, Weinreb NJ, Bembi B (2002) Skeletal aspects of Gaucher disease: a review. Br J Radiol 75(Suppl 1): A2–A12

Wenstrup RJ, Kacena KA, Kaplan P, Pastores GM, Prakash-Cheng A, Zimran A et al (2007) Effect of enzyme replacement therapy with imiglucerase on BMD in type 1 Gaucher disease. J Bone Miner Res 22(1):119–126

JIMD Reports
DOI 10.1007/8904_2015_408

RESEARCH REPORT

Networking Across Borders for Individuals with Organic Acidurias and Urea Cycle Disorders: The E-IMD Consortium

Stefan Kölker • Dries Dobbelaere • Johannes Häberle • Peter Burgard • Florian Gleich • Marshall L. Summar • Steven Hannigan • Samantha Parker • Anupam Chakrapani • Matthias R. Baumgartner • on Behalf of the E-IMD Consortium

Received: 27 August 2014 / Revised: 09 January 2015 / Accepted: 14 January 2015 / Published online: 22 February 2015

Abstract *Background*: Patients with organic acidurias (OAD) and urea cycle disorders (UCD) are at increased risk of disability, impaired quality of life and reduced life expectancy. Clinical care in any one centre is constrained by small patient numbers; and furthermore diagnostic and treatment strategies vary between metabolic centres and countries, resulting in significant inequalities and disparity in patient outcome.

Aims/methods: The overall objective of the EU-funded activity 'European registry and network for intoxication type metabolic diseases' (E-IMD) is to collect systematic data to improve the knowledge of these diseases, to develop consensus care guidelines and to provide detailed information materials for families and professionals.

Results: Within three years E-IMD has (1) established a network of 87 partners in 25 countries (2) set up a patient registry of more than 1,000 individuals with OAD and UCD, (3) launched a website (www.e-imd.org) including detailed information materials in 11 languages, (4) developed guidelines for OAD and UCD, (5) organised two teaching courses and various scientific meetings, (6) extended the IT platform clustering with other inherited metabolic diseases (IMD) and (7) strengthened the collaboration with other international scientific consortia.

Conclusions: E-IMD has made important steps towards improving and sharing knowledge on OAD and UCD and harmonisation of diagnostic and therapeutic strategies. Through the establishment of a modular patient registry, clustering with other IMD and stepwise extension of the network, E-IMD has implemented the core components of a European Reference Network for rare diseases.

Communicated by: Bridget Wilcken

Competing interests: None declared

S. Kölker (✉) · P. Burgard · F. Gleich
Division of Inherited Metabolic Diseases, Department of General Pediatrics, University Children's Hospital Heidelberg, Im Neuenheimer Feld 430, 69120 Heidelberg, Germany
e-mail: Stefan.Koelker@med.uni-heidelberg.de

D. Dobbelaere
Centre de Référence des Maladies Héréditaires du Métabolisme de l'Enfant et de l'Adulte, Hôpital Jeanne de Flandre, Lille, France

J. Häberle · M.R. Baumgartner
Division of Metabolism and Children's Research Centre, University Children's Hospital Zurich, Steinwiesstraße 75, 8032 Zurich, Switzerland

M.L. Summar
Children's National Medical Center, 111 Michigan Avenue, N.W., Washington, DC 20010, USA

S. Hannigan
CLIMB, National Information Centre for Metabolic Diseases, 176 Nantwich Road, Crewe, CW2 6BG, UK

S. Parker
Orphan Europe Sarl, Immeuble "Le Wilson"70 Avenue du Général de Gaulle, 92058 Paris La Défense, France

A. Chakrapani
Birmingham Children's Hospital NHS Foundation Trust, Steelhouse Lane, Birmingham B4 6NH, UK

Abbreviations

EAHC	European Agency for Health and Consumers
E-HOD	European network and registry for homocystinurias and methylation defects
E-IMD	European registry and network for intoxication type metabolic diseases
ERN	European reference network
EUCERD	European Union Committee of Experts on Rare Diseases

GA1	Glutaric aciduria type 1
IMD	Inherited metabolic disease
IVA	Isovaleric aciduria
MMA	Methylmalonic aciduria
MS	Member state
OAD	Organic aciduria
PA	Propionic aciduria
PO	Patient organisation
UCD	Urea cycle disorder
UCDC	Urea cycle disorders consortium

Introduction

Organic acidurias (OAD) and urea cycle disorders (UCD) are rarely inherited metabolic diseases (IMD) with an estimated cumulative incidence of 1 in 35,000 newborns (UCD) or 1 in 14,000–30,000 newborns (OAD), respectively (Kasper et al. 2010; Schulze et al. 2003; Summar et al. 2013; Wilcken et al. 2003). Affected individuals often present with first symptoms in the newborn period or infancy and are at an increased risk of severe disability, impaired quality of life, and reduced life expectancy (Bachmann 2003; Enns et al. 2007; Grünert et al. 2013; Hörster et al. 2007; Kido et al. 2012; Kölker et al. 2006; Nassogne et al. 2005; Pena et al. 2012; Strauss et al. 2003; Summar et al. 2008). Because of their life-threatening character and the permanent risk of metabolic crisis OAD and UCD are also called intoxication type IMD. In some countries these diseases are included in newborn screening programmes, hereby allowing early detection and start of treatment in asymptomatic individuals. This early intervention hopefully leads to improved health outcome, as it has been shown for glutaric aciduria type 1 (GA-1) and isovaleric aciduria (IVA) (Grünert et al. 2012; Heringer et al. 2010; Kölker et al. 2007a).

Patients with rare diseases like intoxication type IMD have become a healthcare priority in developed countries where other causes of infant mortality such as infectious diseases are now treatable (Commission of the European Communities 2008). OAD and UCD patients are scattered across countries and as a result medical expertise for each of these diseases is a scarce resource. Fragmented disease knowledge means that care is not optimal, and there are significant differences in the infrastructure, expertise, diagnostic procedures, time to diagnosis, strategies and outcome. In analogy to other rare diseases it can be expected that this diversity has a negative impact on health outcome and on socio-economics (Brimley et al. 2013; Linertová et al. 2012; López-Bastida et al. 2008; López-Bastida et al. 2009).

The overall aim of the European registry and network for intoxication type metabolic diseases (E-IMD) is to promote health for individuals affected with OAD or UCD by pooling of expertise and networking and by reducing avoidable inequity. E-IMD has two specific objectives: (1) to establish a European patient registry describing the disease course, epidemiology, diagnostic and therapeutic strategies for OAD and UCD and (2) to provide European evidence-based consensus care protocols for patients with OAD and UCD serving as a template for the development of guidelines and patient brochures. This paper describes the establishment of E-IMD, major achievements within the first three years of the project and important obstacles that the consortium learnt to deal with.

Methods and Results

The Network

E-IMD partners have already successfully collaborated for several years in various projects on OAD and UCD. In 2010, a strategic decision was made to formalise cooperation by establishing E-IMD. E-IMD has been partly funded from 1 January 2011 to 30 April 2014 by the European Union [via the European Agency for Health and Consumers (EAHC); agreement no. 2010 12 01], in the framework of the Health Programme 2008-2013. It is coordinated by the University Hospital Heidelberg and started with 28 project partners (coordinator, 12 associated and 15 collaborating partners) from 15 European countries. Associated partners received on average 60% EU co-funding from the grant whilst collaborating partners participated on a voluntary basis. The network has developed beyond expectations and now includes 87 partners from 25 countries on four continents (Fig. 1). Sixteen patient organisations (PO), four industrial partners and 67 clinical partners form the network. Representatives of the adult metabolic and dieticians' groups of the Society for the Study of Inborn Errors of Metabolism, the Urea Cycle Disorders Consortium (UCDC) and the Japanese Consortium for Urea Cycle Disorders are E-IMD partners. Applications for memberships of clinical partners have been evaluated by the steering committee based on the following criteria: (1) clinical and scientific expertise in intoxication type metabolic diseases, (2) metabolic service provided by an interdisciplinary team of experts and (3) capacity and relevant infrastructure to contribute to E-IMD.

The E-IMD advisory board including all network partners is the principal decision-making and arbitration body for the network and registry. The steering group comprising the lead of each work package and a representative of collaborating partners has the overall responsibility

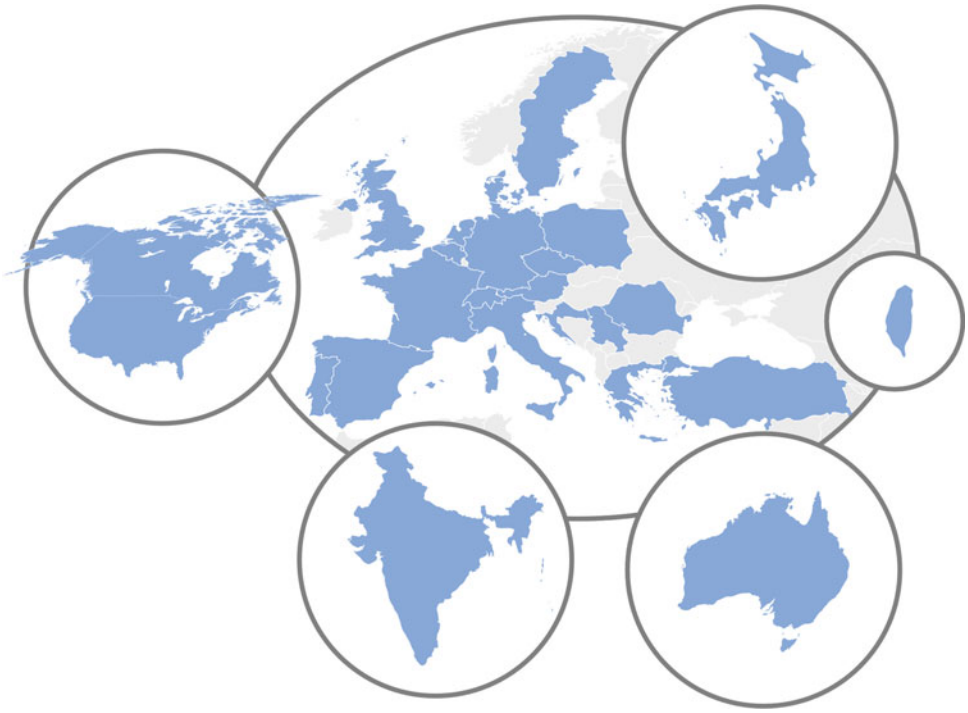


Fig. 1 Crossing borders for patients with rare OAD and UCD. The E-IMD network so far includes 87 partners from 25 countries on four continents. Note that the coloured areas do not reflect the exact geographical coverage of E-IMD

to ensure satisfactory progress of the work and promptly deal with problems. It assists the network director in the implementation of the integrative management of the network. All E-IMD partners have signed a consortium agreement which specifies and defines the organisation, management, responsibilities and tasks of the network.

The Patient Registry

The E-IMD patient registry is a web-based, password-protected registry (https://www.eimd-registry.org) launched on the web in August 2011. It contains comprehensive information on patients with confirmed diagnosis of OAD, i.e. glutaric aciduria type 1 (GA1; OMIM #231670), methylmalonic aciduria (MMA; OMIM #251000, #251100, #251110, #277400, #277410), propionic aciduria (PA; OMIM #606054), IVA (OMIM #243500) and UCD, i.e. inherited deficiency of *N*-acetylglutamate synthase (OMIM #237310), carbamylphosphate synthetase 1 (OMIM #237300), ornithine transcarbamylase (also including heterozygous female carriers; OMIM #311250), argininosuccinate synthetase (OMIM #215700), argininosuccinate lyase (OMIM #207900) and arginase 1 (OMIM #207800), as well as hyperornithinemia-hyperammonemia-homocitrullinuria syndrome (OMIM #238970). Written informed consent is obtained from all study patients before enrolment and baseline visit. The study was approved by the local ethics committee of the coordinating centre (i.e. University Hospital Heidelberg, application no. S-525/2010) on 31 January 2011 and then approved by the ethics committees of clinical partners contributing to the registry ($n = 44$). As of 30 April 2014, 1,009 patients with a confirmed diagnosis of OAD and UCD have been registered. The registry collects prospective data and contains detailed information on 949 baseline, 1,076 regular (annual), 437 emergency and 17 fatal disease course visits, averaging 2.5 visits per patient. A detailed description of the clinical phenotype will be published separately (Kölker et al. 2015a, b).

Forty clinical partners (of the 44 partners with ethical approval) from 20 countries – 17 European countries [15 EU member states (MS), Switzerland and Republic of Serbia], India, Japan and the USA – have so far contributed to the registry. The maximal population size of EU MS covered by clinical E-IMD partners was 453,288,893 citizens, i.e. 89.6% of the population in 28 EU MS according to Eurostat 2013 (http://epp.eurostat.ec.europa.eu). However, since recruitment to E-IMD varies between countries, estimated prevalences of OAD and UCD are likely to be underestimated. For instance, the minimum prevalence of patients with OAD and UCD was 2.10 per million citizens, with a range of 0.15 (Romania) to 8.39 (Denmark) (Table 1). This may skew the results, but with continued registration of patients, this current drawback will diminish.

This variable frequency of UCD and OAD patients is unlikely to reflect true epidemiological differences,

Table 1 Minimum cumulative prevalence of patients with OAD and UCD in Europe

Country	Patients $N_{(pat)}$	Population $N_{(pop)}$	Prevalence $N_{(pat)} \times N_{(pop)}^{-1} \times 10^6$ [a]
Austria	22	8,451,860	2.60
Belgium	22	11,161,642	1.97
Croatia	22	4,262,140	5.16
Czech Republic	29	10,516,125	2.76
Denmark	47	5,602,628	8.39
France	189	65,578,819	2.88
Germany	160	80,523,746	1.99
Greece	8	11,062,508	0.72
Italy	65	59,685,227	1.09
Netherlands	44	16,779,575	2.62
Poland	36	38,533,299	0.93
Portugal	34	10,487,289	3.24
Romania	3	20,020,074	0.15
Republic of Serbia	6	7,181,505	0.84
Spain	145	46,727,890	3.10
Switzerland	24	8,039,060	2.99
UK	129	63,896,071	2.02
Total	985	468,509,458	2.10
Total (EU MS)	955	453,288,893	2.11

EU MS member states of the European Union, *pat* patients, *pop* population

[a] Population size in 2013 according to EUROSTAT. Patients from non-European countries ($n = 24$) are not listed

but merely shows the effect of a number of modulatory factors including the number and capacity of E-IMD partners in different countries as well as the rapidity and success of the activation process in individual centres. The activation process is the time interval from becoming a member of the E-IMD consortium and registering the first patient. The process time has been highly variable reflecting national and local differences and ranged from 2 to 20 months.

An important source of variability has been the ethical review process which includes translation into national languages, adaptation of the application to national and local requirements and submission to the board. The regulations governing this process are highly variable in different countries and local centres. Noteworthily, associated partners who have received EU co-funding have achieved ethical approval and started registering patients earlier than collaborating partners who contributed to the project on a voluntary basis (Fig. 2). Furthermore, the 12 associated clinical partners have registered more patients (74% of total) than 28 collaborating partners (26%). Despite the shortcomings arising from the project funding mechanism, the process of recruiting and registering patients was almost linear with approximately 26 patients with OAD and UCD being newly registered during each month between February 2011 (first patient recruited) and April 2014 (end of the EU funding period). Meanwhile, the follow-up visits outnumber the baseline visits showing that the registry is now increasingly used for systematic follow-up of registered patients (Fig. 3).

Dissemination

Dissemination is an integral part of the E-IMD strategy with targeted distribution of disease information and intervention materials, such as protocols and guidelines. E-IMD developed a website (www.e-imd.org) as the main dissemination vehicle for the network. At present, the website provides information for patients and their family (translated into 11 languages) and for healthcare professionals, newsletters and contact addresses. The number of visitors has been increased to approximately 250 visitors per day and is still increasing. The patient information is the most frequently visited part of the website. In addition, the number of cases can be viewed in real-time mode in the public domain of the patient registry (https://www.eimd-registry.org).

E-IMD has actively sought to publish its achievements in peer-reviewed journals focusing on epidemiology, clinical phenotyping, therapy and guideline development Baumgartner et al. 2014; Boy et al. 2013; Chapman et al. 2012; Häberle et al. 2012; Kölker et al. 2011; Pena et al. 2012; Rüegger et al. 2014; Summar et al. 2013). Four E-IMD advisory board meetings have been held during annual SSIEM and ICIEM symposia between 2011 and 2014. Since 2013, joint meetings with the E-HOD (www.e-hod.org) and UCDC consortia have been organised.

Finally, E-IMD has partnered with the Recordati Rare Diseases Fondation d'entreprise (http://www.rrdacademy.org) to organise three training courses for young doctors in the field of IMD.

Guideline Development

Guidelines are systematically developed statements assisting practitioner and patient decisions in appropriate healthcare for specific clinical circumstances. The Scottish Intercollegiate Guidelines Network (http://www.sign.ac.uk) methodology was employed to develop these guidelines.

European consensus guidelines for UCD (Häberle et al. 2012), GA1 (Kölker et al. 2011), and MMA/PA (Baumgartner et al. 2014) have been published under the umbrella of E-IMD. Those for IVA are still under development. Short guideline versions are available online via the E-IMD website. Based on the available

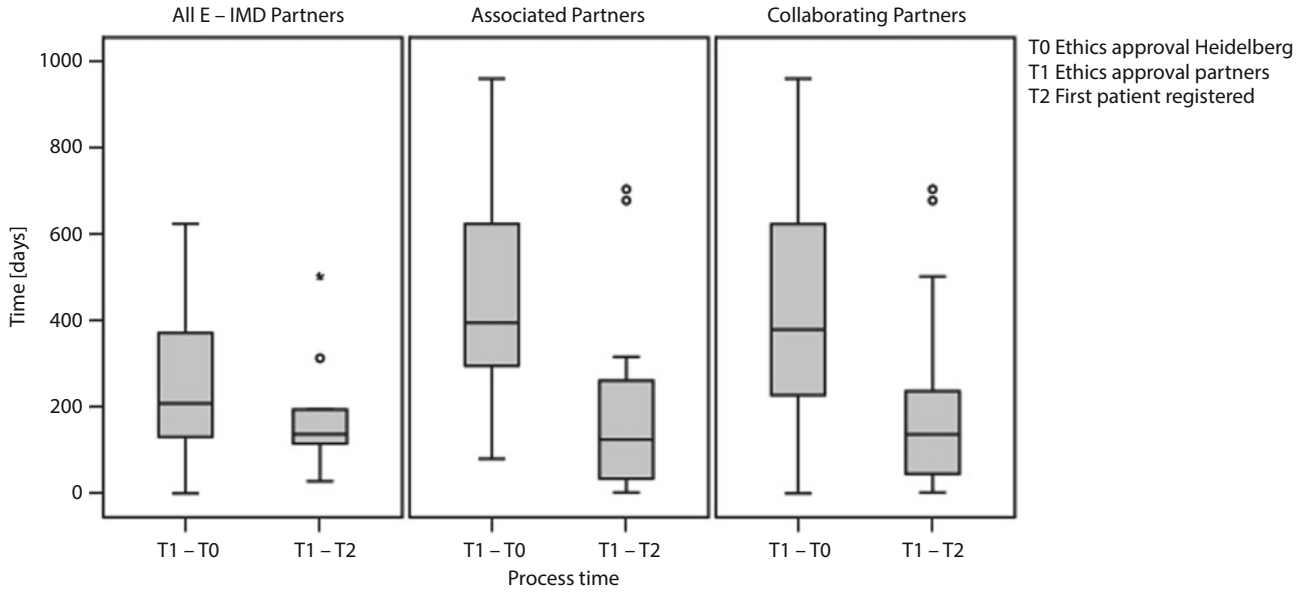


Fig. 2 Milestones of the activation process in associated and collaborating partners. The time required for receiving ethics approval was adjusted to the date when the coordinating partner had received ethics approval and to the individual dates of signing the consortium agreement (for collaborating partners who have joined the consortium after the kick-off meeting)

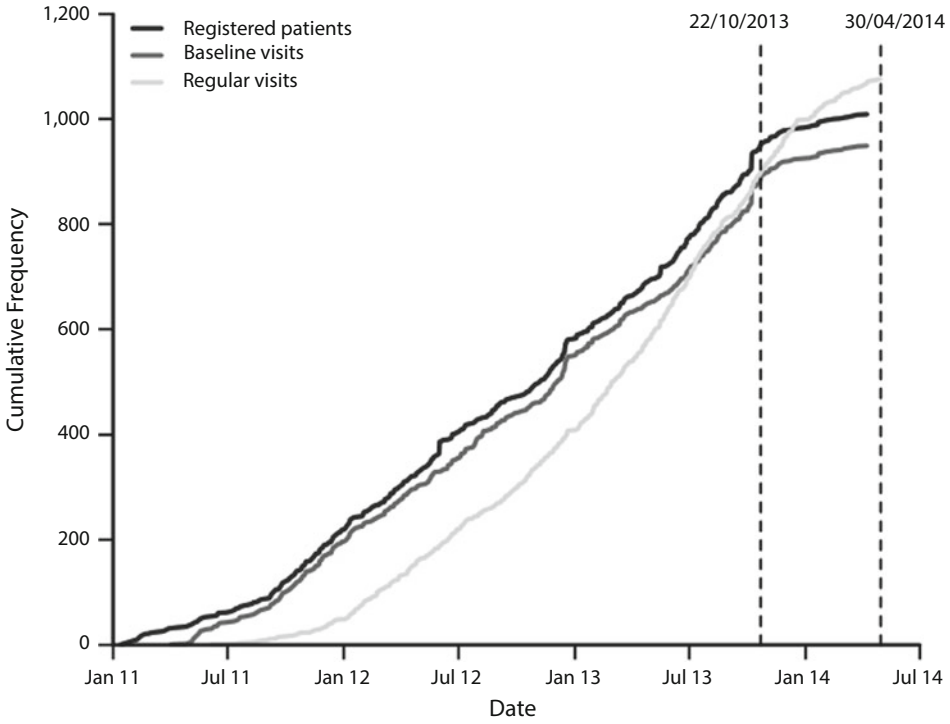


Fig. 3 Timeline of recruiting and registering patients. *Lines* indicate cumulative frequencies of registered patients, baseline visits and regular follow-up visits during the funding period. *Dotted lines* indicate the cut-off date for the interim analysis (22 October 2013) and the end of the EU funding period (30 April 2014)

evidence from literature, the statements for OAD and UCD are mostly graded level C or D. The process of guideline development lasted over several years and can be considered as outstanding in the field of metabolic medicine. Importantly, the use of GA1 guideline recommendations, when evaluated after some years, was shown to improve the neurological outcome and to support normal growth (Boy et al. 2013; Heringer et al. 2010).

Along this, it is anticipated that the achieved publication and dissemination of consensus evidence-based guidelines will further improve care of patients with OAD and UCD.

Evaluation

The evaluation of the project is led by the steering group with strong patient representation. Its overall aim is to appreciate how E-IMD achieved its main goal of building knowledge and whether diagnosis and care improved in the different European countries. The following sources of information and indicators have been used: (1) a survey sent out via national PO, (2) analysis of the use of the E-IMD website and (3) analysis of the patient registry for completeness of geographical coverage, quality and completeness of records and time to diagnosis.

The evaluation highlighted that there is a need to develop care pathways for OAD and UCD patients and to resolve the difficult issue of transition of adolescents into adult care. From the patient perspective there is also a need to develop the PO community for IMD through the establishment of a helpline and online community. E-IMD has been the catalyser in the funding and launching of the European Metabolic Disease Alliance (www.eumda.org).

The website is the principal means of public communication. E-IMD comes up on the first page of Google search engine using the key words ‘organic aciduria’ or ‘urea cycle disorder’; however, it was still difficult to get traffic to the website.

The registry contains data on 1,009 individuals with an OAD or UCD. There must be a concerted effort amongst partners to complete patient records and follow up this unique cohort. Further data is needed in the registry to better understand the outcome of patients diagnosed through newborn screening compared to those diagnosed after the onset of symptoms.

Discussion

In the last three years, E-IMD has started the first collaborative initiative on UCD patients in Europe and the largest initiative for OAD patients worldwide. E-IMD has fostered international collaboration with the American UCDC consortium (Seminara et al. 2010) and the newly established Japanese UCDC. The E-IMD network has developed beyond expectations and now includes 87 partners in 25 countries. The specific objectives of this EU-funded activity are to improve the knowledge base, to develop European consensus guidelines and to foster networking for patients with OAD and UCD in Europe. These goals have all been achieved despite important obstacles and hurdles.

Project Funding Mechanism

A major drawback was the funding mechanism of this project. The activation process time and the number of patients registered differed between associated and collaborating partners. Whereas associated partners received partial EU funding, collaborating partners contributed on a voluntary basis without financial compensation for their working time. Therefore, it can be assumed that the activation process would have been accelerated and the total number of patients would have been significantly increased, if a larger proportion of clinical partners had received project funding. In addition, the shared cost principle and financial mechanism have been a challenge as hospital administrators are often unable to prefinance. In conclusion, the management of this project could have been improved if European regulations had been harmonious on a national level and the financial plan for such projects had been more flexible.

Ethical Review Process

Another source of significant variability in the activation process has been the ethical review. In some countries this is painstakingly long and administrative, whilst in others formal ethical review is not required as the study is regarded as noninterventional audit. It would be of great benefit for rare disease registries to harmonise regulations and to distinguish between noninterventional studies with low or even no risk of potential harm for participating individuals and other types of research, at a European level.

Guidelines for Rare Diseases

Guidelines for OAD and UCD have been developed and published under the umbrella of E-IMD (Baumgartner et al. 2014; Häberle et al. 2012; Kölker et al. 2011). The formal process of guideline development, which was in line with the SIGN methodology, was considered unhelpful by some authors since guidelines for rare diseases often result in low grades of recommendations. It was assumed that such guidelines might be liable to misinterpretation and misuse, not be prescriptive enough and prove to be a hindrance in obtaining funding for treatment (Vockley et al. 2013). However, there are strong refutations to this position:

1. The level of published evidence and grading of a recommendation do not necessarily correlate with its clinical relevance. For instance, although low phenylalanine diet for phenylketonuria has never been tested in a randomised controlled trial, and, therefore, the level of evidence for this intervention has to be formally evaluated as relatively low (Yi and Singh 2008), no

metabolic specialist would doubt that this therapeutic intervention in general is extremely relevant for affected individuals (Blau et al. 2010; Burgard 2000; Camp et al. 2014).

2. The effect size of therapeutic interventions in patients with an IMD is often huge. Therefore, it can be reliably identified by a cohort study with low risk of confounding bias (Kölker et al. 2007b; Heringer et al. 2010). There is no doubt that, if affordable and achievable, randomised controlled trials for rare IMDs should be performed, but this is often not feasible due to low number of patients and would require the interest of the pharmaceutical industry (Enns et al. 2007; Levy et al. 2007; Wraith et al. 2004). However, there are also examples of carefully designed *n* of one trials (Bickel et al. 1953).
3. Low grading helps to identify the gaps in current knowledge thereby setting the scene for further research.
4. Setting standards of practice is important to minimise unnecessary variance or – even worse – trial and error.
5. Identification of alternative approaches is important since these are often required when adverse events occur or a drug is not available in a national health system.
6. Practices based upon expert opinion of single physicians or centres with a long-standing experience do not gain wide appraisal and approval without independent and critical evaluation.

The E-IMD consortium will foster the implementation of the guidelines for OAD and UCD in daily practice and will investigate whether the use of guideline recommendations improves the health outcomes and quality of life of affected individuals. First promising results have already been published for the use of the GA1 guideline on a national level (Heringer et al. 2010) and for the UCD guideline (Häberle and Huemer 2015). To evaluate the effect of newborn screening and adherence to guideline recommendations in rare diseases with a broad clinical spectrum will become challenging, since reliable clinical endpoints and a large number of patients are required for the analysis.

In 2013, after the development of the E-IMD guidelines, the Scottish Intercollegiate Guidelines Network implemented the principles of the GRADE methodology. This change will facilitate the process of recommendation development for rare diseases.

Looking Forward: Towards a European Reference Network for Inherited Metabolic Diseases

Despite some significant challenges, E-IMD has succeeded beyond expectations which will ultimately promote health for individuals with OAD and UCD. However this goal can only be achieved through long-term follow-up, which requires a sustainable funding mechanism. An opportunity for E-IMD could be its establishment as a European Reference Network (ERN) for inherited metabolic diseases. The general concept and implementation of ERNs are defined in Article 12 of the Cross-Border Healthcare Directive (The European Parliament and the Council of the European Union 2011). According to the recommendations of the European Union Committee of Experts on Rare Diseases (EUCERD) on rare disease European Union Committee of Experts on Rare Diseases (2013), which are designed to be complementary to the Cross-Border Healthcare Expert Group on ERNs, a rare disease ERN should cover various core tools and activities, amongst the disease registries, mechanisms for information flow for good practice guidelines, training and education tools and communications infrastructure to ensure visibility. E-IMD has developed these tools and activities.

In addition, E-IMD has proceeded with the concept of disease clustering. Starting with 11 IMD, the modular IT platform has been extended to 26 IMD by inclusion of homocystinurias and methylation defects within the EU-funded project E-HOD (project lead: Prof. Henk Blom, Freiburg, Germany). Since 2013, the IT platform has also been used to gather post-marketing surveillance data for the orphan drug Cystadane™ (betaine anhydrous) which is licensed for adjunctive treatment of homocystinuria caused by deficiencies or defects in cystathionine beta-synthase, 5,10-methylene tetrahydrofolate reductase, and cobalamin cofactor metabolism. The 'Cystadane Surveillance Protocol' project has been realised within a public private partnership between the E-HOD consortium and the drug licence holder, Orphan Europe Sarl. This collaboration maps onto the 2011 recommendations of the European Medicines Agency and EUCERD stressing the need to support public private partnerships in the development of registries and collaboration for post-marketing surveillance (European Union Committee of Experts on Rare Diseases 2011). In 2014, the same IT solution will be used for implementing the IMD group of biogenic amine and pterin biosynthesis and recycling disorders (iNTD, project lead: Dr Thomas Opladen, Heidelberg, Germany; funded by Dietmar Hopp Foundation, Germany).

This clearly shows that disease clustering and the development of new applications using the same IT platform and network have many advantages and are a favourable strategy for both sustainment and extension. The strategy for extending the IT platform and the network towards an ERN for inherited metabolic diseases has been elaborated and shall be realised step by step.

Acknowledgements This publication arises from the project 'European registry and network for intoxication type metabolic diseases (E-IMD)' (EAHC no 2010 12 01) which has received funding from the European Union, in the framework of the Health Programme. After the end of the EU funding period the E-IMD patient registry will be sustained by funding from the Kindness for Kids Foundation (Munich, Germany). MRB and JH are supported by radiz – Rare Disease Initiative Zurich – a clinical research priority programme of the University of Zurich, Switzerland.

We are grateful to the following partners for their valuable contribution to establish the E-IMD consortium and for their fruitful ongoing collaboration (listed in alphabetical order of countries):

Austria: Daniela Karall, Johannes Zschocke and Sabine Scholl-Bürgi (Medizinische Universität Innsbruck, Universitätskinderklinik und Sektionen für Humangenetik und Klinische Genetik, Innsbruck).

Australia: Avihu Boneh (the Murdoch Childrens Research Institute, Royal Children's Hospital, Melbourne)

Belgium: Francois Eyskens and Tine Maes (Universitair Ziekenhuis Antwerpen, Antwerpen), Linda de Meirleir (University Hospital Vrije Universiteit Brussels, Department of Pediatric Neurology, Brussels), Corinne de Laet (Hôpital Universitaire des Enfants Reine Fabiola, Nutrition and Metabolism Unit, Brussels), Etienne Sokal and Florence Defresne (Université Catholique de Louvain, Clinique Universitaires St. Luc, Brussels), Dominique Roland (Institute of Pathology and Genetics, Center for Inherited Metabolic Diseases, Gosselies), Guillaume Debray (Centre Hospitalier Universitaire, Department of Genetics, Liège) and Lut de Baere and Nathalie Stroobant [Belgische Organisatie voor Kinderen en Volwassenen met een Stofwisselingsziekte VZW (BOKS), Melsele, PO group]

Canada: Cheryl R. Greenberg (University of Manitoba, Department of Pediatrics and Child Health, Department of Biochemistry and Medical Genetics, Winnipeg)

Croatia: Ivo Baric, Mario Cuk and Slobodan Galic (Sveuciliste u Zagrebu, Medicinski fakultet, University Hospital Centre, Department of Pediatrics, Zagreb) and Nelia Caric (Hrvatska udruga za rijetke bolesti, PO group)

Czech Republic: Jiri Zeman and Tomas Honzik (Charles University of Prague, First Faculty of Medicine, Department of Pediatrics, Prague)

Denmark: Allan M. Lund, Ernst Christensen, Lise Aksglaede and Malene Bøgehus Rasmussen (Rigshospitalet, Centre for Inherited Metabolic Diseases, Department of Clinical Genetics, Copenhagen) and Annika and Kennet Rovsing (PND – Protein Nedbrydnings Defekt foreningen, PO group)

France: Vassili Valayannopoulos, Jean-Baptiste Arnoux, Pascale de Lonlay, Ulf Aringer, Kim-Hanh Le Quan Sang and Eric Bauchart (Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris, Reference Center for Inherited Metabolic Disease, Necker-Enfants Malades University Hospital and Imagine Institute, Paris); Hélène Ogier de Baulny (Centre de Référence Maladies Héréditaires du Metabolisme, Hôpital Robert Debré, Université Paris VII, Paris); Brigitte Chabrol (Centre de Référence Maladies Héréditaires du Metabolisme, Hôpital d'Enfants Service de Neurologie, Marseille); Pierre Broue (Centre de Référence Maladies Héréditaires du Metabolisme, Hôpital des Enfants – CHU Toulouse, Toulouse); EURO-RDIS, European Organisation for Rare Disease (Paris); and Orphan Europe SARL (Paris)

Germany: S. P. Nikolas Boy, Corinna Bürger, Esther M. Glahn, Friederike Hörster, Gisela Haege, Jana Heringer, Marina A. Morath, Roland Posset, Christian Staufner, Kathrin Zangerl and Matthias Zielonka (Universitätsklinikum Heidelberg, Zentrum für Kinder- und Jugendmedizin, Kinderklinik I); Chris Mühlhausen and René Santer (Universitätsklinikum Hamburg-Eppendorf, Klinik für Kinder- und Jugendmedizin, Hamburg); Regina Ensenauer (Ludwig-Maximilian-Universität München, Dr. von Haunersches Kinderspital, München); Thomas Meissner (Universitätsklinikum Düsseldorf, Zentrum für Kinder- und Jugendmedizin, Düsseldorf); Peter Freisinger (Klinik für Kinder- und Jugendmedizin, Klinikum am Steinenberg, Reutlingen); Sarah Grünert and Ute Spiekerkötter (Universitätsklinikum Freiburg, Zentrum für Kinder- und Jugendmedizin, Freiburg); Martin Lindner (Universitätsklinikum Frankfurt, Klinik für Kinder- und Jugendmedizin, Frankfurt); Markus Ott and Beate Szczerbak (Nutricia Metabolics GmbH, Friedrichsdorf); Hubertus von Voss and Raimund Schmid (Kindernetzwerk e.V., Aschaffenburg); Mandy Kretschmer (Glutarazidurie e.V.); and Reinhild Link (Wiesbaden, representing the SSIEM Dieticians Group)

Greece: Persephone Augoustides-Savvopoulou and Harikleia Ioannou (University A'Pediatric Department, Metabolic Laboratory, 'Hippokration' General Hospital of Thessaloniki, Thessaloniki, and KRIKOS ZOIS, PO group), Evriki Drogari (University of Athens, Aghia Sophia Children's Hospital, Unit of Metabolic Diseases, Athens) and Zarifis Dimitroulis (KRIKOS ZOIS – Society for Patients and Friends of Patients with Inherited Metabolic diseases, PO group)

India: Anil Jalan (N.I.R.M.A.N., Om Rachna Society, Mumbai)

Italy: Alberto B. Burlina, Andrea Bordugo and Francesca Furlan (Azienda Ospedaliera di Padova, U.O.C. Malattie Metaboliche Ereditarie, Dipartimento di Pediatria, Padova); Carlo Dionisi-Vici and Diego Martinelli (Ospedale Pediatrico Bambino Gesù, U.O.C. Patologia Metabolica, Rome); Renza Barbon Galluppi (UNIAMO FIMR, PO group); and Susan Udina (COMETA ASMME – Associazione Studio Malattie Metaboliche Ereditarie – ONLUS, PO group)

Japan: Fumio Endo and Shirou Matsumoto (Kumamoto University Hospital, Department of Pedatrics, Kumamoto, and representing the Japanese Urea Cycle Disorders Consortium)

Netherlands: Frits A. Wijburg and Eveline Langereis (Academisch Medisch Centrum, Department of Pediatrics, Amsterdam), Monique Williams (Erasmus Universiteit Rotterdam, Erasmus MC-Sophia Kinderziekenhuis, Rotterdam) and Hanka Meutgeert (Volwassenen en Kinderen met Stofwisselingsziekten [VKS], Zwolle, PO group)

Poland: Jolanta Sykut-Cegielska (Institute of Mother and Child, Screening Department, Warsaw) and Wanda Gradowska (Instytut 'Pomnik-Centrum Zdrowia Dziecka', The Children's Memorial Health Institute, Department of Metabolic Diseases, Endocrinology and Diabetology, Warsaw)

Portugal: Elisa Teles Leao, Susana Soares and Esmeralda Rodrigues (Unidade de Doenças Metabólicas, Serviço de Pediatria, Hospital de S. João, EPE, Porto); Laura Vilarinho (Newborn Screening Unit, Metabolic Genetics Center, National Institute of Health [INSA], Porto); Ana Gaspar (Unidade de Doenças Metabólicas, Serviço de Pediatria Hospital Santa Maria Lisboa, Lisbon); Isabel Tavares de Almeida (Faculdade de Farmácia da Universidade de Lisboa, Lisbon); Vanessa Ferreira (Associação Portuguesa CDG, PO group); Miguel Macedo (Apofen, PO group); and Sérgio Braz Antão (Rarrisimas, PO group)

Republic of Serbia: Adrijan Sarajlija and Maja Djordjevic (Institut za zdravstvenu zaštitu majke i deteta Srbije, Belgrade)

Romania: Paula Avram (Institute for Mother and Child Care 'Alfred Rusescu', Bucharest)

Spain: Juan-Luque Moreno (CIBERER, Centro de Investigacíon Biomédica en Red de Enfermedades Raras); Angeles Garcia-Cazorla, Jaume Campistol, Carlos Ortez and Elisenda Cortès i Saladelafont (Hospital Sant Joan de Deu, Servicio de Neurologica, Barcelona); Elena Balmaseda and M. Carmen Carrascosa (Complejo Hospitalario Universitario de Albacete, Albacete); Guillem Pintos-Morell (University hospital 'German Trias i Pujol', Badalona); Antonia Ribes (Institut Bioquimica Clinica, Corporacio Sanitaria Clinic, Barcelona); Eduardo Lopéz (Pediatric Neurology Unit Department of Pediatrics, University Hospital Reina Sofia, Cordoba); Immaculada Vives and David Gil Ortega (Hospital Virgen de la Arrixaca de Murcia, El Palmar); Juana Maria de Haro Catellano (Secretaria Tecnica del CEI-Granada (HUVN), Edificio Licinio de la Fuente, Granada); Luis

Pena-Quintana (Hospital Universitario Materno-Infantil de Canarias, Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria); Magdalena Ugarte and Begona Merinero (Universidad Autonoma de Madrid, Madrid); Consuelo Pedròn-Giner (Hospital Infantil Universitario Niño Jesús, Sección de Gastroenterología y Nutrición, Madrid); Javier Blasco-Alonso (Hospital Materno-Infantil de Malaga, Unidad de Gastroenterologia, Hepatologia, Nutricion y Metabolopatias, Malaga); Angeles Ruiz Gómez (Hospital Universitario Son Espases, Palma de Mallorca); Maria L. Couce (Hospital Clinico Universitario de Santiago de Compostela, Santiago de Compostela); Vicente Rubio (Instituto de Biomedicina de Valencia, Valencia); and Sergi Faber (Catalana de Trastorns Metabòlics Hereditaris, PO group)

Sweden: Sofia Nordin (Swedish Orphan Biovitrum AB [SOBI], Stockholm)

Switzerland: Jörn-Oliver Sass (Kinderspital Zürich, Universitäts-Kinderkliniken, Eleonoren-Stiftung, Department of Clinical Chemistry and Biochemistry, Zürich) and Jean-Marc Nuoffer (Universitätsspital Bern, Universitätsklinik für Kinderheilkunde, Bern)

Taiwan: Wu-Liang Hwu, Yin-Hsiu Chien and Ni-Chung Lee (National Taiwan University Hospital, Department of Medical Genetics, Taipei)

Turkey: Mübeccel Demirkol and Gülden Gökcay (Istanbul University, Children's Hospital, Department of Nutrition and Metabolism, Istanbul)

UK: Victoria Riches (Birmingham Children's Hospital NHS Foundation Trust, Birmingham); CLIMB, Children Living with Inherited Metabolic Diseases, National Information Centre for Metabolic Diseases (Crewe); Stephanie Grünewald and Nick Thompson (Great Ormond Street Hospital for Children NHS Trust, London); Robin Lachmann and Elaine Murphy (National Hospital for Neurology and Neurosurgery, Charles Dent Metabolic Unit, London, and representing the SSIEM Adult Metabolic Group); Roshni Vara (Evelina Children's Hospital, St Thomas' Hospital, Department of Inherited Metabolic Disease, London); John Walter and Andrew Morris (Central Manchester and Manchester Children's University Hospital, Willink Biochemical Genetics Unit, Manchester); Bradford Teaching Hospitals NHS Trust, St Luke's Hospital (Bradford); and EMDA, the European Metabolic Disorders Alliance (PO group)

USA: Kimberly Chapman (Children's National Medical Center, Center for Genetic Medicine Research, Washington DC, and representing the Urea Cycle Disorders Consortium) and Jerry Vockley (Children's Hospital of Pittsburgh of UPMC, Pittsburgh)

Synopsis

E-IMD has established an international network, has improved knowledge about organic acidurias and urea cycle disorders and has started harmonising diagnostic and therapeutic strategies. This is the prerequisite for establishing a European Reference Network for inherited metabolic diseases.

Compliance with Ethics Guidelines

Conflict of Interest

Stefan Kölker, Matthias R. Baumgartner, Peter Burgard, Anupam Chakrapani, Dries Dobbelaere, Florian Gleich, Johannes Häberle, Marshall L. Summar, and Steven Hannigan declare that they have no conflict of interest. Samantha Parker is employed by Orphan Europe Sarl being part of the Recordati Group.

Informed Consent

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

Details of the Contributions of Individual Authors

Designing, planning and conducting the study: all authors

Statistical analysis: Peter Burgard, Florian Gleich and Stefan Kölker

Manuscript writing: All authors

Animal Rights

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

References

Bachmann C (2003) Outcome and survival of 88 patients with urea cycle disorders: a retrospective evaluation. Eur J Pediatr 162:410–416

Baumgartner MR, Hörster F, Assoun M et al (2014) Suggested guidelines for the diagnosis and management of methylmalonic and propionic acidemias. Orphanet J Rare Dis 9:130

Bickel H, Gerrard J, Hickmans EM (1953) Influence of phenylalanine intake on phenylketonuria. Lancet 2:812–813

Blau N, Bélanger-Quintana A, Demirkol M et al (2010) Management of phenylketonuria in Europe: survey results from 19 countries. Mol Genet Metab 99:109–115

Boy N, Haege G, Heringer J et al (2013) Low lysine diet in glutaric aciduria type I – effect on anthropometric and biochemical follow-up parameters. J Inherit Metab Dis 36:525–533

Brimley CJ, Lopez J, van Haren K et al (2013) National variation in costs and mortality for leukodystrophy patients in US children's hospitals. Pediatr Neurol 49:156–162

Burgard P (2000) Development of intelligence in early treated phenylketonuria. Eur J Pediatr 159(Suppl 2):S74–S79

Camp KM, Parisi MA, Acosta PB et al (2014) Phenylketonuria scientific review conference: state of the science and future research needs. Mol Genet Metab pii:S1096–7192(14)00085-7. doi:10.1016/j.ymgme.2014.02.013

Chapman KA, Gropman A, MacLeod E et al (2012) Acute management of propionic acidemia. Mol Genet Metab 105:16–25

Commission of the European Communities (2008) Communication from the Commission to the European Parliament, the Council,

the European Economic and Social Committee and the Committee of the regions. http://ec.europa.eu/health/rare_diseases/policy/index_en.htm

Enns GM, Berry SA, Berry GT, Rhead WJ, Brusilow SW, Hamosh A (2007) Survival after treatment with phenylacetate and benzoate for urea-cycle disorders. N Engl J Med 356:2282–2292

European Union Committee of Experts on Rare Diseases (2011) EUCERD/EMA workshop report: Towards a public-private partnership in the field of registries for rare diseases. http://www.eucerd.eu/?post_type=document&p=1234

European Union Committee of Experts on Rare Diseases (2013) EUCERD recommendations on rare disease European Reference Networks (RD ERNS). http://www.eucerd.eu/?post_type=document&p=2207

Grünert SC, Wendel U, Lindner M et al (2012) Clinical and neurocognitive outcome in symptomatic isovaleric acidemia. Orphanet J Rare Dis 7:9

Grünert SC, Müllerleile S, De Silva L et al (2013) Propionic acidemia: clinical course and outcome in 55 pediatric and adolescent patients. Orphanet J Rare Dis 8:6

Häberle J, Huemer M (2015) Evaluation of implementation, adaptation and use of the recently proposed urea cycle disorders guidelines. J Inherit Metab Dis (Reports), doi:10.1007/8904_2014_387

Häberle J, Boddaert N, Burlina A et al (2012) Suggested guidelines for the diagnosis and management of urea cycle disorders. Orphanet J Rare Dis 7:32

Heringer J, Boy SP, Ensenauer R et al (2010) Use of guidelines improves the neurological outcome in glutaric aciduria type I. Ann Neurol 68:743–752

Hörster F, Baumgartner MR, Viardot C et al (2007) Long-term outcome in methylmalonic acidurias is influenced by the underlying defect (mut0, mut-, cblA, cblB). Pediatr Res 62:225–230

Kasper DC, Ratschmann R, Metz TF et al (2010) The national newborn screening program – eight years experience with mass spectrometry. Past, present and future goals. Wien Klin Wochenschr 122:607–613

Kido J, Nakamura K, Mitsubuchi H et al (2012) Long-term outcome and intervention of urea cycle disorders in Japan. J Inherit Metab Dis 35:777–785

Kölker S, Garcia Cazorla A, Valayannopoulos V et al (2015a) The phenotypic spectrum of organic acidurias and urea cycle disorders. Part 1: The initial presentation. J Inherit Metab Dis. doi:10.1007/s10545-015-9817-9

Kölker S, Valayannopoulos V, Garcia Cazorla A et al (2015b) The phenotypic spectrum of organic acidurias and urea cycle disorders. Part 2: The initial presentation. J Inherit Metab Dis. doi:10.1007/s10545-015-9818-8

Kölker S, Garbade SF, Greenberg CR et al (2006) Natural history, outcome, and treatment efficacy in children and adults with glutaryl-CoA dehydrogenase deficiency. Pediatr Res 59:840–847

Kölker S, Christensen E, Leonard JV et al (2007a) Guideline for the diagnosis and management of glutaryl-CoA dehydrogenase deficiency (glutaric aciduria type I). J Inherit Metab Dis 30:5–22

Kölker S, Garbade SF, Boy N et al (2007b) Decline of acute encephalopathic crises in children with glutaryl-CoA dehydrogenase deficiency identified by newborn screening in Germany. Pediatr Res 62:225–260

Kölker S, Christensen E, Leonard JV et al (2011) Diagnosis and management of glutaric aciduria type I – revised recommendations. J Inherit Metab Dis 34:677–694

Levy HL, Milanowski A, Chakrapani A et al (2007) Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomised placebo-controlled study. Lancet 370:504–510

Linertová R, Serrano-Aguilar P, Posada-de-la-Paz M et al (2012) Delphi approach to select rare diseases for a European representative survey. The BURQOL-RD study. Health Policy 108:19–26

López-Bastida J, Perestelo-Pérez L, Montón-Alvarez F, Serrano-Aguilar P (2008) Social economic costs and health-related quality of life in patients with degenerative cerebellar ataxia in Spain. Mov Disord 23:212–217

López-Bastida J, Perestelo-Pérez L, Montón-Alvarez F, Serrano-Aguilar P, Alfonso-Sanchez JL (2009) Social economic costs and health-related quality of life in patients with amyotrophic lateral sclerosis in Spain. Amyotroph Lateral Scler 10:237–243

Nassogne MC, Héron B, Touati G, Rabier D, Saudubray JM (2005) Urea cycle defects: management and outcome. J Inherit Metab Dis 28:407–414

Pena L, Franks J, Chapman KA et al (2012) Natural history of propionic acidemia. Mol Genet Metab 105:5–9

Rüegger CM, Lindner M, Ballhausen D et al (2014) Cross-sectional observational study of 208 patients with non-classical urea cycle disorders. J Inherit Metab Dis 37:21–30

Schulze A, Lindner M, Kohlmüller D, Olgemöller K, Mayatepek E, Hoffmann GF (2003) Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: results, outcome, and implications. Pediatrics 111:1399–1406

Seminara J, Tuchman M, Krivitzky L et al (2010) Establishing a consortium for the study of rare diseases: The Urea Cycle Disorders Consortium. Mol Genet Metab 100:S97–S105

Strauss KA, Puffenberger EG, Robinson DL, Morton DH (2003) Type I glutaric aciduria, part 1: natural history of 77 patients. Am J Med Genet C Semin Med Genet 121C:38–52

Summar ML, Dobbelaere D, Brusilow S, Lee B (2008) Diagnosis, symptoms, frequency and mortality of 260 patients with urea cycle disorders from a 21-year, multi-centre study of acute hyperammonaemic episodes. Acta Paediatr 97:1420–1425

Summar ML, Kölker S, Freedenberg D et al (2013) The incidence of urea cycle disorders. Mol Genet Metab 110:179–180

The European Parliament and the Council of the European Union (2011) Directive 2011/24/EU of the European Parliament and of the Council of 9 March 2011 on the application of patients' rights in cross-border healthcare. Official J Eur Union L 88/45:1–21. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:088:0045:0065:EN:PDF

Vockley J, Chapman KA, Arnold GL (2013) Development of clinical guidelines for inbron errors of metabolism: commentary. Mol Genet Metab 108:203–205

Wilcken B, Wiley V, Hammond J, Carpenter K (2003) Screening methods for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 348:2304–2312

Wraith JE, Clarke LA, Beck M et al (2004) Enzyme replacement therapy for mucopolysaccharidosis I: a randomized, double-blinded, placebo-controlled, multinational study of recombinant human alpha-L-iduronidase (laronidase). J Pediatr 144:581–588

Yi S, Singh RH (2008) Protein substitute for children and adults with phenylketonuria. Cochrane Database Syst Rev 4, CD004731

JIMD Reports
DOI 10.1007/8904_2015_409

CASE REPORT

Two Novel Mutations in the *SLC25A4* Gene in a Patient with Mitochondrial Myopathy

I.M.L.W. Körver-Keularts · M. de Visser · H.D. Bakker · R.J.A. Wanders · F. Vansenne · H.R. Scholte · L. Dorland · G.A.F. Nicolaes · L.M.J. Spaapen · H.J.M. Smeets · A.T.M. Hendrickx · B.J.C. van den Bosch

Received: 20 May 2014 / Revised: 15 January 2015 / Accepted: 16 January 2015 / Published online: 03 March 2015

Abstract In a 28-year-old male with a mild mitochondrial myopathy manifesting as exercise intolerance and early signs of cardiomyopathy without muscle weakness or ophthalmoplegia, we identified two novel mutations in the *SLC25A4* gene: c.707G>C in exon 3 (p.(R236P)) and c.116_137del in exon 2 (p.(Q39Lfs*14)). Serum lactate levels at rest were elevated (12.7 mM). Both the patient's father and brother were heterozygous carriers of the c.707G>C mutation and were asymptomatic. The second mutation causes a 22 bp deletion leading to a frame shift likely giving rise to a premature stop codon and nonsense-mediated decay (NMD). The segregation of the mutations could not be tested directly as the mother had died before. However, indirect evidence from NMD experiments showed that the two mutations were situated on two different alleles in the patient. This case is unique compared to other previously reported patients with either progressive external ophthalmoplegia (PEO) or clear hypertrophic cardiomyopathy with exercise intolerance and/or muscle weakness carrying recessive mutations leading to a complete absence of the SLC25A4 protein. Most likely in our patient, although severely reduced, SLC25A4 is still partially present and functional.

Communicated by: Shamima Rahman

Competing interests: None declared

I.M.L.W. Körver-Keularts (✉) · L. Dorland · L.M.J. Spaapen
Department of Clinical Genetics, Lab of Biochemical Genetics, Maastricht University Medical Center, Maastricht, The Netherlands
e-mail: irene.keularts@mumc.nl

M. de Visser
Department of Neurology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

H.D. Bakker
Department of Paediatrics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

R.J.A. Wanders
Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

F. Vansenne
Department of Clinical Genetics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

H.R. Scholte
Department of Neuroscience, Erasmus Medical Center, Rotterdam, The Netherlands

G.A.F. Nicolaes
Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands

H.J.M. Smeets · A.T.M. Hendrickx · B.J.C. van den Bosch
Department of Clinical Genetics, Unit Clinical Genomics, Maastricht University Medical Center, Maastricht, The Netherlands

Introduction

ANT (adenine nucleotide transporter) is the most abundant protein located in the inner membrane of the mitochondria. It plays a crucial role in the exchange of ADP and ATP between the cytosolic and mitochondrial compartments and is therefore vital for the function of the oxidative phosphorylation (OXPHOS) system. Four isoforms of human ANT can be distinguished (Cozens et al. 1989; Dolce et al. 2005; Li et al. 1989; Ku et al. 1990; Houldsworth and Attardi 1988; Stepien et al. 1992). SLC25A4 (ANT1) is expressed in the heart and skeletal muscle. It has a 91–93% amino acid homology with SLC25A5 (ANT2), SLC25A6 (ANT3) and SLC25A31 (ANT4) of which the first two are expressed in almost all body tissues and the last in the liver, testis, and brain (Dolce et al. 2005; Neckelmann et al. 1987) that

Table 1 Primer sequences for amplification of *SLC25A4*

Exon	Length (bp)	Sequence forward primer	Sequence reverse primer
1	419	5′-CCTCCTCTCGCGAGAGC-3′	5′-GCCTGGCGCAGATTTTC-3′
2A	393	5′-GTCCTCTTCCCTTCTCTCTA-3′	5′-CCAACCTGGTCCTAGCAAAG-3′
2B	452	5′-GGCGCTACTTTGCTGGTAAC-3′	5′-GCACATCACCTCCTCATTCA-3′
3	445	5′-GCAAGGTCAGAGCATGGAG-3′	5′-GTTGAGAACGTTAGGGGGAAT-3′
4	485	5′-GTTGCATGGAGCTGGGACT-3′	5′-CTCAATGAAGCATCTCTTCTGA-3′
cDNA (c.25F-c.270R)	246	5′-CTAAAGGACTTCCTGGCCG-3′	5′-GGCGAAGTTGAGAGCTTG-3′
cDNA (c.533F-c.827R)	295	5′-ACGTCTCTGTCCAAGGCATC-3′	5′-GACCAGGCACCTTTGAAGAA-3′
M13 sequence	18	5′-TGTAAAACGACGGCCAGT-3′	5′-CAGGAAACAGCTATGACC-3′

characterized the cDNA of SLC25A4, which consists of 1,400 nucleotides. The *SLC25A4* gene is located on chromosome 4, spans 5.8 kb, and consists of 4 exons.

Mutations in *SLC25A4* are associated with the presence of multiple mtDNA deletions in different tissues (skeletal and heart muscle, brain, kidney, and liver) (Palmieri et al. 2005). Until now, five different autosomal dominant *SLC25A4* mutations have been described primarily associated with adPEO (autosomal dominant progressive external ophthalmoplegia) (Deschauer et al. 2005; Napoli et al. 2001; Komaki et al. 2002; Kaukonen et al. 2000). An autosomal recessively inherited mitochondrial myopathy and hypertrophic cardiomyopathy was described in two cases (Palmieri et al. 2005; Echaniz-Laguna et al. 2012).

Here, we describe two novel mutations in the *SLC25A4* gene in a patient first reported by Bakker et al. (1993a, b). In the patient (1) immunostaining of Western blots revealed a 4-fold decrease in the concentration of the adenine nucleotide translocator; (2) the activities of complexes I to V catalyzing oxidative phosphorylation and the pyruvate and the 2-oxoglutarate dehydrogenase complexes showed a 2- to 20-fold increase, (3) in the serum, lactate levels up to 12.7 mM at rest were reported; and (4) ^{31}P-nuclear magnetic resonance spectroscopy in the resting muscle showed half the creatine phosphate level as compared to controls. No ragged red fibers were observed in muscle, but electron microscopy revealed abnormal mitochondria. At the age of 28, he was still suffering from only mild exercise intolerance and was using a wheelchair for outdoor transportation. There were no signs of ophthalmoplegia and he only showed mild signs of hypertrophic cardiomyopathy.

Material and Methods

Molecular Studies

DNA Analysis

Total DNA was extracted from blood using the wizard genomic DNA purification kit (Promega, Leiden, The Netherlands) and from fibroblasts using the Puregene kit (Gentra, Minneapolis, Minnesota, USA). Specific intronic primers were designed to amplify the exons and flanking introns of *SLC25A4* (Table 1). All primers contained an additional M13 sequence. Exon 1 was amplified in a volume of 25 µl using 5 ng genomic DNA as template with 0.5 U KAPA2G Fast HotStart DNA polymerase, KAPA2G HotStart Buffer A, 5 mM dNTP-mix (KapaBiosystems, Massachusetts,USA), 3.75 pmol each primer, and 10% DMSO (Merck, Darmstadt, Germany). The reactions for exon 2, 3, and 4 were performed in a 10 µl volume using 5 ng genomic DNA as template, AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, USA), 2 pmol of both primers, and 8% glycerol (Invitrogen, Carlsbad, USA). PCR conditions for all the exons were as follows: first one cycle of 96°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s; and finally one cycle 72°C for 10 min. PCR products were directly sequenced with the PRISM Ready Reaction Sequencing Kit (Perkin-Elmer Life Sciences) on an ABI3100 DNA Analyzer (Applied Biosystems).

cDNA Analysis

The Tripure isolation reagent kit (Roche Applied Science, Mannheim, Germany) was used to extract total RNA from fibroblasts. All extractions were performed according to the instruction manuals. cDNA synthesis was performed by the SuperScript First-Strand Synthesis System for RT-PCR, using oligo(dT) (Invitrogen, Carlsbad, USA). The synthesis was performed according the instruction manual, except for the RNase H step, which was not performed. The PCR was performed in a 10 µl volume with 40 ng cDNA as template, using Phire Hot Start II PCR Master Mix (Finnzymes, Espoo, Finland), 3 pmol each primer, and 3% DMSO (Merck, Darmstadt, Germany) with PCR conditions according to the manual, with the exception of the number of PCR cycles and the annealing temperature, which were respectively 40 cycles and 60°.

Fibroblast Culture

Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM), high glucose, pyruvate, and glutamine (Invitrogen, Carlsbad, USA); 10% fetal bovine serum, 10 U/ml penicillin and 10 U/ml streptomycin; and 0.2 mM uridine in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. To investigate the process of nonsense-mediated decay (NMD) we cultured the cells in duplicate and added 200 µg/ml puromycin to the medium 5 h before harvesting the cells.

Results

Mutation Analysis

In our patient two novel *SLC25A4* mutations were identified. The first mutation is a heterozygous deletion c.116_137del in exon 2 (p.(Q39Lfs*14)), whereas the second mutation is a missense mutation c.707G>C (heterozygous) in exon 3 leading to the substitution of arginine at position 236 into a proline (p.(R236P)) as displayed in Fig. 1. Neither of the mutations were found in a control panel of the same ethnicity ($n = 114$), present in the HGMD® or in SNP databases like dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and the 1000 Genomes Project (Altshuler et al. 2010). In addition, they were not present in the exomes of 61,486 unrelated individuals (Exome Aggregation Consortium, ExAC; http://exac.broadinstitute.org). P.Arg236 is highly conserved among different species including *S. cerevisiae* (baker's yeast). The sequence which harbors the deletion c.116_137del in exon 2 (p.(Q39Lfs*14)) is also highly conserved (up to *Drosophila melanogaster*). Aminoacid alignments are also shown in Fig. 1. Both the patient's father and brother are heterozygous carriers of the c.707G>C mutation and are asymptomatic. We were unable to perform DNA analysis on the patient's mother since she died at 59 years of age from leukaemia.

Q39Lfs*14 Mutation Leads to Partial Nonsense-Mediated Decay in Fibroblasts

The p.(Q39Lfs*14) mutation is caused by a 22 bp deletion, predicted to lead to a frameshift with a premature stop codon. cDNA analysis in fibroblasts with and without puromycin, which suppresses NMD, was performed to test if this mutation would lead to NMD. The results confirm that NMD is at least partially involved.

Discussion

In this paper we report a patient with a severe reduction in *SLC25A4* gene expression with a fourfold decreased expression of SLC25A4 protein (Bakker et al. 1993a). We detected two novel mutations in the *SLC25A4* gene, a c.707G>C in exon 3 leading to the substitution of arginine at position 236 into a proline (p.(R236P)) and a c.116_137del in exon 2 (p.(Q39Lfs*14)).

The p.Arg236 is a highly conserved amino acid, located in the predicted transmembrane region (helix) of the protein involved in maintaining the conformation of the protein. The p.(R236P) mutation is likely to affect the folding of SLC25A4 and its function because proline is known to interfere with alpha-helix formation (Fig. 2). Moreover, previous experiments in yeast, in which position R253 (human SLC25A4 position R236) was substituted for isoleucine, transport function was disrupted (Nelson et al. 1993; Heidkämper et al. 1996). Also, studies in bovine showed that R254 (human SLC25A4 position R236) is the second arginine in the RRRMMM motif, which appears to take part in nucleotide binding (Pebay-Peyroula et al. 2003).

From the asymptomatic patient's father and brother who were carriers of this pathogenic mutation one may infer that the expression of functional SLC25A4 was still sufficient for a normal function (autosomal recessive inheritance). The 22 base pair deletion in exon 2, c.116_137del p.(Q39Lfs*14) leads to a frameshift with a new reading frame which introduces a stop codon at position 13. Both mutations were not found in controls. Our patient is most likely compound heterozygous resulting in little SLC25A4 functional rest activity as the explanation for the clinical signs of exercise intolerance and lactic acidosis. We demonstrated by NMD experiments (the level of mutated vs wild-type RNA levels in presence and absence of puromycin; data not shown) that both mutations are situated on different alleles (*trans* position) indicating that mutations had different origins (paternal/de novo or paternal/maternal) and suggests compound heterozygosity for the mutations. The deletion is most likely inherited from the mother, although this could not be tested as she had died before. Although it would have been interesting to study the presence of mtDNA deletions in muscle, as could be expected based on previous reports of SLC25A4 mutations (Komaki et al. 2002), this has not been performed at the time (Bakker et al. 1993a), and unfortunately no muscle tissue is available anymore. Unfortunately it is also not possible to re-biopsy the patient at this age.

Humans have different tissue-specific isoforms of ADP/ATP translocase (also known as adenine nucleotide translocator or ANT). SLC25A4 is a 60 kD protein encoded by

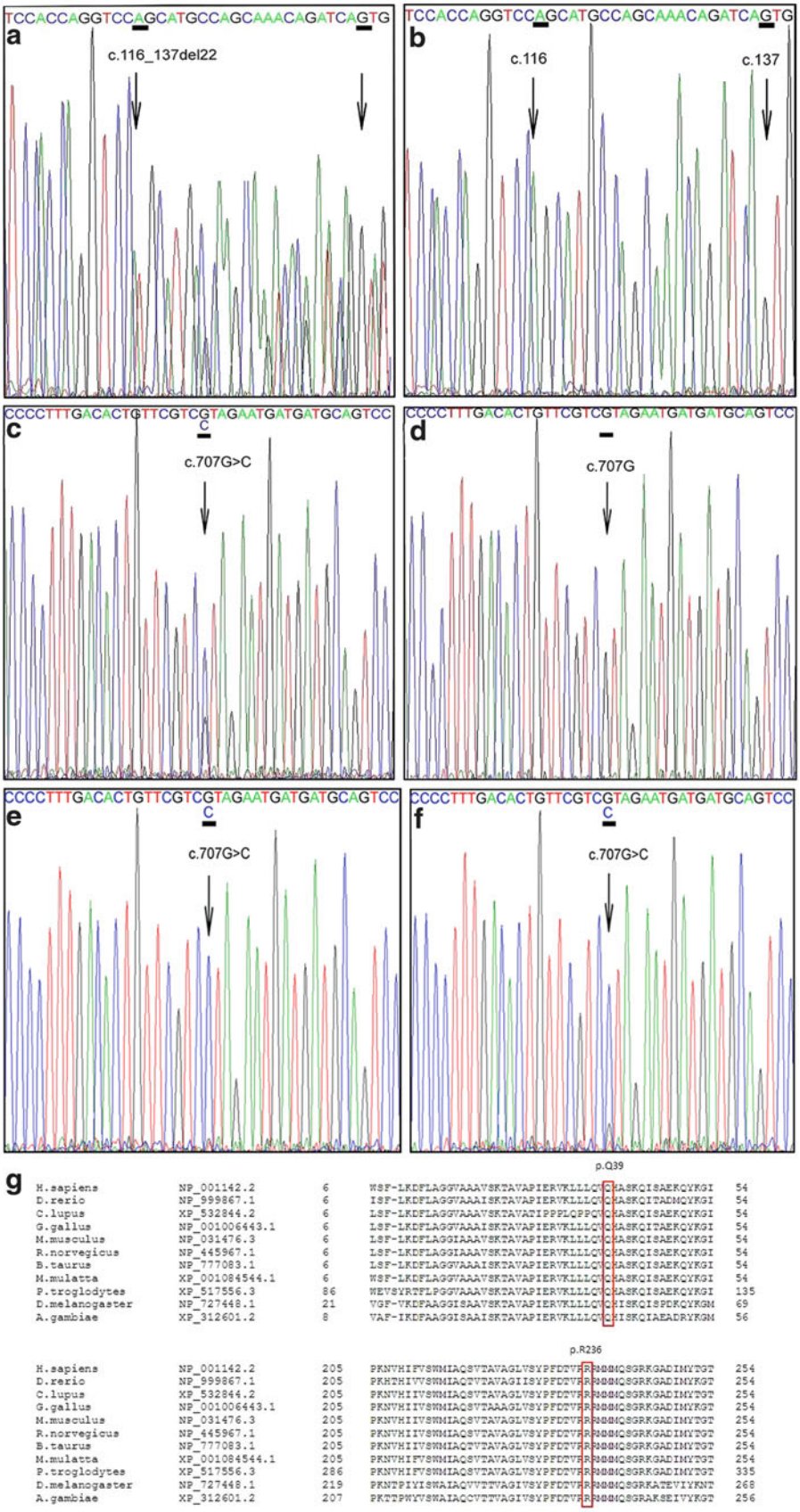


Fig. 1 The c.116_137del p.(Q39Lfs*14) and the c.707G>C p.(R236P) mutation. DNA from fibroblasts of the proband and DNA from blood of the control showing the c.116_137del mutation (A and B) and the c.707G>C mutation (C en D). cDNA from fibroblasts of

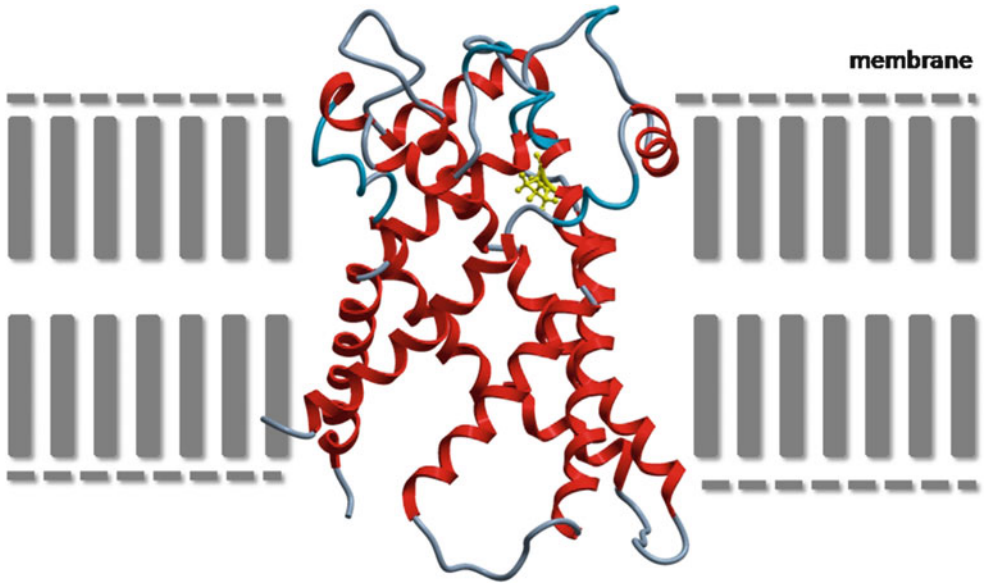


Fig. 2 Model for human SLC25A4. A homology model was built, employing the crystal structure of the mitochondrial ADP/ATP carrier (PDB accession file 1OKC.pdb, sequence identity 96%) with the molecular modeling package ICM Pro (Molsoft, Inc, La Jolla, USA) and was next optimized and minimized following standard parameters. Alpha-helices are depicted in *red*, other areas in *gray*. Location of mutated proline (*yellow*) in SLC25A4 protein is situated in the membrane

the *SLC25A4* gene and is highly expressed in the heart and skeletal muscle, whereas ANT3 is encoded by the *SL2C5A6* and is ubiquitously expressed. The main function of both SLC25A4 and ANT3 is the exchange of ADP and ATP across the inner mitochondrial membrane in order to maintain the cellular bioenergetic state. ANT therefore plays a central role in OXPHOS (Adrian et al. 1986; Klingenberg 1981, 1989; Fiore et al. 1998).

Dysfunction of the ATP/ADP transporter is expected to result in severe defects in OXPHOS, giving rise to considerable energy deficiency primarily in the cytosol and a compensatory induction in mitochondrial proliferation. In a knockout (KO) mouse model (Graham et al. 1997), it was demonstrated that mice lacking SLC25A4 did indeed show progressive hypertrophy of the heart, severe exercise intolerance, a fourfold resting lactate as compared to the wild-type mice, and mitochondrial abnormalities in skeletal muscle.

A patient with a homozygous recessive null mutation p.(A123M) in *SLC25A4* had a similar clinical manifestation, including hypertrophic cardiomyopathy, easy fatigability and exercise-related muscle pain, and episodes of headache associated with vomiting.

Muscle weakness and external ophthalmoplegia were conspicuously absent. Biochemically, lactic acidosis was present and the muscle biopsy showed ragged red fibers (Palmieri et al. 2005). The activities of mtDNA-dependent respiratory chain enzymes were partially reduced.

The other patient with a complete loss of expression of the *SLC25A4* gene due to homozygosity for a new nucleotide variation, c.111+1G>A p.(A123D), was found to have muscle weakness in the arms and legs when examined at age 21 years. Also, the patient showed hypertrophic cardiomyopathy and congenital cataracts and slight mental retardation (Echaniz-Laguna et al 2012). There was no ophthalmoplegia present. Biochemically she had lactic acidosis, ragged red fibers in muscle biopsy and normal activities of mitochondrial respiratory chain complexes.

The patient with a severe SLC25A4 deficiency described in this paper had increased OXPHOS complex activities in muscle as described by Bakker et al. (1993a). This increase was explained as a secondary effect due to the hyperproliferation of mitochondria or an enhanced synthesis of mitochondrial enzymes, which can be a common phenomenon in patients with mitochondrial myopathies and

Fig. 1 (continued) the proband cultured with puromycin, which inhibits NMD (F), shows that the signal of the wild-type nucleotide c.707G is relatively higher compared to cDNA from fibroblasts cultured without puromycin (E). Amino acid alignment of the human SLC25A4 reference sequence versus various species for position 6 to 54 and position 205 to 254. Indicated is the position of the p.(Q39Lfs*14) mutation and the p.(R236P) mutation. The arginine at position 236 is conserved in all species shown (G)

might be an attempt to compensate for the decreased function of individual mitochondria (Bakker et al. 1993a). From the great difference between the coupled and uncoupled respiration activities (in controls these activities were similar), it could be concluded that the patient has a severe defect in the mitochondrial phosphorylation, despite the increased OXPHOS activities. In addition, energy production from pyruvate plus malate was barely normal, further supporting the presence of a phosphorylation defect (Bakker et al. 1993a).

The major effect of SLC25A4 deficiency as demonstrated in SLC25A4-KO mice (with characteristics of a cardiomyopathy and mitochondrial myopathy) is a deficiency of ADP-stimulated but not uncoupled respiration defect resulting from the blockade of ADP/ATP exchange. This defect was most pronounced in muscular and less in heart tissue. In SLC25A4-KO mice, ANT2 could replace SLC25A4 and thus prevent premature heart failure.

At the time of measurement of SLC25A4 activity, no specific isoforms were distinguished during protein analysis (Bakker et al. 1993a). However, it was reported that preliminary results of a transcript analysis of isoform-specific probes showed a specific reduction of the muscle isoform in the patient versus controls (Bakker et al. 1993a). Although it seems unlikely that other isoforms are responsible for residual ANT activity based on these results, we cannot entirely exclude this possibility. We speculate that the patient had still sufficient residual SLC25A4 activity to sustain ATP supply and together with the apparent enhanced mitochondrial function was able to compensate for the defect and prevent transition to a more severe phenotype.

Mitochondria-generated reactive oxygen species which clearly contribute to worsening of the cardiomyopathy was demonstrated in SLC25A4-KO mice (Narula et al. 2011). The residual SLC25A4 activity in combination with the ANT3 backup function might have prevented an excessive ROS production; in addition, the patient described by Bakker et al. (1993b) benefitted from supplementation with the antioxidant vitamin E. Currently, however, the patient is not treated with antioxidants anymore.

In summary, our index patient with only a mild mitochondrial myopathy manifesting as exercise intolerance and early signs of hypertrophic cardiomyopathy did not develop muscle weakness, ophthalmoplegia or cerebellar symptoms over a period of 20 years. In this respect our case is unique as compared to those previously described. In light of this, the absence of ophthalmoplegia seems to contrast with dominant mitochondrial disease due to SLC25A4 mutations, which is also supported by the absence of opthalmoplegia in the patients described by Palmieri et al. (2005) and Echaniz-Laguna et al. (2012). The milder phenotype in our patient is likely correlated with the nature of the two novel recessive mutations which lead to a severely reduced yet still present and partially functioning SLC25A4 protein.

Synopsis

Two novel recessive *SLC25A4* mutations leading to a relatively mild phenotype without progressive external ophthalmoplegia.

Details of the Contributions of Individual Authors

IKK set up the SLC25A4 sequencing and wrote the paper. She is the guarantor. MdV provided medical information of the patient and wrote part of the paper. HB, HS, LD, LS, and RW were involved in the initial investigation and further characterization of the biochemical phenotype of the patient. FV was involved in the patient care. GN created models to assess the impact of the SLC25A4 mutations on protein structure. HS was involved in writing the paper. AH performed the NMD experiments and was involved in the setup of the SLC25A4 sequencing. BB wrote the paper and is also a guarantor. All authors have critically revised the paper.

Name of One Author Who Serves as Guarantor

Irene Körver-Keularts

Conflict of Interest

Irene Körver-Keularts, Marianne de Visser, Henk Bakker, Ronald Wanders, Fleur Vansenne, Jasper Scholte, Bert Dorland, Gerry Nicolaes, Leo Spaapen, Bert Smeets, Alexandra Hendrickx, and Bianca van den Bosch have no conflict of interest.

There are no competing interests.

The author(s) confirm(s) independence from the sponsors.

Compliance with Ethics Guidelines

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

References

Adrian GS, McCammon MT, Montgomery DL, Douglas MG (1986) Sequences required for delivery and localization of the ADP/ATP translocator to the mitochondrial inner membrane. Mol Cell Biol 6:626–634

Altshuler D, Durbin RM, Abecasis GR et al (2010) A map of human genome variation from population-scale sequencing. Nature 467:1061–1073

Bakker HD, Scholte HR, van den Bogert C et al (1993a) Deficiency of the adenine nucleotide translocator in muscle of a patient with myopathy and lactic acidosis: a new mitochondrial defect. Pediatr Res 33:412–417

Bakker HD, Scholte HR, van den Bogert C et al (1993b) Adenine nucleotide translocator deficiency in muscle: potential therapeutic value of vitamin E. J Inherit Metab Dis 16:548–552

Cozens AL, Runswick MJ, Walker JE (1989) DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase. J Mol Biol 206:261–280

Deschauer M, Hudson G, Müller T, Taylor RW, Chinnery PF, Zierz S (2005) A novel ANT1 gene mutation with probable germline mosaicism in autosomal dominant progressive external ophthalmoplegia. Neuromuscul Disord 15:311–315

Dolce V, Scarcia P, Iacopetta D, Palmieri F (2005) A fourth ADP/ATP carrier isoform in man: identification, bacterial expression, functional characterization and tissue distribution. FEBS Lett 579:633–637

Echaniz-Laguna A, Chassagne M, Ceresuela J et al (2012) Complete loss of expression of the ANT1 gene causing cardiomyopathy and myopathy. J Med Genet 49:146–150

Fiore C, Trezeguet V, Le Saux A et al (1998) The mitochondrial ADP/ATP carrier: structural, physiological and pathological aspects. Biochimie 80:137–150

Graham BH, Waymire KG, Cottrell B, Trounce IA, MacGregor GR, Wallace DC (1997) A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. Nat Genet 16:226–234

Heidkämper D, Müller V, Nelson DR, Klingenberg M (1996) Probing the role of positive residues in the ADP/ATP carrier from yeast. The effect of six arginine mutations on transport and the four ATP versus ADP exchange modes. Biochemistry 35:16144–16152

Houldsworth J, Attardi G (1988) Two distinct genes for ADP/ATP translocase are expressed at the mRNA level in adult human liver. Proc Natl Acad Sci USA 85:377–381

Kaukonen J, Juselius JK, Tiranti V et al (2000) Role of adenine nucleotide translocator 1 in mtDNA maintenance. Science 289:782–785

Klingenberg M (1981) Membrane protein oligomeric structure and transport function. Nature 290:449–454

Klingenberg M (1989) Molecular aspects of the adenine nucleotide carrier from mitochondria. Arch Biochem Biophys 270:1–14

Komaki H, Fukazawa T, Houzen H, Yoshida K, Nonaka I, Goto Y (2002) A novel D104G mutation in the adenine nucleotide translocator 1 gene in autosomal dominant progressive external ophthalmoplegia patients with mitochondrial DNA with multiple deletions. Ann Neurol 51:645–648

Ku DH, Kagan J, Chen ST, Chang CD, Baserga R, Wurzel J (1990) The human fibroblast adenine nucleotide translocator gene. Molecular cloning and sequence. J Biol Chem 265:16060–16063

Li K, Warner CK, Hodge JA et al (1989) A human muscle adenine nucleotide translocator gene has four exons, is located on chromosome 4, and is differentially expressed. J Biol Chem 264:13998–14004

Napoli L, Bordoni A, Zeviani M et al (2001) A novel missense adenine nucleotide translocator-1 gene mutation in a Greek adPEO family. Neurology 57:2295–2298

Narula N, Zaragoza MV, Sengupta PP et al (2011) Adenine nucleotide translocase 1 deficiency results in dilated cardiomyopathy with defects in myocardial mechanics, histopathological alterations, and activation of apoptosis. JACC Cardiovasc Imaging 4:1–10

Neckelmann N, Li K, Wade RP, Shuster R, Wallace DC (1987) cDNA sequence of a human skeletal muscle ADP/ATP translocator: lack of a leader peptide, divergence from a fibroblast translocator cDNA, an coevolution with mitochondrial DNA genes. Proc Natl Acad Sci USA 84:7580–7584

Nelson DR, Lawson JE, Klingenberg M, Douglas MG (1993) Site-directed mutagenesis of the yeast mitochondrial ADP/ATP translocator. Six arginines and one lysine are essential. J Mol Biol 230:1159–1170

Palmieri L, Alberio S, Pisano I et al (2005) Complete loss-of-function of the heart/muscle-specific adenine nucleotide translocator is associated with mitochondrial myopathy and cardiomyopathy. Hum Mol Genet 14:3079–3088

Pebay-Peyroula E, Dahout-Gonzalez C, Kahn R, Trézéguet V, Lauquin GJ, Brandolin G (2003) Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. Nature 426:39–44

Stepien G, Torroni A, Chung AB, Hodge JA, Wallace DC (1992) Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. J Biol Chem 267:14592–14597

JIMD Reports
DOI 10.1007/8904_2015_410

CASE REPORT

Combined Sepiapterin Reductase and Methylmalonyl-CoA Epimerase Deficiency in a Second Patient: Cerebrospinal Fluid Polyunsaturated Fatty Acid Level and Follow-Up Under L-DOPA, 5-HTP and BH4 Trials

Michel Mazzuca · Marie-Anne Maubert · Léna Damaj · Fabienne Clot · Marylène Cadoudal · Christele Dubourg · Sylvie Odent · Jean François Benoit · Nadia Bahi-Buisson · Laurence Christa · Pascale de Lonlay

Received: 14 October 2014 / Revised: 16 January 2015 / Accepted: 19 January 2015 / Published online: 13 March 2015

Abstract *Objective/context*: We describe the second patient presenting the combination of two homoallelic homozygous nonsense mutations in two genes distant from 1.8 Mb in the chromosome 2p13-3, the methylmalonyl-CoA epimerase gene (*MCEE*) and the sepiapterin reductase gene (*SPR*).

Case report: The patient was born from consanguineous parents. He has presented a moderate but constant methylmalonic acid (MMA) excretion in urine associated with a mental retardation. The first homozygous mutation was identified in the *MCEE* gene (c.139C>T; p.Arg47*). Progressive dystonia and cataplexy narcolepsy led to diagnose the second homozygous mutation in the *SPR* gene: c.751A>T; p.Lys251*. Sepiapterin reductase deficiency (SRD) was characterized by a defect in tetrahydrobiopterin (BH4), the cofactor of several hydroxylases needed for the synthesis of neurotransmitters. A treatment with L-DOPA/carbidopa and 5-HTP dramatically improved the dystonic posture, the mood and the hypersomnia, proving that the pathogenesis was due to SRD. A supplementation with BH4 did not induce additional clinical benefit, although HVA and HIAA increased in CSF. The polyunsaturated fatty acids were measured in CSF as the markers of the neuronal stress. We have shown that DHA and its precursor EPA were high before and during the time course of the different treatments.

In conclusion: The patient has inherited two copies of the two mutations from his consanguineous parents in the *MCEE* and *SPR* genes in the chromosome 2p13-3. DHA and EPA increased in CSF as a response to the neuronal stress induced by the defect in neurotransmitters or the altered metabolism of the odd-chain fatty acids and cholesterol.

Communicated by: Nenad Blau, PhD

Competing interests: None declared

M. Mazzuca · N. Bahi-Buisson · P. de Lonlay
Neurometabolism Department, Reference Center for Inherited Metabolic Diseases, Assistance Publique des Hôpitaux de Paris et Université, Necker, Paris, France

M.-A. Maubert
Assistance Publique des Hôpitaux de Paris et Université Saint-Antoine, Biochemistry Laboratories, Paris, France

L. Damaj · S. Odent
Neurometabolism Department, Reference Center for Inherited Metabolic Diseases, Rennes Hospital, Rennes, France

F. Clot
Assistance Publique des Hôpitaux de Paris et Université, La Pitié Salpétrière-Charles Foix, Molecular and Cellular Neuro Genetic Department, Paris, France

C. Dubourg
Rennes Hospital, Molecular and Cellular Neuro Genetic Department, Rennes, France

J.F. Benoit
Assistance Publique des Hôpitaux de Paris et Université, Robert Debré, Biochemistry Laboratories, Paris, France

M. Cadoudal · L. Christa (✉)
Assistance Publique des Hôpitaux de Paris et Université Necker, Biochemistry Laboratories, Paris, France
e-mail: Laurence.christa@parisdescartes.fr

Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine/serotonin
5-HTP	5-Hydroxytryptophan

BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
BP	Biopterin
CSF	Cerebrospinal fluid
DA	Dopamine
DHA	Docosahexaenoic acid (22:6n-3)
EPA	Eicosapentaenoic acid (20:5n-3)
GC-MS	Gas chromatography-mass spectrometry
HGH	Human growth hormone
HVA	Homovanillic acid
L-DOPA	3,4-Dihydroxyphenylalanine
MCEE	Methylmalonyl-CoA epimerase gene
MMA	Methylmalonic acid
MTHF	Methyltetrahydrofolate
NOS	Nitric oxide synthase
OMD	3-Orthomethyl-Dopa or 3-methoxytyrosine
PUFA	Polyunsaturated fatty acid
RV	Reference value
SPR	Sepiapterin reductase gene
SRD	Sepiapterin reductase deficiency

Introduction

Sepiapterin reductase (*SPR* MIM 612716) and methylmalonyl-CoA epimerase (*MCEE* MIM 608419) genes are mapped on chromosome 2p13-3, 1.8 Mb one of the other (Fig. 1). Catabolic pathways for the amino acids isoleucine, valine, methionine and threonine as well as for the odd-chain fatty acids and cholesterol proceeded via propionyl-CoA which is converted to (2S)-methylmalonyl-CoA by the propionyl-CoA carboxylase. Methylmalonyl-CoA is then isomerized into its (2R)-enantiomer by the action of the MCEE enzyme. In contrast to the (2R)-methylmalonyl-coenzyme A ([2R]-methylmalonyl-CoA) mutase (MIM 609058) deficiency which leads to a high excretion of methylmalonic acid, MCEE deficiency is responsible for a mild increase of methylmalonic aciduria, probably because of an in vivo shunt from the propionate-to-succinate pathway. Mutations in the *MCEE* gene were described in only five patients, identified by a moderate methylmalonic acid excretion in urine (Gradinger et al. 2007).

On the contrary, sepiapterin reductase deficiency (SRD) is a severe autosomal recessive disorder of tetrahydrobiopterin (BH4) metabolism (Friedman et al. 2012). BH4 is synthesized de novo from guanosine triphosphate (GTP) by three enzymatic steps: GTP cyclohydrolase I, 6-pyruvoyl-tetrahydrobiopterin synthase and the SPR enzyme to reduce 6-pyruvoyl-tetrahydropterin into BH4. BH4 is an essential cofactor required by phenylalanine, tyrosine and tryptophan hydroxylases which catalyse the rate-limiting steps in the biosynthesis of neurotransmitters, dopamine and serotonin, respectively. A defect in BH4 explains the neurological features. Most of the patients present symptoms in the first years of age, but the diagnosis is delayed when neurotransmitters in CSF are missed. Commonly, patients exhibit progressive psychomotor retardation, tremor, seizures, oculogyric crises and notably dystonia with diurnal fluctuations (Clot et al. 2009). Sleep disorders and marked hypersomnolence are also described (Friedman et al. 2006). To date, 13 different mutations for 44 subjects have been reported in the database (http://www.biopku.org) (Koht et al. 2014). SRD is a potentially treatable inborn error of pterin metabolism with a response to L-DOPA/carbidopa and 5-hydroxytryptophan (5-HTP).

Polyunsaturated long-chain fatty acids (PUFAs) were analysed in CSF as markers of neuronal deterioration (Engstrom et al. 2009) (Fig. 2). Here, we present the second patient described with combined SRD and MCEE deficiencies (Abeling et al. 2006; Bikker et al. 2006). Permanent but moderate methylmalonic aciduria led to firstly identify MCEE deficiency. A progressive neurological deterioration with a global hypotonia and hypersomnia led to secondarily diagnose SRD with neurotransmitters in CSF.

Methods

Neurotransmitter Analysis in CSF

Biogenic amines, pterins, methyltetrahydrofolate and sepiapterin were analysed as previously described (Ormazabal et al. 2005; Zorzi et al. 2002).

Molecular Analysis

DNA was extracted from the white blood cells of the patient and his parents, after the informed consent. After the amplification, all the exons of the *SPR* and *MCEE* genes were sequenced and compared to the reference sequences (Big Dye TM Terminator v3.0 kit, cycle sequencing on the ABI 3130, Applied Biosystems Forster City, CA). Karyotype and sub-telomeric MLPA (kit SALSA P036) were used for genetic investigations.

GC-MS PUFA Analysis

Total lipids were extracted from CSF (100 µl) by 10 ml of chloroform/methanol (1:2 v/v) (Bligh and Dyer 1959). The chloroform lipid-rich lower layer was evaporated to dryness. Dried lipids were derivatized to fatty acid methyl esters with 6 ml of methanol/sulphuric acid 2% (v/v) for 2 h at 70°C and then extracted by hexane. The extract (2 µl) was separated and quantified by GC-MS (Trace DSQ2, Thermo Electron, Les Ulis, France) using the

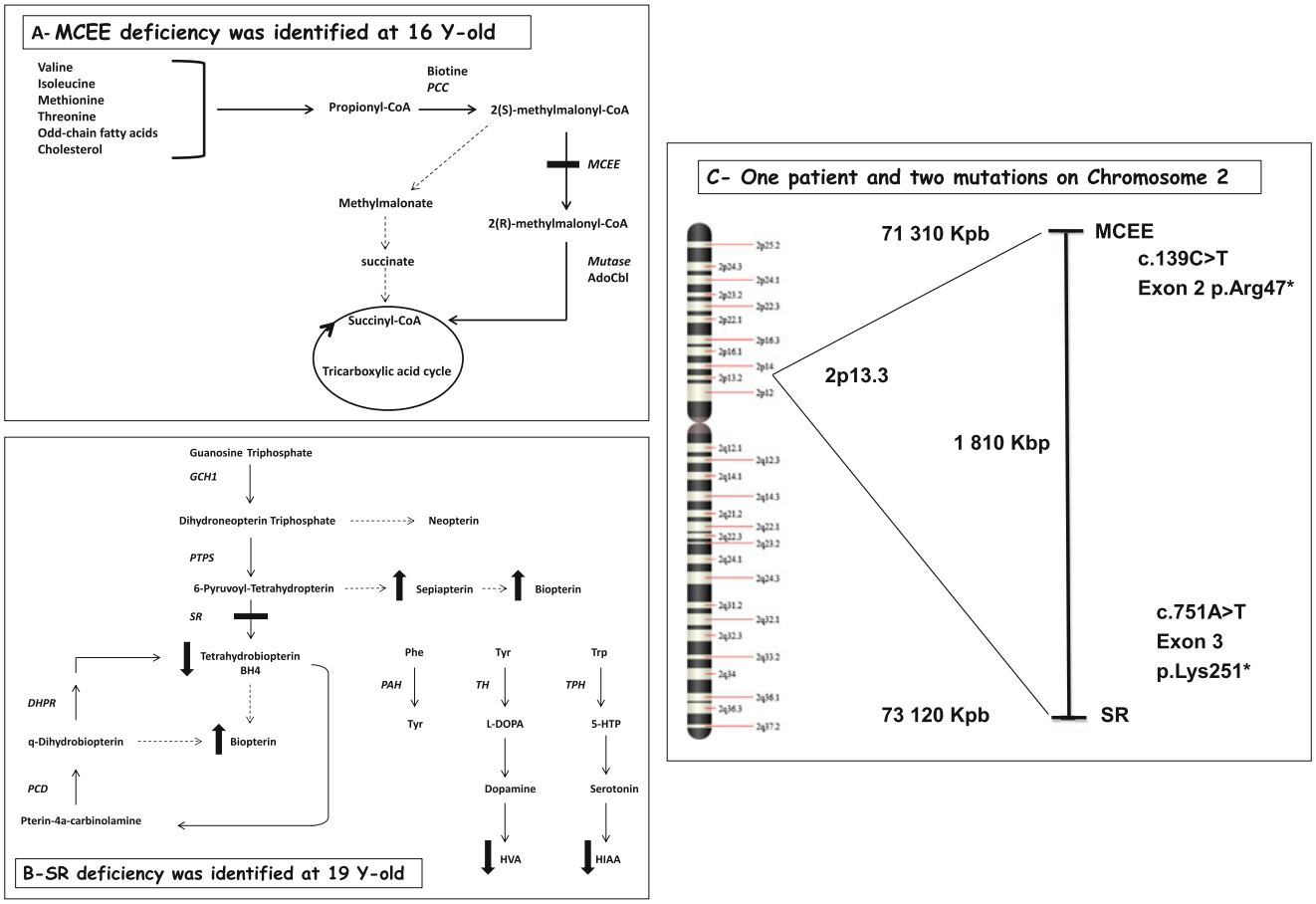


Fig. 1 (**a**) The propionate-to-succinate pathway, propionyl-CoA carboxylase (PCC), 5′-deoxyadenosylcobalamin (AdoCbl); (**b**) metabolism of the BH4 cofactor, GTP cyclohydrolase I (GCH1), dihydropteridine reductase (DHPR), 6-pyruvoyl tetrahydropterin synthase (PTPS), pterin 4α-carbinolamine dehydratase (PCD), phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH); (**c**) localization of the SR and MCEE genes on chromosome 2. The *hatched line* represents a non-enzymatic step or an unknown shunt pathway

positive chemical ionization mode. Fatty acid methyl esters ($n = 17$) were presently detected. The data were analysed using *Qual Browser*® software (Xcalibur® version 2.0.7, Thermo Electron, Les Ulis, France). The results were statistically analysed with a comparison of a single case to control normative samples by developing the Bayesian approach (Crawford and Garthwaite 2007).

Case Story

The patient was the second son born from French consanguineous parents. Two cousins presented methylmalonyl-CoA deficiency with neonatal coma at birth. At the age of 12 months, renal cysts led to the surgical ablation of the patient's left kidney. From the first month of life, he was a floppy baby with many episodes of eyes rolling up. He walked at the age of 21 months. Cognitive impairment with an intelligence quotient of 55 and limited speech were noted at the age of 5. At the age of 7, axial hypotonia, postural instability, oculogyric crisis and fatigability with sleep disorders were persistent. A moderated but persisting excretion of urinary methyl malonic acid was identified as soon as the age of 9 (60 µmol/mmol creatinine/mmol creatinine; rv <2). C^{14} propionate incorporation into macromolecules was slightly decreased, suggesting a defect in the propionate-to-succinate pathways (data not shown). However, the methylmalonic-CoA mutase activity was normal.

From the age of 7 to 18, the patient presented hypersomnolence requiring daily long sleeps. The circadian rhythm was distorted with an ultradian sleep-wake activity. The abnormal movements induced dystonia with peripheral hypertonia worsening at the evening. The neurological deteriorations progressively induced limb blockades and abnormal eye movements. The boy became wheelchair-bound at the age of 16. Cystic thyroid nodules led to thyroidectomy. The brain MRI was normal. X-linked

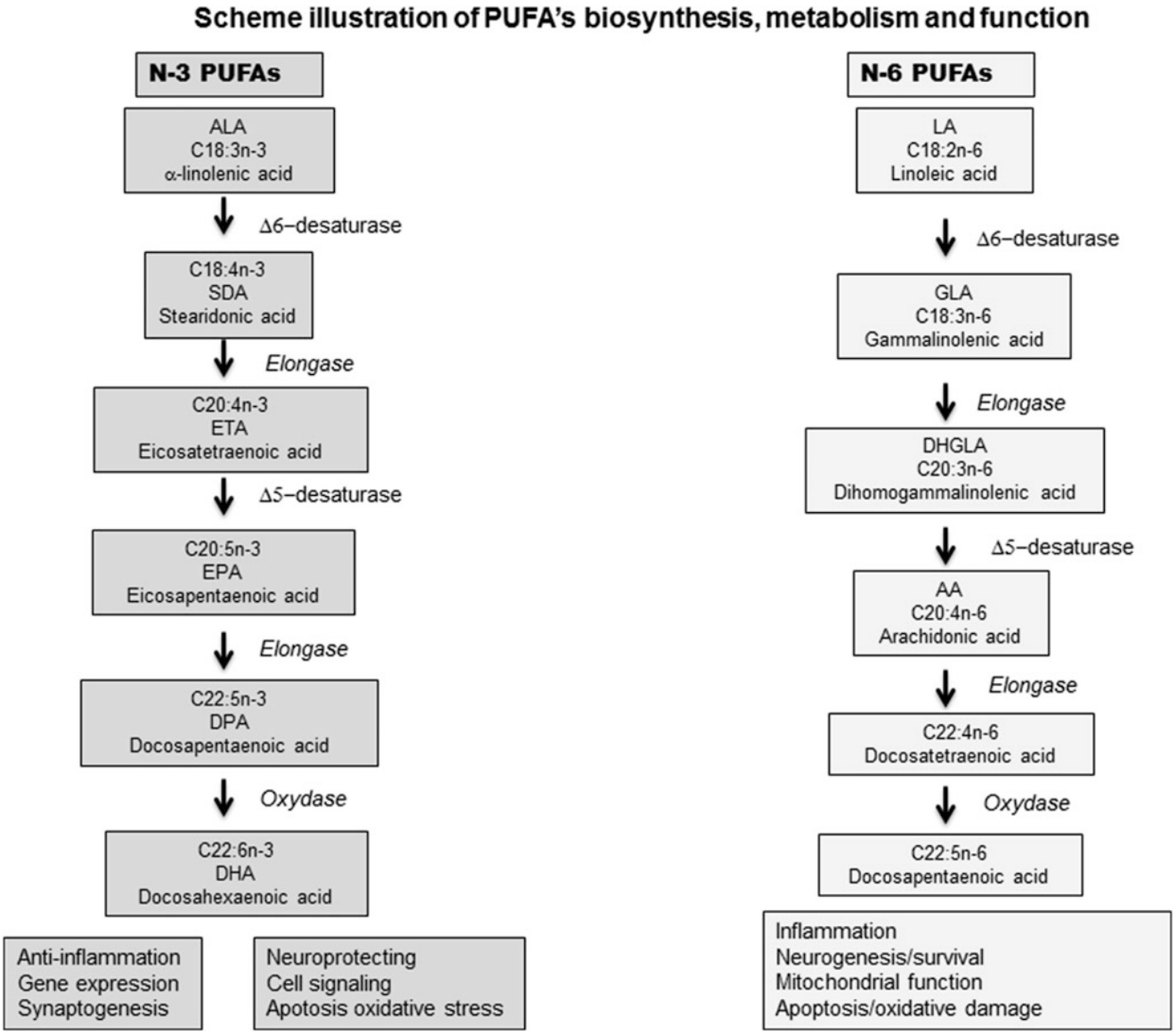


Fig. 2 Scheme illustrating PUFA biosynthesis, metabolism and function. PUFA can be provided directly by the diet or can be synthesized from their respective essential dietary precursors, α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6). For example, DHA (22:6w3) is composed of 22 carbon atoms and six double bonds, and its upstream metabolic precursor is EPA (20:5w3). Because the first double bond, as counted from the methyl terminus, is at position 3, they belong to the omega-3 group. Two key enzymes, the Δ6 and Δ5 desaturases, catalyse the desaturation and elongation of ALA and LA in the endoplasmic reticulum and in one terminal cycle of β-oxidation in the peroxisome

mental retardation, Smith-Magenis and Willi-Prader syndromes, chromosomal rearrangement and CGH array abnormalities were excluded. At the age of 16, a homozygous nonsense mutation in the *MCEE* gene, c139C>T inducing an early terminating signal (p.R47*) in the MCEE enzyme, led to identify MCEE deficiency, an unclassified form of methylmalonic aciduria. Both parents were heterozygous for the mutation and excreted normal amounts of methylmalonic acid. The supplementations with carnitine 4 g/day (Levocarnil®), oral hydroxocobalamin 1 mg/day and the antiepileptic sodium valproate up to 30 mg/kg/day did not improve the clinical evolution. The hypotonia, the psychomotor retardation and the fatigability were not considered to be suggestive of a neurotransmitter defect. At the age of 19, the sleep disturbances led to investigate long-term EEG and CSF neurotransmitters. A long-duration video-EEG sleep study (data not shown) clearly showed some asymptomatic spike wave discharges and an ultradian sleep-wake rhythm. The rapid eye movement sleeps represented more than 50% of the total sleep activity, and cataplexic narcolepsy was identified. Orexin A/hypocretin level was normal in CSF. A light elevation of prolactin

Table 1 CSF neurotransmitter analysis before SRD diagnosis and during the time course of the different treatments. Biogenic amines, pterins and MTHF were analysed in CSF from 4 successive lumbar punctures: (1) before SRD diagnosis, (2) 1 year after L-DOPA/5-HTP therapy, (3) 4 months after the combined therapy of L-DOPA/5-HTP and BH4 (5 mg/kg/day) and (4) 4 months after the combined therapy of L-DOPA/5-HTP and BH4 (20 mg/kg/day). The MTHF level has decreased from the beginning of the treatment by L-DOPA because no Lederfolin was introduced. Supplementation by Lederfolin was then introduced

Values (nM)	At the time of SRD diagnosis	1 year after L-DOPA/ 5-HTP therapy	4 months after L-dopa/5-HTP and BH4 5 mg/kg/day	4 months after L-DOPA/5-HTP and BH4 20 mg/kg/day	Reference values
5-HTP	2	141	10	5	3–12
HIAA	7	16	**26**	**29**	63–185
3-OMD	3	192	288	251	3–54
HVA	64	78	**129**	**123**	156–410
MHPG	2	14	11	16	11–46
Neopterin	25	19	37	28	10–24
Biopterin	51	37	73	54	14–36
Sepiapterin	15	nd	nd	nd	0
MTHF	75	62	56	39	>44

level (31 ng/ml; normal range 2–20) was noted. We measured the low levels of HIAA and HVA associated with a high level of BP in CSF. We also quantified sepiapterin (15 nM) which is the specific marker for SRD, and thus, we have confirmed the diagnosis of SRD, explaining the aetiology of cataplexic narcolepsy (Table 1 and Fig. 1b). The homozygous nonsense mutation was identified in the *SR* gene c.751A>T and led to a truncated protein (p.Lys251*). The parents were also both heterozygous for this mutation. L-DOPA/carbidopa/benserazide 1.5 mg/kg/day (Modopar®, Sinemet®) and 5-HTP (Levotonine® 0.75 mg/kg/day) were gradually increased to 100 mg/day and were combined with sertraline (50–150 mg/day) and selegiline (5–10 mg/day). The treatment induced spectacular clinical improvements in respect to dystonia, fatigability and sleep. From the wheelchair condition, the boy was able to walk and practise sports. Circadian sleep-wake rhythm was restored. Despite a good response with an improvement in motor ability and in mood, he has continued to have learning difficulties with drug-induced mild dyskinesia. The treatment led to a gradual increase in the levels of HVA and HIAA which however did not reach the normal range after 18 months of treatment (Table 1). In SRD, the initial defect was the absence of BH4 with a low BH4/BH2 ratio. We have introduced BH4 (Kuvan® or sapropterin dihydrochloride) in addition to L-DOPA/5-HTP therapy. After the first dose of 5 mg/kg/day for 4 months followed by a lumbar puncture, the second dose reached 20 mg/kg/day for a further 4 months followed by the last lumbar puncture. HIAA and HVA increased by 60% under BH4 (Table 1). However, the treatment by BH4 was stopped since no additional clinical benefits were objectively noted. At the age of 20, the atonic episodes disappeared and he was self-sufficient, but he remained highly fatigable, not being able to work. However, the drug induced dyskinesia of the mouth and of the hands.

PUFA Analysis

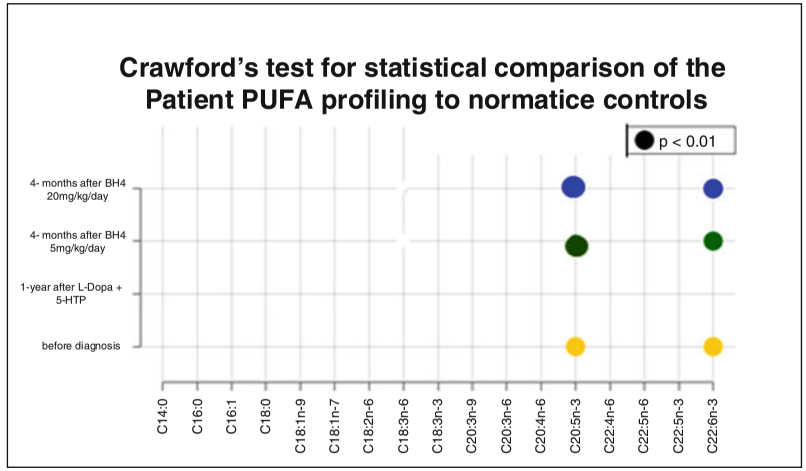
The plasma and erythrocyte PUFA levels were in the normal range (data not shown). The PUFA analysis has been set in CSF, and the reference values have been established in controls, excluding patients with neurotransmitter or BH4 defects. A statistical test comparing a single case to normative samples allowed us to find that DHA and EPA increased in 4/4 lumbar punctures of the patient, with a statistical significance in 3/4 ($p < 0.01$) and a trend for 1/4 lumbar punctures (Table 2). The other n-3 or n-6 fatty acids were never significantly different from controls. However, the levels of DHA and EPA did not normalize under different treatments.

Discussion

Our patient has turned out to be homozygous for two different autosomal recessive disorders, i.e. *MCEE* deficiency and *SRD*. Both diseases are extremely rare. The consanguineous parents were heterozygous for both mutations. One coincidence was the fact that the two genes are mapped in the same region of the chromosome 2p13-3 and distant for only 1.8 Mb. More surprisingly, our patient was the second patient identified with combined *MCEE* and *SPR* mutations: the *MCEE* gene mutations were similar, whereas the mutations in the *SR* gene were different (Abeling et al. 2006; Bikker et al. 2006). The possibility of a contiguous gene syndrome was ruled out by CGH

Table 2 PUFAs were analyzed in CSF obtained from four successive lumbar punctures: (1) before the SRD diagnosis, (2) 1 year after L-DOPA/5-HTP therapy, (3) 4 months after the combined therapy by L-DOPA/5-HTP and BH4 (5 mg/kg/day), and (4) 4 months after the combined therapy by L-DOPA/5-HTP and BH4 (20 mg/kg/day)

	Patient				Controls $n = 11$	
PUFAs (%)	Before SRD diagnosis	1 year after L-DOPA +5-HTP	4 months after L-DOPA/5-HTTP and BH4 5 mg/kg/day	4 months after L-DOPA/5-HT and BH4 20 mg/kg/day	Mean	SD
C14:0	1.05	1.35	1.39	1.41	2.36	1.11
C16:0	29.89	28.93	28.35	32.32	30.98	5.99
C16:1	3.43	2.3	2.77	2.22	4.47	2.26
C18:0	22.12	25.39	23.41	24.97	19.95	4.08
C18:1 n-9	24.65	27.23	26.18	22.85	26.18	4.45
C18:1 n-7	5.1	2.94	3.38	3.37	4.32	1.42
C18:2 n-6 LA	6.45	7.69	7.83	5.98	8.11	2.95
C18:3 n-6	0.003	0.01	0.04	0.04	0.04	0.03
C18:3 n-3 ALA	0.18	1.23	0.24	0.15	0.51	0.46
C20:3 n-9	0.06	0.05	0.05	0.05	0.06	0.02
C20:3 n-6	0.28	0.1	0.29	0.27	0.17	0.09
C20:4n-6 ARA	2.71	1.15	2.62	2.75	1.51	0.66
C20:5 n+3 EPA	**0.08***	**0.03**	**0.07***	**0.06***	**0.02**	**0.01**
C22:4 n-6	0.32	0.11	0.31	0.3	0.22	0.18
C22:5 n-6	0.34	0.56	0.23	0.61	0.51	0.29
C22:5 n-3	0.12	0.05	0.12	0.11	0.08	0.07
C22:6 n-3 DHA	**3.19***	**0.92**	**2.73***	**2.53***	**0.53**	**0.27**



Significance was analysed by Crawford's test: EPA and DHA $p < 0.01$ was indicated by circles

Fatty acids of the main interest are in bold. DHA (C22 6n-3) and EPA (C20:5n-3) were significantly higher in the patient than in controls. Crawford's test was used for statistical comparison of the patient PUFA profiling to normative controls: EPA and DHA $p < 0.01$ was indicated by*

array. Uniparental disomy was excluded since both parents were heterozygous for the two mutations. However, their union was consanguineous, and the closer is the parental relationship, the greater is the risk of the child inheriting 2 copies of a deleterious gene mutation from his parents (Kearney et al. 2011). The mild methylmalonic aciduria and the decrease in [14C] propionate incorporation were attributed to the *MCEE* gene mutation. Five other cases were reported without clinical feature (Gradinger et al. 2007). A functional role for *MCEE* or epimerase has been debated in humans (Montgomery et al. 1983). For our patient, the *MCEE* defect has no clinical effect and the treatment by vitamins was inefficient. So MCEE deficiency can be considered as a genetic variant. In contrast, the establishment of the SRD fully explained the neurological picture. All the clinical symptoms resulting from the low production of dopamine and serotonin in SRD dramatically improved under treatment by L-DOPA and 5-HTP. The dystonia has regressed; the excessive sleepiness and the narcolepsy were suppressed. The addition of BH4 led to an increase in HVA and HIAA by 60%. The amount of BH4 being able to cross the uninjured brain blood barrier is still under debate. SRD is characterized by a high level of BH2 and a low level of BH4. However, we have only measured BP which is the sum of BH4 and BH2. In these conditions, we could not interpret the increment due to BH4. Finally, the clinical benefits did not objectively improve and BH4 supplementation was stopped. To further investigate the neuronal deterioration of our patient, we have pointed out that the PUFA status in CSF represented a marker of its neuronal stress. We have shown that the levels of DHA and its precursor EPA, but not the other n-3 or n-6 PUFA, increased in CSF. The only study of fatty acid levels in CSF was recently reported in Alzheimer's patients (Fonteh et al. 2014). An increase in the PLA2 activity has liberated DHA from the breakdown of the neuronal lipid membrane and was related to the abnormal oxidative metabolism (Fonteh et al. 2013). The Alzheimer disorder is a neurodegenerative disorder thus radically different in terms of pathogenesis and genetic background. Thus, our results represented the first study of CSF PUFA status in an inherited metabolic disease. Some further investigations will be necessary to elucidate whether the increase in DHA was a common response to a neuronal stress, itself coming from either the odd-chain fatty acids and the cholesterol metabolism or the neurotransmitter disorders.

The diet has been reported to modulate the brain PUFA level and consequently biogenic amine metabolism (Lavialle et al. 2008; Jiang et al. 2009). The high level of EPA and DHA in red blood cells is associated with a slowed hippocampal and overall brain atrophy in humans (Pottala et al. 2014). A supplementation with DHA and EPA has been reported to enhance memory and cognitive function though DHA treatment remains somewhat unproven (Bauer et al. 2014). However, the peripheral status of PUFA in blood was normal in our patient. Thus, the high level of DHA in CSF could better result from a remodelling of PUFA metabolism in the brain. DHA mainly esterifies the brain and the retina phospholipids and thus plays a structural role for the membranes (Kim 2007). DHA is also the precursor for bioactive signalling molecules such as neuroprotectin D1, identified as a neuroprotective mediator to counteract neuronal apoptosis (Bazan 2006; Hong et al. 2014). DHA is thus considered to be essential for proper neuronal development and function.

Conclusion

We have reported the second case with the double *MCEE/SPR* homozygous mutations: the MCEE mutation was similar, but the *SPR* mutations were different. Our observation underlined that the pathogenesis was not due to the *MCEE* variant, but to the SRD which induced a defect in BH4 and consequently a defect in neurotransmitter metabolism. Indeed, the L-DOPA/carbidopa and 5-HTP treatment dramatically improved the clinical outcome. The supplementation with BH4 did not induce additional clinical benefits, although the level of HVA and HIAA increased in CSF. The high level of DHA and EPA in CSF probably constituted a marker in response to its neuronal stress.

Acknowledgements We thank Clotilde Robin for correcting the English language. Claude Wolf for helpful discussions about PUFA and Rafel Laboissiere for statistical advising.

Take-Home Message

We present a complete description of the medical and the genetic history for the patient with a combination of the two rare MCEE/SPR homozygous mutations, and we present the first investigation of the level of DHA and EPA in CSF for metabolic diseases.

Disclosure

The authors have nothing to disclose.

Compliance with Ethics Guidelines

Conflict of Interest

Michel Mazzuca, Marie-Anne Maubert, Léna Damaj, Fabienne Clot, Marylène Cadoudal, Christele Dubourg, Sylvie Odent, Jean François Benoit, Nadia Bahi-Buisson, Laurence Christa and Pascale de Lonlay declare that they have no conflict of interest.

Informed Consent

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

Details of the Contributions of Individual Authors

For medical care of the patient: Michel Mazzuca, Léna Damaj, Nadia Bahi-Buisson and Pascale de Lonlay were the referring physicians.

For biochemical analysis: Marie-Anne Maubert (PUFA analysis) and Marylène Cadoudal and Laurence Christa (neurotransmitter analysis) designed and performed the experiments.

For molecular genetic analysis: Fabienne Clot, Christele Dubourg, Sylvie Odent and Jean François Benoit designed and performed the experiments.

Conduct and reporting of the work described in the article: Michel Mazzuca, Sylvie Odent, Laurence Christa and Pascale de Lonlay analysed the data and wrote the manuscript.

All these authors equally contributed to this work.

References

Abeling NG, Duran M, Bakker HD, Stroomer L, Thony B, Blau N, Booij J, Poll-The BT (2006) Sepiapterin reductase deficiency an autosomal recessive DOPA-responsive dystonia. Mol Genet Metab 89:116–120

Bauer I, Crewther S, Pipingas A, Sellick L, Crewther D (2014) Does omega-3 fatty acid supplementation enhance neural efficiency? A review of the literature. Hum Psychopharmacol 29:8–18. doi:10.1002/hup.2370

Bazan NG (2006) Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. Trends Neurosci 29:263–271. doi:10.1016/j.tins.2006.03.005

Bikker H, Bakker HD, Abeling NG, Poll-The BT, Kleijer WJ, Rosenblatt DS, Waterham HR, Wanders RJ, Duran M (2006) A homozygous nonsense mutation in the methylmalonyl-CoA epimerase gene (MCEE) results in mild methylmalonic aciduria. Hum Mutat 27:640–643. doi:10.1002/humu.20373

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Clot F, Grabli D, Cazeneuve C, Roze E, Castelnau P, Chabrol B, Landrieu P, Nguyen K, Ponsot G, Abada M, Doummar D, Damier P, Gil R, Thobois S, Ward AJ, Hutchinson M, Toutain A, Picard F, Camuzat A, Fedirko E, San C, Bouteiller D, LeGuern E, Durr A, Vidailhet M, Brice A (2009) Exhaustive analysis of BH4 and dopamine biosynthesis genes in patients with Dopa-responsive dystonia. Brain 132:1753–1763. doi:10.1093/brain/awp084

Crawford JR, Garthwaite PH (2007) Comparison of a single case to a control or normative sample in neuropsychology: development of a Bayesian approach. Cogn Neuropsychol 24:343–372. doi:10.1080/02643290701290146

Engstrom K, Saldeen AS, Yang B, Mehta JL, Saldeen T (2009) Effect of fish oils containing different amounts of EPA, DHA, and antioxidants on plasma and brain fatty acids and brain nitric oxide synthase activity in rats. Ups J Med Sci 114:206–213. doi:10.3109/03009730903268958

Fonteh AN, Chiang J, Cipolla M, Hale J, Diallo F, Chirino A, Arakaki X, Harrington MG (2013) Alterations in cerebrospinal fluid glycerophospholipids and phospholipase A2 activity in Alzheimer's disease. J Lipid Res 54:2884–2897. doi:10.1194/jlr.M037622

Fonteh AN, Cipolla M, Chiang J, Arakaki X, Harrington MG (2014) Human cerebrospinal fluid fatty acid levels differ between supernatant fluid and brain-derived nanoparticle fractions, and are altered in Alzheimer's disease. PLoS One 9:e100519. doi:10.1371/journal.pone.0100519

Friedman J, Hyland K, Blau N, MacCollin M (2006) Dopa-responsive hypersomnia and mixed movement disorder due to sepiapterin reductase deficiency. Neurology 67:2032–2035. doi:10.1212/01.wnl.0000247274.21261.b4

Friedman J, Roze E, Abdenur JE, Chang R, Gasperini S, Saletti V, Wali GM, Eiroa H, Neville B, Felice A, Parascandalo R, Zafeiriou DI, Arrabal-Fernandez L, Dill P, Eichler FS, Echenne B, Gutierrez-Solana LG, Hoffmann GF, Hyland K, Kusmierska K, Tijssen MA, Lutz T, Mazzuca M, Penzien J, Poll-The BT, Sykut-Cegielska J, Szymanska K, Thony B, Blau N (2012) Sepiapterin reductase deficiency: a treatable mimic of cerebral palsy. Ann Neurol 71:520–530. doi:10.1002/ana.22685

Gradinger AB, Belair C, Worgan LC, Li CD, Lavallee J, Roquis D, Watkins D, Rosenblatt DS (2007) Atypical methylmalonic aciduria: frequency of mutations in the methylmalonyl CoA epimerase gene (MCEE). Hum Mutat 28:1045. doi:10.1002/humu.9507

Hong SH, Belayev L, Khoutorova L, Obenaus A, Bazan NG (2014) Docosahexaenoic acid confers enduring neuroprotection in experimental stroke. J Neurol Sci 338:135–141. doi:10.1016/j.jns.2013.12.033

Jiang LH, Shi Y, Wang LS, Yang ZR (2009) The influence of orally administered docosahexaenoic acid on cognitive ability in aged mice. J Nutr Biochem 20:735–741. doi:10.1016/j.jnutbio.2008.07.003

Kearney H.M., Kearney J.B., Conlin L.K. (2011) Diagnostic implications of excessive homozygosity detected by SNP-based microarrays: consanguinity, uniparental disomy, and recessive single-gene mutations. Clin Lab Med 31:595-613, ix. DOI: 10.1016/j.cll.2011.08.003.

Kim HY (2007) Novel metabolism of docosahexaenoic acid in neural cells. J Biol Chem 282:18661–18665. doi:10.1074/jbc.R700015200

Koht J, Rengmark A, Opladen T, Bjornara KA, Selberg T, Tallaksen CM, Blau N, Toft M (2014) Clinical and genetic studies in a family with a novel mutation in the sepiapterin reductase gene. Acta Neurol Scand Suppl 7–12. doi:10.1111/ane.12230

Lavialle M, Champeil-Potokar G, Alessandri JM, Balasse L, Guesnet P, Papillon C, Pevet P, Vancassel S, Vivien-Roels B, Denis I (2008) An (n-3) polyunsaturated fatty acid-deficient diet disturbs daily locomotor activity, melatonin rhythm, and striatal dopamine in Syrian hamsters. J Nutr 138:1719–1724

Montgomery JA, Mamer OA, Scriver CR (1983) Metabolism of methylmalonic acid in rats. Is methylmalonyl-coenzyme a racemase deficiency symptomatic in man? J Clin Invest 72: 1937–1947. doi:10.1172/JCI111158

Ormazabal A, Garcia-Cazorla A, Fernandez Y, Fernandez-Alvarez E, Campistol J, Artuch R (2005) HPLC with electrochemical and fluorescence detection procedures for the diagnosis of inborn errors of biogenic amines and pterins. J Neurosci Methods 142:153–158

Pottala JV, Yaffe K, Robinson JG, Espeland MA, Wallace R, Harris WS (2014) Higher RBC EPA+DHA corresponds with larger total brain and hippocampal volumes: WHIMS-MRI study. Neurology 82:435–442. doi:10.1212/WNL.0000000000000080

Zorzi G, Redweik U, Trippe H, Penzien JM, Thony B, Blau N (2002) Detection of sepiapterin in CSF of patients with sepiapterin reductase deficiency. Mol Genet Metab 75:174–177

JIMD Reports
DOI 10.1007/8904_2015_411

RESEARCH REPORT

Audit of the Use of Regular Haem Arginate Infusions in Patients with Acute Porphyria to Prevent Recurrent Symptoms

Joanne T. Marsden • Simon Guppy • Penelope Stein • Timothy M. Cox • Michael Badminton • Tricia Gardiner • Julian H. Barth • M. Felicity Stewart • David C. Rees

Received: 17 September 2014 / Revised: 08 January 2015 / Accepted: 22 January 2015 / Published online: 12 March 2015

Abstract The National Acute Porphyria Service (NAPS) provides acute care support and clinical advice for patients in England with active acute porphyria requiring haem arginate treatment and patients with recurrent acute attacks.

This audit examined the benefits and complications of regular haem arginate treatment started with prophylactic intent to reduce the frequency of recurrent acute attacks in a group of patients managed through NAPS. We included 22 patients (21 female and 1 male) and returned information on diagnosis, indications for prophylactic infusions, frequency and dose, analgesia, activity and employment and complications including thromboembolic disease and iron overload.

The median age at presentation with porphyria was 21 years (range 9–44), with acute abdominal pain as the predominant symptom. Patients had a median of 12 (1–400) attacks before starting prophylaxis and had received a median of 52 (0–1,350) doses of haem arginate. The median age at starting prophylaxis was 28 years (13–58) with a median delay of 4 years (0.5–37) between presentation and prophylaxis. The frequency of prophylactic haem arginate varied from 1 to 8 per month, and 67% patients were documented as having a reduction in pain frequency on prophylaxis. Only one patient developed clinically significant iron overload and required iron chelation, but the number of venous access devices required varied from 1 to 15, with each device lasting a median of 1.2 years before requiring replacement. Six patients stopped haem arginate and in three this was because their symptoms had improved. Prophylactic haem arginate appears to be beneficial in patients with recurrent acute porphyria symptoms, but maintaining central venous access may prove challenging.

Communicated by: John Christodoulou, MB BS PhD FRACP FRCPA

Competing interests: None declared

J.T. Marsden (✉)
Department of Clinical Biochemistry, King's College Hospital, NHS Foundation Trust, Denmark Hill, London SE5 9RS, UK
e-mail: joannemarsden1@nhs.net

S. Guppy · D.C. Rees
Haematological Medicine, King's College Hospital, London SE5 9RS, UK

P. Stein · T.M. Cox
Department of Medicine, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK

T.M. Cox
University of Cambridge, Cambridge CB2 0QQ, UK

M. Badminton · T. Gardiner
Department of Medical Biochemistry and Immunology, University Hospital of Wales, Cardiff CF14 4XW, UK

J.H. Barth
Department of Clinical Chemistry, Leeds General Infirmary, Leeds LS1 3EX, UK

M.F. Stewart
Department of Clinical Biochemistry, University of Manchester, Manchester Academic Health Sciences Centre, Salford Royal NHS Foundation Trust, Salford M6 8HD, UK

Introduction

The porphyrias are rare metabolic disorders caused by defects in the synthesis of haem and are usually associated with either acute or cutaneous symptoms or a mixture of both. The three main types of acute porphyria are acute intermittent porphyria (AIP), variegate porphyria (VP) and

hereditary coproporphyria (HCP). Acute porphyrias cause sudden-onset, unpredictable episodes of illness, characterised by severe abdominal pain, gastrointestinal symptoms, hypertension, hyponatraemia and varying degrees of additional neurological impairment (Puy et al. 2010). Typically the diseases are inherited as an autosomal dominant trait, although many people with acute porphyria have few or no symptoms with penetrance within affected families being reported as between 10 and 50%. AIP is the most common of the acute porphyrias and, unlike VP and HCP, is not associated with bullous skin lesions. Most symptomatic patients have only one attack, but approximately 5% women and 3% men with porphyria suffer recurrent and frequent attacks which persist for many years (Elder et al. 2013).

An acute porphyria attack can be life threatening, and severe episodes usually require hospital admission and treatment with a preparation of intravenous solubilised haem, by supplying the end product of the biosynthetic pathway. Haem therapy decreases the formation of porphyrin precursors, overproduction of which is believed to be responsible for the acute clinical compensation. In Europe, haem therapy is available in the form of haem arginate (Normosang®). Patients may also require high doses of opiate medication to relieve the pain suffered during the attack (Stein et al. 2013).

Recurrent severe attacks are rare and affect about 5% of patients with acute porphyria; typically recurrent attacks occur in women in association with the menstrual cycle and usually improve after the menopause. Severe attacks may occur more frequently than monthly and can be associated with progressive neuropathy, chronic pain, renal impairment and increased risk of hepatocellular carcinoma (Sardh et al. 2013). Frequent attacks result in severe disability, making normal working, family and social life very difficult. It is thought that 20–30 individuals in the UK currently suffer from this pattern of frequent recurrent attacks.

There is little evidence published on how to manage these patients optimally; the therapeutic options include suppression of menstruation with gonadorelin (GnRH) analogues (Anderson et al. 1990), regular haem arginate infusions (Stein and Cox 2011) and, in a few cases, liver transplantation (Soonawalla et al. 2004; Dowman et al. 2011).

There is some evidence that haem arginate infusions are of benefit in shortening the duration and severity of single acute attacks of porphyria, with a rapid fall in urinary concentrations of porphobilinogen (PBG) and other metabolites indicative of an acute attack (Herrick et al. 1989; Ma et al. 2011). Based on this acute effect and licenced use, regular haem arginate infusions have been used in an attempt to prevent recurrent attacks for at least 20 years. As many as six infusions have been given each month usually via an indwelling central line. Anecdotally some patients benefit from this, although published data are lacking. Conversely, significant side effects can occur, including central venous thrombosis and iron overload, and chronic pain may persist.

The aim of this audit was to evaluate the benefits and complications of haem arginate prophylaxis in patients with recurrent acute attacks of porphyria and where possible to develop preliminary evidence-based guidelines for its use.

Design and Methods

Patients were identified from the records of the three acute porphyria centres in England and Wales. Any patient with a diagnosis of acute porphyria who had started haem arginate infusions with prophylactic intent from 1999 to 2012 was included.

All patients in England who started regular prophylactic haem arginate were identified from records of the National Acute Porphyria (NAPS) centres in Cambridge, Cardiff and London and outreach clinics in Leeds and Salford and from the records of Orphan Europe, who are exclusive suppliers of haem arginate in the UK. Audit data was collected using an agreed proforma (Table 1) through a combination of examining patient records both at NAPS centres and local hospitals. Qualitative data on activity levels and employment before, during and after prophylaxis was also collected.

Results

Patients

Twenty-three patients (21 females, 2 males) (Cardiff/Salford, 7 patients; Cambridge/Leeds, 12 patients; and King's College Hospital, 4 patients) had received prophylactic haem arginate treatment. One patient had variegate porphyria (VP), one hereditary coproporphyria (HCP), twenty acute intermittent porphyria (AIP) and one post-liver transplant (who was not included in subsequent analyses). All diagnoses were confirmed by biochemical analysis, with increased urine porphobilinogen (PBG) measurements at the time of presentation; the differential diagnosis of VP, HCP or AIP was established by biochemical porphyrin investigations and genetic analysis.

Presenting Features

The median age of symptomatic presentation and diagnosis of acute porphyria was 21 years (range 9–44 years). Most

Table 1 Parameters studied during the audit

	Parameters studied
Diagnosis and history before prophylaxis	Type of porphyria and biochemical diagnosis
	Date, place of diagnosis and presenting symptoms
	Age of first acute symptoms and precipitants
	Number of hospital admissions, haem arginate doses and regular analgesia
	Treatment with GnRH agonist: type, duration, reason for stopping
	Iron indices: ferritin, serum iron and transferrin saturation
	Other relevant co-morbidities
	Quality of life (QOL): employment, activity, exercise
Prophylactic haem arginate	Frequency and dose of haem arginate and route of administration
	Duration of treatment including reason for stopping treatment
	Frequency of acute and chronic symptoms
	Number of hospital admissions, haem arginate doses and regular analgesia
	Iron indices: ferritin, serum iron and transferrin saturation
	Other relevant co-morbidities
	QOL: employment, activity, exercise
After prophylactic haem arginate	Treatment with GnRH agonist: type, duration, reason for stopping
	Iron indices: ferritin, serum iron and transferrin saturation
	Regular analgesia
	QOL: employment, activity, exercise

presented with an acute attack, with abdominal pain as the dominant feature. Three patients were asymptomatic when diagnosed as part of family screening but subsequently developed acute symptoms. The presenting features of porphyria in patients with recurrent attacks do not differ obviously from the initial symptoms of porphyria patients in general, including those who have only had a single attack.

History Before Prophylaxis

The number of acute attacks requiring hospital admission before prophylactic treatment varied, with a median of 12 (range 1–400). One patient started prophylactic haem arginate after a single admission lasting 7 months. The number of days in hospital was also very high with a median 94 days (range 20–2,000). Patients received a median of 52 doses (range 0–1,350) of haem arginate before being started on prophylaxis, given as treatment for acute attacks.

GnRH agonists were prescribed for 15/21 (71%) of the female patients before starting prophylaxis with haem arginate. Three patients were also given oestrogen replacement during treatment, and the median time of treatment was 6 months (1 month to 3 years). Eleven patients stopped GnRH agonists because the treatment was ineffective and acute attacks persisted; one patient continued on GnRH agonists with regular haem arginate. The other reasons for stopping treatment are shown in Table 2.

Prophylactic Haem Arginate Treatment

The median age of starting prophylactic haem arginate treatment was 28 years (range 13–58), and the median time between onset of symptoms and starting prophylaxis was 4 years (0.5–37 years). No standard treatment regimen was used, and the dose was 3 mg/kg for each patient referred to as "dose" in the text. Approaches differed for each patient and varied depending on physician choice, the frequency of acute attacks and linkage to the menstrual cycle. Four patients were prescribed one dose of haem arginate monthly, three patients twice per month, thirteen patients four times per month, one patient six times per month and one patient eight times per month. Fifty percent of the patients were also on regular opiate analgesia because of chronic pain. The duration of prophylaxis varied with a median of 50 months (range 1–150) and a median of 150 (2–1,000) doses of haem arginate used; 16 patients continued on haem prophylaxis beyond the end of the audit period, into January 2013. Table 3 shows the dose

Table 2 Reasons for stopping GnRH treatment

Agonist	Length of treatment	Oestrogen	Reason for stopping treatment
Leuprorelin	4 months	No	TAH and BSO
Not known	3 months	Patch	No benefit
Goserelin	1 month	No	Exacerbated an acute attack
Goserelin	<12 months	Tibolone	Ineffective
Goserelin	8 months	No	Side effects, attacks continued
Not known	Not known	No	Ineffective
Goserelin	6 months	No	Acute attacks continued
Buserelin then Goserelin	Not known	No	Not known
Goserelin	3 years	No	Monthly attacks continued
Goserelin	6 months	No	Attacks stopped then relapsed
Not known	9 months	No	Precipitated several acute attacks
Goserelin	6 months	No	Attacks persisted
Goserelin	4 months	No	Ineffective
Goserelin	7 months	Tibolone	Acute attacks continued
Goserelin	<12 months	No	Ineffective. Attacks persisted

schedule and cumulative doses given to each patient over the treatment period.

While receiving these prophylactic measures, eight (36%) patients had no hospital admissions, eleven (50%) were admitted between 1 and 5 times/year, and three (14%) were admitted more than 5 times per year. The median number of admissions was 9 (range 0–100). Fourteen (67%) patients showed a decrease in pain frequency on haem prophylaxis compared with before it had been started; six (29%) reported no change; one (5%) had an increased frequency of symptoms; and it was too early to evaluate one patient.

The activity and exercise capacity were assessed qualitatively; the activity level of one (5%) patient was worse following prophylactic haem treatment, while 8 (40%) patients showed no change and 11 (40%) noted an improvement. Seven (35%) patients showed improved work capacity on haem prophylaxis, 12 (60%) showed no change, and 1 (5%) showed reduced capacity.

One of the potential effects of haem treatment is iron overload. Each ampoule of haem arginate (10 mL, 250 mg of human haemin) contains 22 mg of iron. Serum ferritin concentrations were available in 19 patients and showed a median of 208 μg/L (reference range: 20–200), but there was a wide range (21–3,165 μg/L). Transferrin saturations were available in 16 patients, with a median of 29% (reference range: 20–50%) and a range of 9–100%. One patient was receiving iron chelation, but as far as could be ascertained, no patients had organ damage related to iron overload. There was a statistically significant correlation between serum ferritin concentration and the number of doses administered ($r = 0.884$, $p < 0.001$), and the data is shown in Fig. 1. Ferritin concentrations were proportionately more elevated than transferrin saturation, with 53% (10/19 patients) having serum ferritin determinations greater than the upper reference limit, compared with only 23% with raised transferrin saturations; two patients had transferrin saturations below the normal reference range.

The number of semi-permanent central venous access devices used during prophylactic haem treatment and the reasons for removal of a device were available from 20 patients. The number of devices used per patient ranged from 1 to 15. The median number of devices used per patient was two, and eight patients had only one device fitted. There were a total of 52 devices removed, and the average lifespan for a single device was 1.2 years. There was a correlation between the number of devices used and cumulative dose received, but this was not statistically significant possibly as a result of the limited number of patients in the study (Fig. 2). The reasons for removal of the devices were infection (8%), blockage (35%) and a mixture of infection/blockage (38%) and single cases of thrombosis, infection/thrombosis, difficulties with venous access and pain.

Table 3 Dosage schedule and cumulative doses of haem arginate with complications

Patient	Dosage schedule	Total monthly dose (3 mg/kg/dose)	Total doses	Treatment period (months)	Number of devices used	Complications	Ferritin ug/L (normal: 20–300)
1	MX2	2	8	4	1	None	21
2	M	1	40	40	1	None	50
3	MX4, MX2, M	4	73	30	3	Location	Unknown
4	MX4	4	250	62	10	Three infected, one blocked	Unknown
5	MX3, MX2, MX4	4	58	29	1	Blocked but okay	140
6	MX4	4	30	7	1	None	84
7	MX2	2	100	50	1	None	208
8	M	1	144	144	Unknown	None	37
9	MX4	4	2	1	1	None	89.2
10	MX2, M	1	200	132	1	Unknown	44.9
11	M, MX2, MX4	4	450	132	5	Infection/ thrombosis	258.2
12	MX2	2	50	25	4	Infection/ thrombosis	45.9
13	MX4, MX8, MX4	4	275	69	Unknown	Unknown	701
14	MX6	6	1,000	150	15	Infection/ thrombosis	3,165
15	M	1	150	150	2	Not worked	675
16	MX4	4	170	43	1	None	549.3
17	MX4	4	300	75	4	Infection/ blocked	423.1
18	MX4	4	20	5	2	Blocked	311.9
19	MX4	4	19	5	3	Blocked	97
20	MX4	4	150	37	2	Blocked	Unknown
21	MX2, MX4, MX8	8	170	60	6	Blocked	854
22	M	4	250	60	5	Blocked	248

Key: *M* monthly, *MX2* twice per month, *MX3* three times per month, *MX4* four times per month, *MX6* six times per month, *MX8* eight times per month

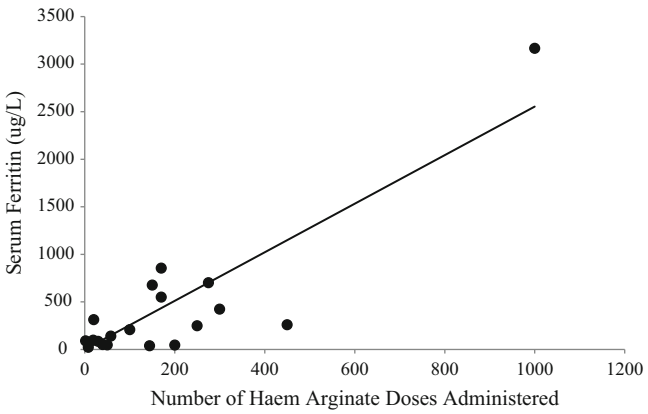


Fig. 1 Graph showing the correlation between the number of haem arginate vials and serum ferritin concentration

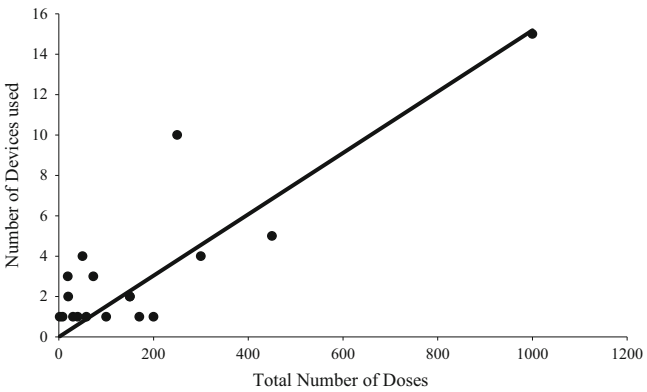


Fig. 2 Graph showing total number of doses of haem arginate and indwelling devices used

Table 4 Number of hospital admissions, acute attacks and co-morbidities before and during prophylaxis haem treatment

	Before prophylaxis				During prophylaxis				
Patient	Hospital admissions	Number of acute attacks	Number of years	Doses per year	Hospital admissions	No of acute attacks	Number of years	Doses per year	Co-morbidities
1	4	4	6	2	0	0	4 months	0	None
2	360	Acute symptoms	36	0	0	0	4	0	Depression
3	6	Acute symptoms	1	Unknown	0	0	2	0	Depression
4	Unknown	Unknown	Unknown	Unknown	35	35	8	4	None
5	5	5	2	10	15	15	3	5	None
6	6	6	3	8	0	0	7 months	1	None
7	7 months	15	1	60	0	0	5	0	None
8	4	4	32	<1	0	0	13	0	Renal impairment
9	5	5	2	10	1	1	1	1	None
10	20	20	6	13	11	11	11	1	Depression
11	140	140	7	80	63	63	11	6	Renal impairment
12	84	84	7	48	1	1	5	1	Depression
13	50	50	4	48	100	100	5	20	Renal impairment
14	60	60	4	75	28	28	14	2	Iron overload
15	18	18	9	8	0	0	10	0	Depression
16	12	12	2	24	0	0	4	0	None
17	50	50	4	50	7	7	7	1	None
18	12	12	1	48	10	10	2	5	Renal impairment depression
19	14	14	3	18	11	11	1	11	Depression
20	14	14	4	7	20	20	8	2	Renal impairment
21	Unknown	Unknown	6	Unknown	5	5	5	1	None
22	400	400	21	64	30	25	6	5	Renal impairment

Co-morbidities while the patients were on haem treatment included six patients (27%) with renal impairment and seven patients (32%) with depression. One patient had received a liver transplant and was not included in the study after the procedure.

Table 4 shows summary data on the patients detailing the hospital admissions, acute attacks and haem dosage before and during prophylactic haem treatment.

Stopping Prophylactic Haem Arginate Treatment

Six patients (27%) stopped prophylactic regular haem treatment, after a duration which varied from 8 months to 8 years. The reasons for stopping haem treatment are given in Table 5. Of the six who stopped treatment, prophylaxis had been effective in half.

Table 5 Reasons for stopping prophylactic haem arginate treatment

	Duration of treatment	Reason for stopping
1	2.5 years	Improved condition
2	13 years	Improved condition
3	5 years	Ineffective: liver transplant
4	8 months	Ineffective: treatment restarted
5	8 years	Improved condition
6	6 years	Loss of venous access

Discussion

Haem arginate (Normosang®) is usually administered at a dose of 3 mg/kg once daily for 4 days by infusions into a central vein delivered over at least 30 min. There is reasonable evidence for using haem arginate for patients having a single acute attack or infrequent attacks (Mustajoki et al. 1986; Kostrzewska et al. 1991). However, there is an important group of patients, predominantly as here women in the reproductive age group who have recurrent acute attacks usually requiring admission to hospital, opiate analgesia and emergency administration of haem arginate. These attacks severely disrupt home life, and many patients are no longer able to work; their quality of life is greatly impaired.

Although prophylactic haem is increasingly used, there is little or no published evidence to support its use to date. A recent observational study in the USA on patients with acute porphyrias using a different alkaline preparation (Panhematin®) reported that it had been effective in preventing recurrent attacks (Bonkovsky et al. 2014).

Our audit suggests that initiating regular haem arginate coincided with clinical improvement in 50–70% patients. There was an improvement in physical activity in 11 patients (55%), and 7 patients (35%) reported that their work attendance had improved. However there was still a requirement for regular analgesia with opiates in half of the patients, although 14 (67%) had fewer episodes of acute pain.

Suppression of menstruation is a therapeutic option in women with recurrent attacks, particularly if there is a link between the premenstrual period and acute pain. Seventy-one percent of the women in this audit had experienced GnRH agonists before starting haem therapy, although the hormonal intervention was ineffective in the majority. There are reports of beneficial effects of suppressing the menstrual cycle in some women, although these patients were unwilling to undergo treatment with regular haem arginate and were not included in this study.

As expected, we found a correlation between serum ferritin concentration and the number of haem arginate doses administered. However, in most cases, ferritin was only modestly elevated, and only one patient had unequivocal evidence of iron overload. This patient had received ~1,000 doses of haem arginate over a 10-year period; while there was hepatic iron loading, there was no evidence of cardiac iron loading on T2* magnetic resonance imaging. The pattern of iron loading in these patients was unusual, with relatively high serum ferritins associated with low or normal transferrin saturation, possibly reflecting the manner in which iron derived from paternally administered haem is metabolised.

The most frequent serious unwanted side effect concerned the loss of venous access, with an indwelling device typically lasting only 1–2 years. The main problem seems to be occlusion of the central catheter, and this appears to result from aggregation of haem arginate. Haem may also induce marked phlebitis and so activate local coagulation pathways leading to thrombus formation. One patient had to stop haem prophylaxis because of a complete loss of central and peripheral venous access, and another has already had 15 different semi-permanent venous access devices. Progressive damage to central veins is potentially serious and can preclude liver transplantation. The injurious effects on venous endothelium may be reduced by the use of albumin to dilute the haem arginate; while this is also an unlicenced procedure, it is the one adopted for nearly all the patients in this group.

Other important co-morbidities reported were renal impairment in six patients (27%); seven patients (32%) reported feeling depressed, and one patient had required a liver transplant. Renal impairment in patients, usually associated with hypertension and active porphyria, is well documented (Marsden et al. 2008; Frei et al. 2012). The causal factors are unclear although it may be due to toxicity of the precursor 5-aminolaevulinic acid or other haem precursors filtered in the urine that are formed in excess in the body during periods of activity; it is also possible in some patients that the effect is compounded by the use of nonsteroidal anti-inflammatory drugs. Depression is a prominent feature in patients with acute porphyria with seven patients (32%) reported feeling depressed during the period in which they were receiving treatment with prophylactic haem. No formal assessment was used, and it is not possible to assess from this retrospective audit data whether prophylactic haem arginate has any effect on co-morbidities such as depression or renal impairment.

Six patients stopped the treatment, and in three (50%) there were no further reported acute attacks, suggesting that prophylactic haem infusion is an effective long-term strategy in some, but not all, patients.

We noticed that stopping or reducing the frequency of haem arginate was difficult and associated with an exacerbation of symptoms in some patients. This “dependence”

has been plausibly linked to the induction of hepatic haem oxygenase-1 expression reported in healthy subjects (Doberer et al. 2010). Other studies have provided evidence of significant toxicity associated with intravenous haem, with induction of endothelial activation and a procoagulant state, and it is uncertain whether regular administration over many years is actually beneficial.

In the past, prophylactic haem arginate infusions were almost always administered in hospital through day-case admissions. However in the last 2 years, patients in England have been offered the alternative option of receiving haem arginate at home through a contracted homecare nursing provider as part of the National Acute Porphyria Service (NAPS). Twenty patients currently receive home infusions in this way. Our initial experience suggests that home treatment is safe, and there may even be a lower complication rate of vascular access, since home-care nurses work to a strictly agreed protocols and are experienced in administration techniques. Administration of haem arginate at home results in cost saving to the health service of up to 50% compared with hospital-based infusions. In addition, homecare allows more flexibility with infusion frequency and the potential for early treatment to avert acute attacks that would otherwise result in hospital admission.

In this study, few patients become symptom-free on prophylaxis, and only 14% were able to stop treatment successfully. This audit is retrospective and based on a small number of patients' experience with the use of prophylactic haem. For this reason, it is difficult to give any firm recommendations for treatment based on the findings so far. Prophylaxis seems to offer significant short- to medium-term benefit to more than half of the patients in this study with recurrent attacks. However, patients should be fully counselled about the potentially long-term nature of haem prophylaxis before starting, and potential side effects should be discussed. Ideally, prospective, randomised controlled trials are needed to address the value of prophylaxis, although the rarity of the condition makes this difficult.

Other therapeutic options for treating these patients are limited, and liver transplantation has been used with varying success. Dowman et al. 2012 reported a survival rate of 80% from UK Transplant Registry data collected between 2002 and 2010, but in that series, there was a high percentage of hepatic artery stenosis (40%). We consider that this procedure should be reserved for patients with severe recurrent acute attacks and greatly impaired quality of life.

Recent studies have shown that experimental targeting of 5-aminolevulinic acid synthase 1 (ALAS 1), the principal regulatory enzyme of haem biosynthesis in the liver by administering small interfering RNAs, is highly effective in preventing and aborting acute porphyric episodes in a mouse model of acute intermittent porphyria (Yasuda et al. 2014). Preclinical studies have been completed by Alnylam® Pharmaceuticals and phase I clinical studies are planned for 2015.

Synopsis

Prophylactic haem arginate treatment can be beneficial to patients with recurrent attacks of acute porphyria.

Compliance with Ethics Guidelines

Conflict of Interest

Joanne Marsden, Simon Guppy, Penelope Stein, Timothy Cox, Michael Badminton, Tricia Gardiner, Julian Barth, M Felicity Stewart and David Rees declare that they have no conflict of interest.

Informed Consent

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

Details on Contributions of Individual Authors

Joanne Marsden drafted and revised the article and analysed and interpreted the data.

Simon Guppy was involved in the design of the audit, collated and analysed the data and had input into revision of the article.

Penelope Stein was involved in the design of the audit, collated data and had input into revision of the article.

Timothy Cox was involved in the design of the audit, collated data and had input into revision of the article.

Michael Badminton was involved in the design of the audit, collated data and had input into revision of the article.

Tricia Gardiner was involved in the design of the audit, collated data and had input into revision of the article.

Julian Barth was involved in the design of the audit, collated data and had input into revision of the article.

M Felicity Stewart was involved in the design of the audit, collated data and had input into revision of the article.

David Rees was involved in the design of the audit, collated and analysed data and had input into the revision of article. He is Guarantor for the article.

References

Anderson KE, Spitz IM, Bardin CW, Kappas A (1990) A gonadotropin releasing hormone analogue prevents cyclical attacks of porphyria. Arch Intern Med 150(7):1469–1474

Bonkovsky HL, Maddukuri VC, Yazici C et al (2014) Acute porphyrias in the USA: features of 108 subjects from porphyria consortium. Am J Med. doi:10.1016/j.amjmed.2014.06.036

Doberer D, Haschemi A, Andreas M et al (2010) Haem arginate infusion stimulates haem oxygenase-1 expression in healthy subjects. Br J Pharmacol 161:1751–1762

Dowman JK, Gunson BK, Mirza DF, Badminton MN, Newsome PN (2011) UK experience of liver transplantation for erythropoietic protoporphyria. J Inherit Metab Dis 34(2):539–545

Dowman JK, Gunson BK, Mirza DF et al (2012) Liver transplantation for acute intermittent porphyria is complicated by a high rate of hepatic artery thrombosis. Liver Transpl 34:195–200

Elder G, Badminton M, Harper P, Sandberg S, Deybach JC (2013) The incidence of inherited porphyrias in Europe. J Inherit Metab Dis 36(5):849–857

Frei P, Minder EI, Corti N et al (2012) Liver transplantation because of acute liver failure due to heme arginate overdose in a patient with acute intermittent porphyria. Gastroenterology 6 (1):190–196

Herrick AL, McColl KE, Moore MR, Cook A, Goldberg A (1989) Controlled trial of haem arginate in acute hepatic porphyria. Lancet 10(1):1295–1297

Kostrzewska E, Gregor A, Tarczynska-Nosal S (1991) Heme arginate (Normosang) in the treatment of attacks of acute hepatic porphyrias. Mater Med Pol 23(4):259–262

Ma E, Mar V, Varigos G, Nicoll A, Ross G (2011) Haem arginate as effective maintenance therapy for hereditary coproporphyria. Australas J Dermatol 52(2):135–138

Marsden JT, Chowdhury P, Wang J et al (2008) Acute intermittent porphyria and chronic renal failure. Clin Nephrol 69(5):339–346

Mustajoki P, Tenhunen R, Tokoa O, Gothoni G (1986) Haem arginate in the treatment of acute hepatic porphyrias. Br Med J (Clin Res Ed) 293:538–539

Puy H, Gouya L, Deybach JC (2010) Porphyrias. Lancet 375:924–937

Sardh E, Wahlin S, Bjornstedt M, Harper P, Andersson DE (2013) High risk of primary liver cancer in a cohort of 179 patients with acute hepatic porphyria. J Inherit Metab Dis 36(6):1063–1071

Soonawalla ZF, Orug T, Badminton MN et al (2004) Liver transplantation as a cure for acute intermittent porphyria. Lancet 363:705–706

Stein PE, Cox TM (2011) Homecare delivery of haem arginate in acute porphyria International Porphyrins and Porphyrias Meeting, 10-14 April 2011, Cardiff, UK. Br J Dermatol 164:1125–1176

Stein P, Badminton M, Barth J, Rees D, Stewart MF (2013) Best practice guidelines on clinical management of acute attacks of porphyria and their complications. Ann Clin Biochem 50:217–223

Yasuda M, Gan L, Chen B et al (2014) RNAi-mediated silencing of hepatic Alas 1 effectively prevents and treats the induced acute attacks in acute intermittent porphyria mice. Proc Natl Acad Sci U S A 111(21):7777–7782

JIMD Reports
DOI 10.1007/8904_2015_413

RESEARCH REPORT

Normal Cerebrospinal Fluid Pyridoxal 5′-Phosphate Level in a PNPO-Deficient Patient with Neonatal-Onset Epileptic Encephalopathy

Alina Levtova · Stephane Camuzeaux · Anne-Marie Laberge · Pierre Allard · Catherine Brunel-Guitton · Paola Diadori · Elsa Rossignol · Keith Hyland · Peter T. Clayton · Philippa B. Mills · Grant A. Mitchell

Received: 27 October 2014 / Revised: 23 January 2015 / Accepted: 26 January 2015 / Published online: 12 March 2015

Abstract Deficiency of pyridox(am)ine 5′-phosphate oxidase (PNPO, OMIM 610090) is a treatable autosomal recessive inborn error of metabolism. Neonatal epileptic encephalopathy and a low cerebrospinal fluid (CSF) pyridoxal 5′-phosphate level are the reported hallmarks of PNPO deficiency, but its clinical and biochemical spectra are not fully known. Case presentation: A girl born at 33 3/7 weeks of gestation developed seizures in the first hours of life. Her seizures initially responded to GABAergic agonists, but she subsequently developed a severe epileptic encephalopathy. Brain MRI and infectious and metabolic evaluations at birth, including urinary alpha-aminoadipic semialdehyde (AASA), were normal. Lumbar puncture at age 3 months showed: pyridoxal 5′-phosphate, 52 nmol/L (normal, 23–64); homovanillic acid, 392 nmol/L (normal, 450–1,132); 5-hydroxyindoleacetic acid, 341 nmol/L (normal, 179–711); and 3-ortho-methyldopa, 30 nmol/L (normal, below 300). The patient was not being treated with pyridoxine nor with pyridoxal 5′-phosphate at the time of the lumbar puncture. She died at age 14 months. A sequencing panel targeting 53 epilepsy-related genes revealed a homozygous missense mutation in *PNPO* (c.674G>A, p.R225H). Homozygosity was confirmed by parental testing. Expression studies of mutant p.R225H PNPO revealed greatly reduced activity. In conclusion, a normal CSF level of pyridoxal 5′-phosphate does not rule out PNPO deficiency.

Communicated by: Cornelis Jakobs, PhD

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2015_413) contains supplementary material, which is available to authorized users.

A. Levtova · A.-M. Laberge · P. Allard · C. Brunel-Guitton · P. Diadori · E. Rossignol · G.A. Mitchell (✉)
Divisions of Medical Genetics (AL, AML, CBG, GM) and Neurology (PD, ER), Department of Paediatrics, Biochemical Genetics Laboratory (CBG, PA), CHU Sainte-Justine and Université de Montréal, 3175 Côte-Sainte-Catherine, Montreal, QC, Canada H3T 1C5
e-mail: grant.mitchell@recherche-ste-justine.qc.ca

S. Camuzeaux · P.T. Clayton · P.B. Mills
Genetics and Genomic Medicine Programme, UCL Institute of Child Health, London, UK

E. Rossignol
Department of Neurosciences, Université de Montréal, Montreal, QC, Canada

K. Hyland
Medical Neurogenetics Atlanta, Atlanta, Georgia

Introduction

Pyridox(am)ine 5′-phosphate oxidase deficiency (PNPO deficiency, OMIM 610090) is a treatable cause of neonatal epileptic encephalopathy (Mills et al. 2005), clinically resembling pyridoxine-dependent epilepsy due to antiquitin deficiency (OMIM 266100). Since PNPO is necessary for conversion of pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate into pyridoxal 5′-phosphate (PLP; the only active form of vitamin B6), as well as for the recycling of PLP (Musayev et al. 2003), seizures in PNPO deficiency are usually resistant to pyridoxine but respond to pyridoxal 5′-phosphate (di Salvo et al. 2011), although several pyridoxine-responsive patients have been reported (Mills et al. 2014; Plecko et al. 2014).

Given the catastrophic natural history of PNPO deficiency (Mills et al. 2005) and the possibility of satisfactory outcomes in some cases with prompt diagnosis and treatment (Schmitt et al. 2010; Goyal et al. 2013), the development of reliable diagnostic tools for the disease is crucial.

PLP in cerebrospinal fluid has been proposed as an important diagnostic marker in PNPO deficiency, and reference ranges have been established for this purpose (Ormazabal et al. 2008). It is known that the finding of low CSF PLP is not in itself diagnostic of PNPO deficiency. Low CSF PLP has been reported in inborn errors of metabolism associated with the accumulation of metabolites that inactivate PLP such as antiquitin deficiency and molybdenum cofactor deficiency (Footitt et al. 2011), as well as in epileptic encephalopathy with no specific diagnosis (Goyal et al. 2013).

On the other hand, until the present report, all six patients with confirmed PNPO deficiency in whom pretreatment CSF PLP has been measured in the absence of PLP or pyridoxine supplementation have had low levels (cf. Table 1). Given the role of PNPO in the synthesis and recycling of PLP, the finding of low CSF PLP levels in deficient patients is also intuitively plausible. Nevertheless, as we show in the following report, the presence of a normal PLP level is insufficient to rule out PNPO deficiency.

We describe the first known patient with PNPO deficiency and a normal CSF PLP level, and we place her clinical presentation and diagnostic findings within the context of previously reported PNPO-deficient patients with a targeted literature review.

Methods

Cerebrospinal Fluid Analysis

Reversed phase HPLC and fluorescence detection were used for measurement of PLP, as described by Ormazabal

Table 1 Analysis of pretreatment CSF biochemical findings in previous descriptions of patients with PNPO deficiency

	PLP			5-HIAA			HVA			3-Ortho-methyldopa	
Patients	Low	Lower limit of normal	Normal	Low	Normal	High	Low	Normal	High	Normal	High
This report			✓		✓		✓			✓	
1 (A)[a]		✓[a]			✓[a]			✓[a]		NA	NA
2 (B)	✓				✓			✓		NA	NA
5 (E)	✓				✓		✓				✓
6 (F)	NA	NA	NA			✓			✓		✓
9 (H)	NA	NA	NA		✓			✓		NA	NA
10 (H)	NA	NA	NA		✓			✓		NA	NA
11 (H)	NA	NA	NA			✓			✓	NA	NA
14 (I)	NA	NA	NA	✓			✓				✓
15 (I)	NA	NA	NA	✓			✓				✓
17 (J)	✓			✓			✓				✓
18 (K)	✓			✓				✓			✓
19 (K)	✓			✓			✓				✓
33 (X)	✓					✓			✓		✓
34 (Y)	NA	NA	NA	✓			✓			NA	NA
35 (Z)	NA	NA	NA	✓			✓			NA	NA
37 (AA)	NA	NA	NA			✓		✓			✓
39 (BB)	NA	NA	NA		✓			✓		✓	
45 (FF)	NA	NA	NA		✓			✓		✓	
46 (GG)[a]	NA	NA	NA		✓[a]			✓[a]		✓[a]	
Info available	7	7	7	19	19	19	19	19	19	12	12
n	6	1	0	7	8	4	7	9	3	3	9
%	86	14	0	37	42	21	37	47	16	25	75

Information concerning our patient is included in the table for the sake of comparison, but was not used in the analysis.
5-HIAA 5-hydroxyindolacetate, *HVA* homovanillic acid, *hz* homozygous, *NA* not available, *PLP* pyridoxal 5′-phosphate
[a] These patients were receiving pyridoxine at the time of sampling.

et al. (2008), and reversed phase HPLC with electrochemical detection was used for measurement of neurotransmitter metabolites, as described by Hyland et al. (1993). Reference values for CSF pyridoxal 5′-phosphate were obtained using CSF from over 100 infants and children with neurological disease in whom seizures were not present. Values obtained were similar to published values (Ormazabal et al. 2008). CSF values decrease with age and the appropriate age-matched control group was used for comparison with the value obtained from our patient.

Mutant PNPO Expression Studies

The p.R225H mutant was created by site-directed mutagenesis and expressed in an in vitro translation system using a HeLa cell lysate (Thermo Scientific 1-Step Human Coupled IVT Kit) according to manufacturer's instructions. The reaction was incubated for 6 h at 30°C. PNPO enzyme activity was measured with pyridoxamine 5′-phosphate (PMP) as substrate using an HPLC-mass spectrometry assay for B6 vitamers (Footitt et al. 2013). These methods are described in full elsewhere (Mills et al. 2014).

Literature Review

PubMed was searched for "PNPO", "pyridoxal 5′-phosphate", "pyridoxal 5′-phosphate-dependent epilepsy" and variants. Articles containing case descriptions of patients diagnosed with PNPO deficiency were retained for further analysis.

Duplicate descriptions of the same patient were combined, and patient descriptions that provided no clinical information and/or did not provide molecular proof of PNPO deficiency were excluded. In particular, two patients (Footitt et al. 2011) were excluded as no clinical information, apart from posttreatment plasma PLP levels, was provided. Likewise, one patient (Kuo and Wang 2002) was excluded, as the diagnosis was confirmed neither by molecular nor by biochemical methods. We also excluded patients G3 and G4 described by Mills et al. (2005), as it is unclear whether they had clinical symptoms of epileptic encephalopathy or died of complications of extreme prematurity. Finally, one patient (Veerapandiyan et al. 2011) was excluded as *PNPO* sequencing was normal.

The remaining 46 patient descriptions were compared with our patient using the following parameters: gestational age, age at seizure onset, presenting features, neuroimaging and EEG findings, history of treatment with and response to pyridoxine and PLP and outcome.

Patient descriptions were numbered 1–46, as shown in Table 2.

Table 2 Previously published cases of PNPO deficiency

Patient	Reference
1 (A)[a]	Pearl et al. (2013)
2 (B)	Goyal et al. (2013) (pt 4)
3 (C)	Veerapandiyan et al. (2011) (case 1)
4 (D)	Schmitt et al. (2010) (pt 5); Hoffmann et al. (2007) (pt 3)
5 (E)	Ruiz et al. (2008); Ormazabal et al. (2008) (pt 1)
6 (F)	Khayat et al. (2008)
7 (G)	Hoffmann et al. (2007) (pt 1)
8 (G)	Hoffmann et al. (2007) (pt 2)
9 (H)	Hoffmann et al. (2007) (pt 4); Bagci et al. (2008)
10 (H)	Hoffmann et al. (2007) (pt 5); Bagci et al. (2008)
11 (H)	Hoffmann et al. (2007) (pt 6); Bagci et al. (2008)
12 (I)	Mills et al. (2005) (pt G1)
13 (I)	Mills et al. (2005) (pt G2)
14 (I)	Mills et al. (2005) (pt G5)
15 (I)	Mills et al. (2005) (pt G6)
16 (J)	Mills et al. (2005) (pt J1)
17 (J)	Mills et al. (2005) (pt J2); Ormazabal et al. (2008) (pt 2)
18 (K)	Mills et al. (2005) (pt K1); Ormazabal et al. (2008) (pt 3)
19 (K)	Mills et al. (2005 (pt K2); Ormazabal et al. (2008) (pt 4)
20 (L)	Mills et al. (2014) (pt 1)
21 (M)	Mills et al. (2014) (pt 2)
22 (N)	Mills et al. (2014) (pt 3)
23 (N)	Mills et al. (2014) (pt 4)
24 (O)	Mills et al. (2014) (pt 5)
25 (P)	Mills et al. (2014) (pt 6)
26 (Q)	Mills et al. (2014) (pt 7)
27 (R)	Mills et al. (2014) (pt 8)
28 (S)	Mills et al. (2014) (pt 9)
29 (T)	Mills et al. (2014) (pt 10)
30 (U)	Mills et al. (2014) (pt 11)
31 (V)	Mills et al. (2014) (pt 12)
32 (W)	Mills et al. (2014) (pt 14)
33 (X)	Porri et al. (2014)
34 (Y)	Ware et al. (2014) (case 1)
35 (Z)	Ware et al. (2014) (case 2)
36 (AA)	Plecko et al. (2014) (pt 1a)
37 (AA)	Mills et al. (2014) (pt 15); Plecko et al. (2014) (pt 1b)
38 (BB)	Plecko et al. (2014) (pt 2a)
39 (BB)	Plecko et al. (2014) (pt 2b)
40 (BB)	Plecko et al. (2014) (pt 2c)
41 (CC)	Plecko et al. (2014) (pt 3)
42 (DD)	Plecko et al. (2014) (pt 4a)
43 (DD)	Plecko et al. (2014) (pt 4b)
44 (EE)	Plecko et al. (2014) (pt 5)
45 (FF)	Mills et al. (2014) (pt 13); Plecko et al. (2014) (pt 6)
46 (GG)	Plecko et al. (2014) (pt 7)

[a] Letters in parentheses mark sibships.

Results

Case Description

Our patient, a girl, was born at $33^{3/7}$ weeks gestation to healthy non-consanguineous French-Canadian parents with no relevant family history. Before the premature onset of labour, the pregnancy had been uneventful apart from the sensation of abnormal "vibrating" fetal movements. Caesarean section was performed for breech presentation. Meconial fluid was observed, but Apgar scores were 9-9-9 (at 1, 5 and 10 min, respectively). Birth weight (2.080 kg) was normal for gestational age.

Myoclonic or multifocal seizures with abnormal movements of the head, eyes and mouth began at 2 h of life, and initially responded to benzodiazepines, with seizure-free periods of up to 9 days in the neonatal period. EEGs performed in the first 2 weeks of life displayed immature rhythms with inter-hemispheric asynchrony and prolonged intervals of attenuation of background rhythms lasting up to 60 s, compatible with *tracé discontinu* (discontinuous pattern), which failed to improve with maturation (Supplementary Figure 1a). Pyridoxine treatment was attempted on days 7–14 (100 mg intravenously, then 50 mg orally twice a day) and day 22 (50 mg twice a day); there was no clear effect on seizure activity, but somnolence and increased apnoeic episodes were observed. PLP was not administered. Initial lumbar puncture did not provide enough fluid for neurotransmitter analysis; subsequent attempts (five overall) were unsuccessful. Cerebral magnetic resonance imaging and infectious and metabolic evaluations, including urinary alpha-aminoadipic semialdehyde (AASA), were normal.

The baby was discharged on clobazam 9 mg/kg/day with satisfactory seizure control at 6 weeks of chronological age. Myoclonic seizures and episodes of tonic flexion spasms unresponsive to topiramate and ACTH occurred 3 weeks later. EEGs revealed slow background activity with episodes of rhythm desynchronisation and attenuation (electrodecremental patterns) without hypsarrhythmia. At 4 and 5 months, she was hospitalised for benzodiazepine-responsive status epilepticus.

CSF was obtained by lumbar puncture under fluoroscopic guidance at 4 months of age. Cell count, protein content, glucose, lactate, amino acids, 5-methyltetrahydrofolate and pterin metabolites were normal. The CSF pyridoxal 5′-phosphate was 52 nmol/L (normal, 23–64); homovanillic acid, 392 nmol/L (normal, 450–1,132); 5-hydroxyindoleacetic acid, 341 nmol/L (normal, 179–711); and 3-orthomethyldopa, 30 nmol/L (normal, below 300). An additional early eluting unknown compound was seen in the chromatogram obtained from the analysis of CSF pyridoxal 5′-phosphate (not shown). A likely candidate is pyridoxine phosphate (Pearl et al. 2013), but unfortunately we have been unable to obtain a reference compound for verification studies. Pyridoxamine has been reported to be elevated in plasma and CSF in PNPO deficiency in patients receiving pyridoxal 5′-phosphate or pyridoxine (Ware et al. 2014). Our patient was not receiving B6 therapy at the time of lumbar puncture so the unknown compound is unlikely to be pyridoxamine although this remains to be verified.

After the second episode of status epilepticus at age 5 months, she suffered from refractory epilepsy (multifocal and tonic seizures, spasms) and repeated status epilepticus (responsive to IV lorazepam or phenobarbital).

She had better seizure control from 7½ to 12 months of age (one seizure a week or fewer), which a trial of ketogenic diet did not improve further. In retrospect, this period coincided with the administration of pyridoxine (100 mg twice a day) and ended after pyridoxine was discontinued; she was, however, also being treated with phenobarbital, clobazam and clonazepam, and the relationship between pyridoxine and seizure control was not clinically apparent at the time. She had severe psychomotor delay, and her head circumference declined from the 50th percentile at birth to between the second and fifth percentiles.

At the chronological age of 12 months, she presented with a refractory status epilepticus. She had tonic seizures (Supplementary Figure 1b), followed by refractory subclinical continuous multifocal epileptic activity on EEG accompanied by hypoventilation and apnoea; multifocal seizure activity on EEG responded to midazolam perfusion but recurred when the perfusion was stopped. Cerebral MRI was still normal apart from atrophic changes and delays in myelination, both mild. She died at 14 months of age, having been taken off life support.

A sequencing panel targeting 53 epilepsy-related genes (Medical Neurogenetics) revealed an apparently homozygous missense mutation in *PNPO* (c.674G>A, p.R225H). Homozygosity was confirmed by parental testing. Samples from this patient were also included in an exome-sequencing study of unexplained infantile spasms, which simultaneously revealed the same *PNPO* mutation as our investigation (Michaud et al. 2014).

The p.R225H mutation was characterised in several fashions. Arginine 225 is conserved from yeast to humans (Supplementary Figure 2), is located at the active site of PNPO and interacts directly with the phosphate moiety of pyridoxal 5′-phosphate (Musayev et al. 2003). In silico analysis using PolyPhen-2, Proveen and Mutation Taster predicted p.R225H to be pathogenic. During the preparation of this article, the p.R225H mutation was reported in other patients with PNPO deficiency (Mills et al. 2014; Plecko et al. 2014; Ware et al. 2014). Finally, in vitro expression of a mutant *PNPO* cDNA containing p.R225H produced 8% of the PNPO activity of wild-type controls.

Targeted Literature Review

Clinical Findings

We reviewed the clinical presentation, response to treatment and outcome of the 48 previously described cases of PNPO deficiency (summarised in Table 3 and detailed in Supplementary Table 1) and their pretreatment biochemical findings (summarised in Table 1 and detailed in Supplementary Table 2).

Our patient's clinical presentation was consistent with previous reports. Prematurity was a finding in 27/44 (61%) of patients for whom gestational age was known, and in at least 5/46 (11%), abnormal movements were noted in utero. 27/44 (61%) of patients presented with seizures on the first day of life and all but two (96%) before 1 month of age; the exceptions were a child treated from birth and therefore asymptomatic (patient 23) and a child first presenting with infantile spasms at age 5 months (patient 26). Clinical descriptions of the seizures were available in 33/46 cases (72%); of these, myoclonus was reported in 20/33 (61%) and abnormal oral, ocular or head movements in 9/33 (27%). Metabolic acidosis was present in 13/46 (28%) of cases. Burst suppression was reported in 20 of the 35 patients for whom EEG data were available (57%), but various other EEG patterns were also described, including at least 7/35 (20%) with a discontinuous pattern.

Our patient had an ambiguous response to pyridoxine in the neonatal period – no change in seizure frequency but increased apnoeic episodes and lethargy, which have been associated with pyridoxine responsiveness in antiquitin deficiency (Stockler et al. 2011) – and later had a period of relatively good seizure control that coincided with pyridoxine administration; in retrospect, she may well have been partially pyridoxine responsive. 37/46 (80%) of previously described patients underwent a pyridoxine trial; of these, 17/37 (46%) showed a clear clinical response. Our patient's p.R225H genotype is one of several reported to be associated with pyridoxine responsiveness (Mills et al. 2014; Plecko et al. 2014). Five patients homozygous for p.R225H and seven homozygous for a mutant allele containing p.R225H and p.R116Q have been identified. Of these 12 patients, 10 had a clear response to pyridoxine. Furthermore, each of the two non-responsive patients (patients 36 and 38) had younger affected siblings who were pyridoxine responsive but who were noted to respond gradually. It is therefore conceivable that patients 36 and 38 might also have responded to longer pyridoxine trials. The correlation between genotype and pyridoxine responsiveness is imperfect for other mutations as well. For instance, only 4/7 patients with one or two p.D33V mutant alleles were classified as pyridoxine responsive, suggesting that genotype alone cannot accurately predict pyridoxine responsiveness.

PLP was administered to 24/46 patients (52%) and was associated with a clear positive response in 18/24 (75%). Surprisingly, in 4/24 (17%) of cases, pyridoxine-responsive patients reacted poorly to a transition to PLP (with status epilepticus in two cases and an increase or recurrence of seizures in two others).

Clinical outcome was influenced by treatment and response to treatment. All 17 pyridoxine-responsive patients survived, as did all but one (93%) of the 15 patients who either did not receive pyridoxine or did not respond but *did* receive PLP. On the other hand, all 13 patients who either did not receive pyridoxine or did not respond, and also did not receive PLP, died: 9 in the first weeks of life (69%) and the rest between 5 and 7 months (31%). One patient survived for approximately 3 years before PLP treatment was begun (Patient 9); therefore, survival beyond 1 year is possible, though rare, in non-pyridoxine-responsive patients without PLP treatment. Overall, 14/46 patients (30%) died, at a median age of 33 days (range 7 days to 7 months). Information (often limited) about psychomotor development was available for all 32 survivors: 12/32 (38%) had significant developmental and/or neurological sequelae, while 20/32 (63%) had a normal or mildly delayed development.

Findings in Cerebrospinal Fluid

Our patient had normal CSF levels of PLP, 3-ortho-methyldopa and 5-HIAA, with mildly decreased levels of HVA.

In the literature, PLP was measured before PLP administration in only 7/46 patients (15%). In 6/7 (86%), PLP levels were below the reference range. The exception, a patient with a low-normal CSF PLP level, was receiving high-dose pyridoxine, with a partial clinical and biochemical response (Patient 1).

3-Ortho-methyldopa levels were elevated, as expected, in 9/12 (75%) patients; 3/12 (25%) of patients, like ours, had normal levels (one of these, however, was receiving pyridoxine at the time of sampling). HVA and 5-HIAA levels were reported in 19/46 patients (41%). Of these, 5-HIAA was low in seven (37%), normal in eight (42%) and high in 4 (21%); Likewise, HVA was low in seven cases (37%), normal in nine (47%) and high in three (16%). Of the patients with normal 5-HIAA and HVA, however, two were receiving pyridoxine at the time of sampling.

Table 3 Analysis of clinical findings in previous descriptions of patients with PNPO deficiency

		GA	Sz onset	Seizure type			Acidosis	EEG	Pyridoxine		PLP		Outcome		
Patients	*PNPO* mutations	<37 weeks	≤24 h	Any	Myoclonic	Abnormal mvts of the head/ eye/ mouth	Metabolic acidosis	Burst suppression	Received	Response	Received	Response	Death	Mild delay or normal development	Alive but significant sequelae
This report	hz R225H	✓	✓	✓	✓	✓			Yes	+/−	No		✓		
1 (A)	hz G118R		✓	✓	✓	✓			Yes	+	Yes	+		✓	
2 (B)	hz D33V			✓		✓			Yes	−	Yes	+		✓	
3 (C)	hz R225C		✓	✓				✓	Yes	−	Yes	+		✓	
4 (D)	D33V+c.246delT		✓	✓		✓			Yes	−	Yes	+		✓	
5 (E)	hz A174X	✓	✓	✓	✓	✓		✓	Yes	−	Yes	+/−	✓		
6 (F)	hz R95H	✓	✓	✓	✓			NA	Yes	+/−	No		✓		
7 (G)	Presumably hz R95C	✓		✓	NA	NA		NA	No		No		✓		
8 (G)	hz R95C			✓	✓			✓	Yes	−	Yes	+			✓
9 (H)	hz R95C	✓		✓	NA	NA	✓	✓	Yes	−	Yes	+			✓
10 (H)	Presumably hz R95C	✓		✓	NA	NA	✓		No		No		✓		
11 (H)	hz R95C	✓		✓			✓	NA	Yes	−	No		✓		
12 (I)	Presumably hz R229W	✓		✓	NA	NA		NA	No		No		✓		
13 (I)	Presumably hz R229W	✓		✓	NA	NA		NA	No		No		✓		
14 (I)	hz R229W	✓	✓	✓	✓	✓	✓	✓	Yes	+/−	No		✓		
15 (I)	hz R229W	✓	✓	✓	✓	✓	✓	✓	Yes	+/−	No		✓		
16 (J)	Presumably hz IVS3-1G>A	✓	✓	✓	NA	NA	✓	NA	No		No		✓		
17 (J)	hz IVS3-1G>A	✓	✓	✓		✓	✓	✓	Yes	−	Yes	+			✓
18 (K)	hz X262Q	✓	✓	✓	NA	NA	✓		Yes	−	No		✓		
19 (K)	hz X262Q	✓	✓	✓	NA	NA	✓	✓	Yes	−	No		✓		
20 (L)	R95H+E50K, c.364-1G>A (splice errors)	✓	✓	✓	NA	NA	✓	✓	Yes	−	Yes	+			✓
21 (M)	hz D33V		✓	✓				NA	Yes	−	Yes	+		✓	
22 (N)	hz P213S		✓	✓				✓	Yes	−	Yes	+		✓	
23 (N)	hz P213S	✓	None						No		Yes	+		✓	
24 (O)	hz R95C	✓	✓	✓		✓		✓	No		Yes	+		✓	
25 (P)	Q214fs+?	✓	✓	✓			✓	✓	Yes	−	Yes	+		✓	
26 (Q)	hz R116Q			✓					No		Yes	+		✓	
27 (R)	hz R116Q			✓					Yes	+	No			✓	
28 (S)	hz D33V		✓	✓	✓	✓	✓		Yes	+	Yes	Irritability, increase in sz		✓	
29 (T)	hz D33V			✓	NA	NA			Yes	+	No			✓	
30 (U)	D33V+c.264-21_264-1 delinsC (splice errors)			✓	NA	NA			Yes	+	Yes	+			✓

31 (V)	D33V; R116Q; R225C (no parental data)	✓	✓	✓	NA	NA		NA	Yes	+	No			✓	
32 (W)	R116Q; R225H+R116Q; R225H	NA	✓	✓				NA	Yes	+	Yes	Recurrence of sz			✓
33 (X)	hz IVS2+2T>C	✓	✓	✓	NA	NA	✓	✓	Yes	–	Yes	+		✓	
34 (Y)	hz R225H	✓	✓	✓	✓			✓	Yes	+	Yes	+			✓
35 (Z)	hz R229Q	✓	✓	✓	✓			✓	No		Yes	+			✓
36 (AA)	Presumably R116Q; R225H +R116Q; R225H	✓	✓	✓	✓			✓	Yes	–	No		✓		
37 (AA)	R116Q; R225H+R116Q; R225H	✓	✓	✓	✓			✓	Yes	+	Yes	SE			✓
38 (BB)	Presumably hz R225H	NA	NA	✓	✓			✓	Yes	–	No		✓		
39 (BB)	hz R225H		✓	✓	✓			NA	Yes	+	Yes	SE			✓
40 (BB)	hz R225H	✓		✓	✓			✓	Yes	+	No			✓	
41 (CC)	R141C+S93S; A94_L97del			✓	✓				Yes	+	No			✓	
42 (DD)	hz R225H	✓	NA	✓	✓			✓	Yes	+	No				✓
43 (DD)	hz R225H		✓	✓	✓				Yes	+	No			✓	
44 (EE)	hz R225H			✓	✓				Yes	+	No			✓	
45 (FF)	hz R225H	✓		✓	✓			NA	Yes	+	No				✓
46 (GG)	hz R225H		✓	✓	✓				Yes	+	No			✓	
Info available		44	44	46	33	33	46	35	46		46		46	46	46
n		27	27	45	20	9	13	20	37	17	24	18	14	20	12
%		61	61	98	61	27	28	57	80	46	52	75	30	43	26

Information concerning our patient is included in the table for the sake of comparison, but is not used in the analysis.

EEG electroencephalogram, *GA* gestational age, *hrs* hours, *hz* homozygous (in some publications, it is not clear whether this was verified by parental studies), *mvts* movements, *NA* not available, *PLP* pyridoxal-5′-phosphate, treatment with, *presumably* genotype not reported, but presumed to be the same as that of an affected sibling, *SE* status epilepticus, *sz* seizure(s), *wks* weeks

Discussion

To our knowledge, this is the first PNPO-deficient patient to present with normal CSF PLP levels in the absence of PLP or pyridoxine supplementation.

There is no evidence that this counterintuitive finding is explicable by error or artefact. The diagnosis of PNPO deficiency in our patient has been confirmed by both molecular and enzymatic methods. Likewise, there was no evidence of sample mix-up or laboratory error. Lumbar ultrasound showed no pockets of abnormal fluid accumulation nor anatomical abnormalities that might affect CSF dynamics. Repeat testing of the CSF specimen confirmed the initial observations, yielding an inter-assay difference of less than 1%. Our patient died before the results of *PNPO* sequencing became available, and repeat lumbar puncture or a therapeutic trial with PLP was not performed.

In retrospect our patient was probably partially pyridoxine responsive, like other reported p.R225H homozygotes. She had received no pyridoxine for 3 months before the lumbar puncture, however, making it unlikely that the previous treatment with pyridoxine could have altered PLP concentrations at the time of CSF sampling.

Pretreatment PLP levels have not been reported for other patients with p.R225H *PNPO* mutations; indeed, pretreatment PLP levels have been reported for only seven patients in the literature. The fraction of PNPO-deficient patients with normal CSF PLP levels is therefore unknown. A channelling mechanism has been suggested for the transfer of newly formed PLP from pyridoxal kinase and PNPO to the various enzymes that require it as a cofactor (di Salvo et al. 2011). If this is the case, certain *PNPO* mutations might adversely affect this transfer step as well as the known enzymatic activity of PNPO. We speculate that, under such circumstances, a normal CSF PLP level would be insufficient to ensure adequate delivery of cofactor to PLP-dependent enzymes, leading a patient to become symptomatic despite a normal CSF PLP level.

By showing that a normal CSF PLP level does not suffice to rule out PNPO deficiency, this report highlights the difficulties involved in diagnosing this condition. The clinical spectrum of PNPO deficiency has now been broadened, ranging from in utero presentations to a hitherto asymptomatic child presenting at 5 months with infantile spasms (Mills et al. 2014). As seen above, neurotransmitter metabolite levels may be variable in PNPO deficiency. A low level of CSF PLP is not a specific marker for PNPO deficiency (Footitt et al. 2011; Goyal et al. 2013); the present report shows that neither is it a sensitive diagnostic marker. A trial of treatment with PLP has the advantage of being potentially both therapeutic and diagnostic. PLP responsiveness, however, may also indicate other causes of PLP deficiency (Goyal et al. 2013). PLP may also exert a nonspecific anticonvulsant effect (Wang et al. 2005), although the responsive patients were not tested for PNPO deficiency in this study. Surprisingly, treatment with PLP may even exacerbate symptoms, at least in some pyridoxine-responsive patients (Mills et al. 2014; Plecko et al. 2014). Finally, while *PNPO* sequencing and deletion/duplication analysis is specific to PNPO deficiency and is likely to identify most affected patients, its utility is at present limited by the difficulty of interpreting sequence variants of uncertain significance and the possibility of unidentified pathogenic mutations in noncoding sequence.

In the absence of a diagnostic gold standard, a combination of approaches is likely to yield the best results. As we have shown, a normal CSF PLP level does not rule out PNPO deficiency. The present report highlights the critical importance of a timely therapeutic trial of pyridoxal 5′-phosphate in the context of a neonatal epileptic encephalopathy; in the presence of a positive response, molecular testing can in most cases clarify the diagnosis.

Acknowledgments GAM and AML are supported by the Canadian Institutes for Health Research (CIHR).

PTC and PBM are supported by funding from Great Ormond Street Children's Charity.

Synopsis

Pyridoxal 5′-phosphate levels in cerebrospinal fluid may be normal in neonatal epileptic encephalopathy caused by pyridox(am)ine 5′-phosphate oxidase (PNPO) deficiency.

Compliance with Ethics Guidelines

Conflicts of Interest

Alina Levtova, Stephane Camuzeaux, Anne-Marie Laberge, Pierre Allard, Catherine Brunel-Guitton, Paola Diadori, Elsa Rossignol, Peter T. Clayton, Philippa B. Mills and Grant A. Mitchell declare no conflict of interest.

Keith Hyland is Vice President of Medical Neurogenetics, a company that measures pyridoxal 5′-phosphate in cerebrospinal fluid and performs sequencing of the *PNPO* gene.

Informed Consent

The investigations performed were done on a clinical basis. Consent was obtained from the family for the publication of this patient's history.

Details of the Contributions of Individual Authors

- Writing and preparation of the manuscript: AL and GM
- Clinical management and investigation of the patient: AL, AML, PD, ER, GM
- Detailed review of the patient's neurological and electrophysiological presentation: PD, ER
- cDNA cloning and expression studies of the mutant PNPO enzyme: SC, PC, PM
- Measurement of neurotransmitter metabolites and pyridoxal 5′-phosphate in cerebrospinal fluid and analysis of fluorescence chromatograms: KH
- Critical review of the manuscript: All authors.

References

Bagci S, Zschocke J, Hoffmann GF et al (2008) Pyridoxal phosphate-dependent neonatal epileptic encephalopathy. Arch Dis Child Fetal Neonatal Ed 93(2):F151–F152

di Salvo ML, Contestabile R, Safo MK (2011) Vitamin B(6) salvage enzymes: mechanism, structure and regulation. Biochim Biophys Acta 1814(11):1597–1608

Footitt EJ, Heales SJ, Mills PB, Allen GF, Oppenheim M, Clayton PT (2011) Pyridoxal 5′-phosphate in cerebrospinal fluid; factors affecting concentration. J Inherit Metab Dis 34(2):529–538

Footitt EJ, Clayton PT, Mills K et al (2013) Measurement of plasma B6 vitamer profiles in children with inborn errors of vitamin B6 metabolism using an LC-MS/MS method. J Inherit Metab Dis 36 (1):139–145

Goyal M, Fequiere PR, McGrath TM, Hyland K (2013) Seizures with decreased levels of pyridoxal phosphate in cerebrospinal fluid. Pediatr Neurol 48(3):227–231

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

Hyland K, Surtees RA, Heales SJ, Bowron A, Howells DW, Smith I (1993) Cerebrospinal fluid concentrations of pterins and metabolites of serotonin and dopamine in a pediatric reference population. Pediatr Res 34(1):10–14

Khayat M, Korman SH, Frankel P et al (2008) PNPO deficiency: an under diagnosed inborn error of pyridoxine metabolism. Mol Genet Metab 94(4):431–434

Kuo MF, Wang HS (2002) Pyridoxal phosphate-responsive epilepsy with resistance to pyridoxine. Pediatr Neurol 26(2):146–147

Michaud JL, Lachance M, Hamdan FF et al (2014) The genetic landscape of infantile spasms. Hum Mol Genet 23(18):4846–4858

Mills PB, Surtees RA, 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

Mills PB, Camuzeaux SS, Footitt EJ et al (2014) Epilepsy due to PNPO mutations: genotype, environment and treatment affect presentation and outcome. Brain 137(Pt 5):1350–1360

Musayev FN, Di Salvo ML, Ko TP, Schirch V, Safo MK (2003) Structure and properties of recombinant human pyridoxine 5′-phosphate oxidase. Protein Sci 12(7):1455–1463

Ormazabal A, Oppenheim M, Serrano M et al (2008) Pyridoxal 5′-phosphate values in cerebrospinal fluid: reference values and diagnosis of PNPO deficiency in paediatric patients. Mol Genet Metab 94(2):173–177

Pearl PL, Hyland K, Chiles J, McGavin CL, Yu Y, Taylor D (2013) Partial pyridoxine responsiveness in PNPO Deficiency. JIMD Rep 9:139–142

Plecko B, Paul K, Mills P et al (2014) Pyridoxine responsiveness in novel mutations of the PNPO gene. Neurology 82 (16):1425–1433

Porri S, Fluss J, Plecko B et al (2014) Positive outcome following early diagnosis and treatment of pyridoxal 5-phosphate oxidase deficiency: a case report. Neuropediatrics 45(1):64–68

Ruiz A, García-Villoria J, Ormazabal A et al (2008) A new fatal case of pyridox(am)ine 5′-phosphate oxidase (PNPO) deficiency. Mol Genet Metab 93(2):216–218

Schmitt B, Baumgartner M, Mills PB et al (2010) Seizures and paroxysmal events: symptoms pointing to the diagnosis of pyridoxine-dependent epilepsy and pyridoxine phosphate oxidase deficiency. Dev Med Child Neurol 52(7):e133–e142

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

Veerapandiyan A, Winchester SA, Gallentine WB et al (2011) Electroencephalographic and seizure manifestations of pyridoxal 5′-phosphate-dependent epilepsy. Epilepsy Behav 20(3):494–501

Wang HS, Kuo MF, Chou ML et al (2005) Pyridoxal phosphate is better than pyridoxine for controlling idiopathic intractable epilepsy. Arch Dis Child 90(5):512–515

Ware TL, Earl J, Salomons GS et al (2014) Typical and atypical phenotypes of PNPO deficiency with elevated CSF and plasma pyridoxamine on treatment. Dev Med Child Neurol 56 (5):498–502

JIMD Reports
DOI 10.1007/8904_2015_414

RESEARCH REPORT

Bladder and Bowel Dysfunction Is Common in Both Men and Women with Mutation of the ABCD1 Gene for X-Linked Adrenoleukodystrophy

Johann Hofereiter • Matthew D. Smith • Jai Seth • Katarina Ivana Tudor • Zoe Fox • Anton Emmanuel • Elaine Murphy • Robin H. Lachmann • Jalesh Panicker

Received: 24 September 2014 / Revised: 20 January 2015 / Accepted: 26 January 2015 / Published online: 13 March 2015

Abstract *Background*: X-linked adrenoleukodystrophy (X-ALD) is a disorder caused by mutations in the ABCD1 gene. The commonest phenotype of X-ALD is adrenomyeloneuropathy (AMN), which is characterised by involvement of the spinal cord and peripheral nerves. The aim of this study was to evaluate bladder and bowel symptoms in men with AMN and female X-ALD carriers.

Methods: In this cross-sectional study, patients with confirmed mutation of the ABCD1 gene attending a tertiary care service were approached about bladder and bowel complaints and completed the Urinary Symptom Profile (USP), Qualiveen Short Form (SF-Qualiveen), International Prostate Symptom Score (IPSS) and Neurogenic Bowel Dysfunction (NBD) questionnaires. Neurological disability was assessed using the Expanded Disability Status Scale (EDSS).

Results: Forty-eight patients participated, 19 males (mean EDSS score ($n = 16$) 5.0 (95% CI ± 1.03)) and 29 females (mean EDSS score ($n = 25$) 3.2 (95% CI ± 0.98)). Overactive bladder (OAB) symptoms were reported in both males (100%, $n = 19$) and females (86.2%, $n = 25$). There was no significant gender difference in severity of OAB symptoms ($P = 0.35$) and impact on quality of life ($P = 0.13$). Furthermore, there was no significant difference in OAB severity when symptoms were compared between female carriers and a cohort of women ($n = 17$) with spinal cord damage due to multiple sclerosis ($P = 0.27$). Twenty-one percent ($n = 4$) of males and 10% ($n = 3$) of females had moderate to severe bowel dysfunction.

Conclusions: Bladder and bowel complaints are common in both men with AMN and female carriers. They have a significant impact on the quality of life yet are under-reported and under-treated. Though having an X-linked pattern of inheritance, female carriers may experience overactive bladder symptoms which are as severe as in male patients and are likely to be neurological in origin.

Communicated by: Carla E. Hollak, M.D.

Competing interests: None declared

J. Hofereiter (✉)
Department of Neurosurgery, University Hospital Munich, Ludwig-Maximilians-University (LMU), Marchioninistraße 15, 81377 Munich, Germany
e-mail: johann.hofereiter@med.uni-muenchen.de

M.D. Smith · J. Seth · K.I. Tudor · J. Panicker
Department of Uro-Neurology, National Hospital for Neurology and Neurosurgery, UCL Institute of Neurology, Queen Square, London, UK

Z. Fox
UCL Biostatistics Unit, University College London, London, UK

A. Emmanuel
GI Physiology Unit, University College London Hospital, London, UK

E. Murphy · R.H. Lachmann
Charles Dent Metabolic Unit, National Hospital for Neurology and Neurosurgery, Queen Square, London, UK

Introduction

X-linked adrenoleukodystrophy (ALD) (OMIM #300100) is a disorder caused by mutations in the ABCD1 (ATP-binding cassette, subfamily D, member 1) gene which encodes a peroxisomal membrane protein. This results in an accumulation of saturated very long-chain fatty acids (VLCFAs) in plasma and tissues as a consequence of impaired peroxisomal β-oxidation. The disorder primarily affects the adrenal cortex and the central nervous system

(Moser et al 2007). There are two main phenotypes of this X-linked condition in males: adrenomyeloneuropathy (AMN), which is the most frequent phenotype, and cerebral ALD. In contrast to cerebral ALD, which is characterised by severe inflammatory demyelination within the brain and typically presents in young boys with rapidly progressive neurological disability, AMN usually develops in the third or fourth decade as a syndrome of slowly progressive spastic paraparesis with sensory disturbances and results from noninflammatory axonopathy involving the ascending and descending tracts of the spinal cord (Moser et al 2007; Kemp et al 2012). AMN has an X-linked pattern of inheritance and typically affects males. Female X-ALD carriers are either asymptomatic or may develop a milder, more slowly progressive neurological disability (O'Neill et al 1984; Jangouk et al 2012; Lourenco et al 2012).

Bladder and bowel dysfunction has been reported in men with AMN in case reports or short series (Griffin et al 1977; O'Neill et al 1981; Tezuka et al 1991; Walther and Cutler 1997; Sakakibara et al 1998; Silveri et al 2004). To some extent, urinary and faecal incontinence have recently been reported in female X-ALD carriers (Engelen et al 2014) but have not been evaluated in greater detail using validated questionnaires. The early identification of the carrier status because of better availability of screening facilities for family members has uncovered a wide spectrum of symptoms and signs amongst female carriers (Jangouk et al 2012). The objective of this study was therefore to evaluate and compare the prevalence, severity and impact on health-related quality of life with regard to bladder and bowel symptoms in a cohort of men and women carrying the ABCD1 mutation.

Methods

This was a cross-sectional study of male patients with AMN and female X-ALD carriers attending a dedicated unit for inherited metabolic diseases at a tertiary-level neurology centre. Of 77 patients (32 males, 45 females) attending the unit, 76 were contacted by telephone. One male patient developed cerebral ALD and was therefore not included (Fig. 1). In this service evaluation, patients agreeing to take part were asked about the extent and duration of their neurological symptoms, as well as bladder and bowel complaints.

Four validated self-administered questionnaires were sent by post, including the Urinary Symptom Profile (USP), International Prostate Symptom Score (IPSS), Qualiveen Short Form (SF-Qualiveen) and the Neurogenic Bowel Dysfunction (NBD) score. These were intended to evaluate specific domains of bladder and bowel complaints and the effects these had on quality of life. The USP

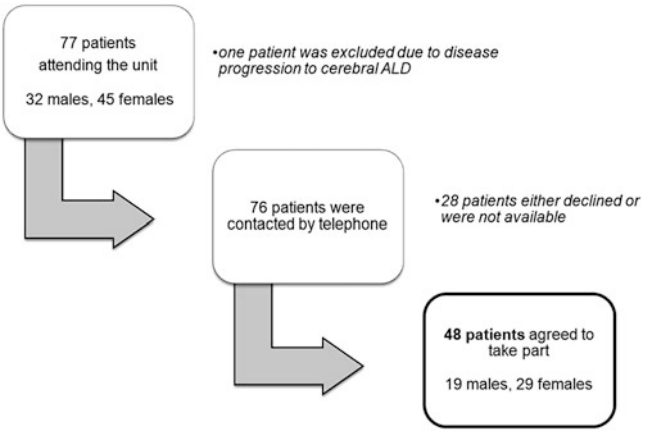


Fig. 1 Flow chart illustrating the recruitment process of patients with AMN attending a tertiary unit for inherited metabolic diseases

questionnaire provides a comprehensive evaluation of urinary symptoms and their severity in males and females, assessing three domains: overactive bladder (OAB) (score range 0 to 21), stress urinary incontinence (SUI) (score range 0–9) and low stream (LS) (score range 0–9). A higher score indicates greater severity (Haab et al 2008). The IPSS questionnaire is used to assess bladder symptoms and provides an indicator of severity (Barry et al 1992). Even though the questionnaire was initially developed to assess voiding symptoms in men with benign prostatic hyperplasia (BPH), it is neither prostate nor gender specific (Chai et al 1993; Chancellor and Rivas 1993; Lepor and Machi 1993). The SF-Qualiveen questionnaire is a health-related quality of life questionnaire (HRQoL) for urinary disorders. The questionnaire is composed of 8 items distributed in four domains: "bother with limitations" (two items), "frequency of limitations" (two items), "fears" (two items) and "feelings" (two items). Patients are asked to recall their experience and respond to each question on a 5-point scale (ranging from 0 indicating no impact on HRQoL to 4 indicating high impact on HRQoL). Each Qualiveen domain score is calculated as an average of the scores for the items in each domain (Bonniaud et al 2008). The Neurogenic Bowel Dysfunction (NBD) questionnaire covers bowel complaints including both constipation and faecal incontinence, weighing each symptom of NBD according to impact on the quality of life, and provides a symptom-based score (maximum score 47) (Krogh et al 2006). Completed questionnaires were sent back in self-stamped envelopes.

Additionally, overactive bladder (OAB) scores from the USP questionnaire were compared to the scores from a cohort of women with another disorder affecting the spinal cord, multiple sclerosis (MS) ($n = 17$, mean age 46.5 years [range 24–65]), who had completed the questionnaire separately.

Neurological disability was assessed from the medical records using the Expanded Disability Status Scale (EDSS) (Kurtzke 1983).

Categorical data were compared using chi-square tests or Fisher's exact tests depending on the frequency. Numerical data was examined and compared between groups using either an independent *t*-test or Mann–Whitney *U* test depending on the distribution. The correlation between EDSS and OAB scores was measured using Pearson's correlation coefficient (0 = no agreement, 0.01–0.20 = slight, 0.21–0.40 = fair, 0.41–0.60 = moderate, 0.61–0.80 = substantial and 0.81–1 almost perfect agreement). All analyses were performed using Stata 11.2, StataCorp LP, College Station, Texas. Differences were considered statistically significant at $P < 0.05$. For the individual comparisons of the SF-Qualiveen scores, Bonferroni correction was applied considering a *P*-value <0.01 to be significant.

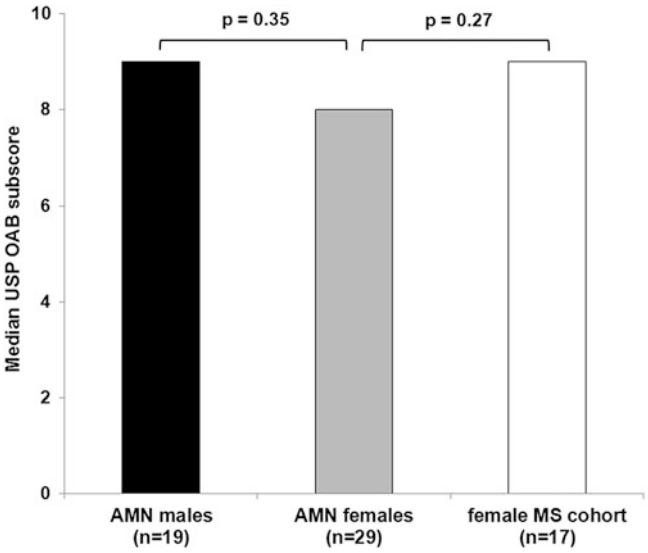


Fig. 2 Comparison of median OAB scores between different cohorts. A *P*-value <0.05 is considered to be statistically significant

Results

Forty-eight patients (19 males, mean age 47.5 years [range 21–68], and 29 females, mean age 46.8 years [range 20–69]) completed the questionnaires, giving an overall response rate of 63.2%. The mean EDSS score ($n = 41$) was 3.9 (95% CI ± 0.77): 5.0 (95% CI ± 1.03) for males ($n = 16$) and 3.2 (95% CI ± 0.98) for females ($n = 25$).

Pattern of Bladder Symptoms and Severity

Overactive bladder (OAB) symptoms were reported in both males (100%, $n = 19$) and females (86.2%, $n = 25$) (median duration 4 years [IQR: 3.5–8 years] in males and 2.5 years [IQR: 0–8 years] in females) according to the Urinary Symptom Profile (USP) questionnaire. Less frequently, patients reported stress urinary incontinence or low stream. The pattern of bladder symptoms and scores on the USP questionnaire are presented in Table 1A and 1B, respectively. Notably, all males and 17 (58.6%) females with overactive bladder symptoms also reported problems with mobility (median duration 9 years [IQR: 5.5–16 years] in males and 1 year [IQR: 0–9 years] in females). There is a fair correlation ($r = 0.4044$ [22 females, 15 males]) between OAB score and EDSS.

When the OAB subscores were compared between males and females, there was no significant difference between the groups ($P = 0.35$) (Fig. 2). The OAB subscores in the female cohort were compared with scores in a cohort of women with spinal cord damage due to multiple sclerosis (MS) ($n = 17$), and no significant difference was noted between the two groups ($P = 0.27$) (Fig. 2).

The mean score on the IPSS for males was 16.3 (95% CI ± 4.8) and 9.1 (95% CI ± 2.7) for females. The severity of bladder symptoms, as determined by the IPSS score, is shown in Fig. 3. Eighty-four percent ($n = 16$) of males and 55% ($n = 16$) of females reported moderate or

Table 1 Pattern of bladder symptoms and scores in patients with AMN as assessed by the USP questionnaire

	A		B	
	Number of patients with scores ≥ 1		Median score and IQR	
USP questionnaire domain	Male ($n = 19$)	Female ($n = 29$)	Male ($n = 19$)	Female ($n = 29$)
Overactive bladder (OAB)	19 (100%)	25 (86.2%)	9 (5–14)	8 (3–10)
Stress urinary incontinence (SUI)	8 (42.1%)	19 (65.5%)	0 (0–3)	2 (0–4)
Low stream (LS)	16 (84.2%)	16 (55.2%)	2 (1–3)	2 (0–2)

Possible score range for USP domains: OAB (0–21), SUI (0–9), LS (0–9)
USP Urinary Symptom Profile, *IQR* interquartile range

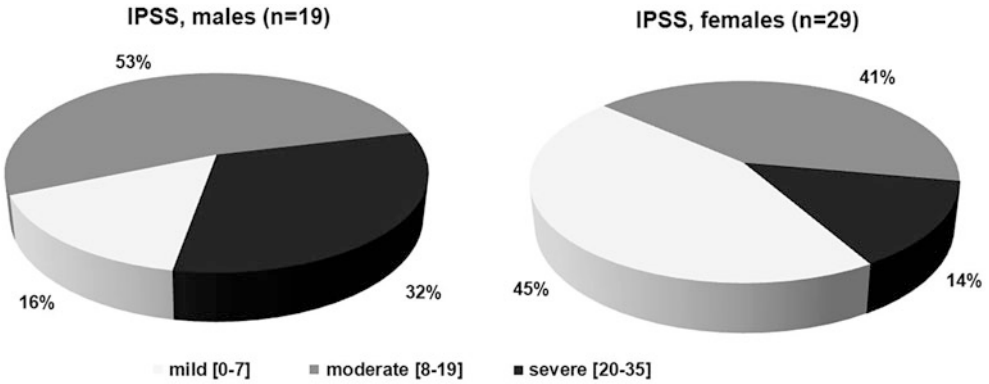


Fig. 3 Severity of bladder symptoms in patients with AMN, graded mild [0–7], moderate [8–19] or severe [20–35] according to the IPSS

Table 2 Scores on the SF-Qualiveen questionnaire, comparing between males and females

	Mean score (95% CI)		
SF-Qualiveen domain	Male ($n = 19$)	Female ($n = 29$)	*P*-value
Bother with limitations	2.1 (1.5–2.7)	1.3 (0.9–1.7)	0.06
Frequency of limitations	2.1 (1.5–2.7)	1.5 (1.1–1.9)	0.10
Fears	1.8 (1.2–2.4)	1.5 (1.1–1.9)	0.42
Feelings	1.7 (1.1–2.3)	1.2 (0.8–1.6)	0.17
Overall score	1.9 (1.4–2.4)	1.4 (1.0–1.8)	0.13

For the individual comparisons, Bonferroni correction was applied considering a *P*-value <0.01 to be statistically significant
CI confidence interval

severe bladder symptoms of which 6 male (32%) and 4 female patients (14%) had bladder symptoms graded as severe. Despite this, only 42.1% ($n = 8$) of male and 24.1% ($n = 7$) of female patients were receiving treatment for bladder symptoms at the time of the study. There is a moderate correlation ($r = 0.4094$ [22 females, 15 males]) between IPSS and EDSS.

Impact of Bladder Symptoms on Health-Related Quality of Life

The impact on health-related quality (HRQoL) of life as assessed by the SF-Qualiveen questionnaire is presented in Table 2. The overall mean score for males was 1.9 (95% CI $\pm$ 0.5), whilst it was 1.4 (95% CI $\pm$ 0.4) for the female cohort, suggesting an impact on HRQoL but no significant difference between groups ($P = 0.13$).

Pattern of Bowel Complaints

During the telephone interview, 11 (57.9%) male and 10 (34.5%) female patients reported bowel complaints, the predominant symptom being constipation. Most patients, 13 (68.4%) males and 22 (75.9%) females, had none or "very minor" bowel symptoms on the NBD questionnaire. However, four males (21%) and three females (10%) reported moderate to severe bowel symptoms (Fig. 4).

Discussion

Lower urinary tract dysfunction has been described in the ALD spectrum of disorders. Patients most often report overactive bladder symptoms, and urodynamic testing demonstrates detrusor overactivity (Griffin et al 1977; O'Neill et al 1981; Tezuka et al 1991; Walther and Cutler 1997; Sakakibara et al 1998; Shinbo et al 2001; Silveri et al 2004). Adrenomyeloneuropathy (AMN) represents a more slowly progressive form of the condition and presents predominantly as a spinal cord syndrome with spastic paraparesis and sensory disturbances in men. There have been a few case reports of bladder dysfunction in AMN, and urodynamics demonstrate the presence of detrusor overactivity and detrusor sphincter dyssynergia (Griffin et al 1977; Tezuka et al 1991; Walther and Cutler 1997; Sakakibara et al 1998), a pattern distinctive of spinal cord dysfunction.

Through a series of standardised validated questionnaires, we have established that lower urinary tract symptoms are common in male patients with AMN and in X-ALD carriers. Urinary urgency, frequency, nocturia and incontinence, collectively known as overactive bladder symptoms, were most commonly reported, and 67% of patients reported symptoms to be of moderate or severe grade and having a significant impact on the quality of life. Despite this high figure, only a third of patients were receiving treatment for managing bladder symptoms at the time of the study.

Adrenoleukodystrophy, respectively, adrenomyeloneuropathy, has an X-linked pattern of inheritance and therefore is classically a disorder affecting only males, whereas

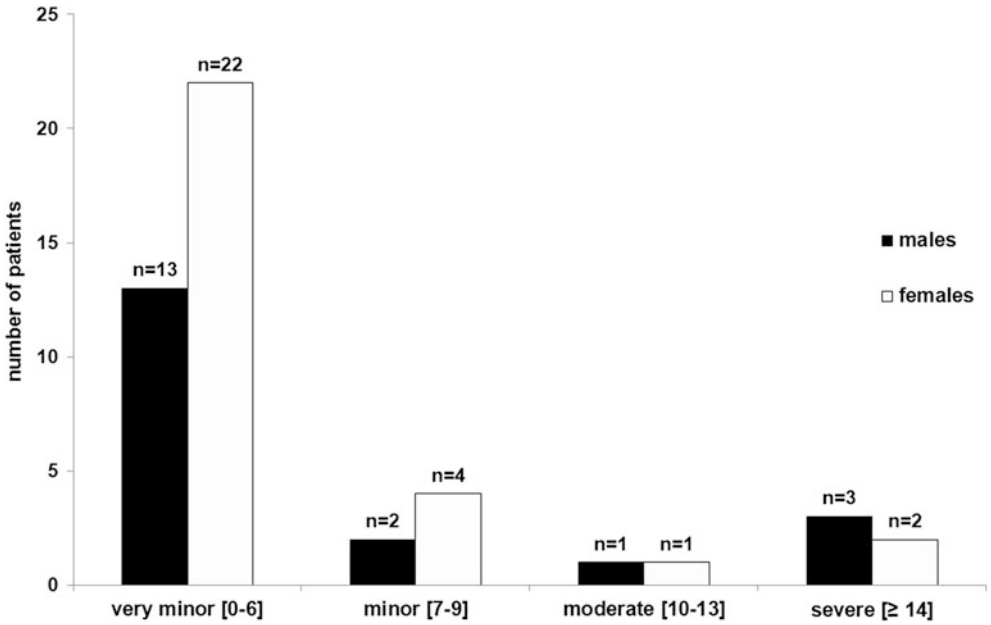


Fig. 4 Bowel dysfunction in males ($n = 19$) and females ($n = 29$) with AMN. Patients categorised into standardised severity grades (very minor [0–6], minor [7–9], moderate [10–13] and severe [≥14]) according to their NBD score

females carrying only one copy of the mutation should not express the phenotype. However, it is now recognised that female carriers of the mutation also manifest with symptoms (O'Neill et al 1984; Jangouk et al 2012; Engelen et al 2014). Females may develop a range of neurological deficits including hyperreflexia, impaired vibration sense and also spastic paraparesis, deficits reported also in our cohort, and may erroneously be diagnosed initially as having some other neurological condition such as multiple sclerosis before being found to be carrying the ABCD1 gene mutation (Dooley and Wright 1985; Stockler et al 1993; Krenn et al 2001; Di Filippo et al 2011).

To our knowledge, bladder and bowel symptoms have never been evaluated in greater detail using validated questionnaires in female carriers of the ABCD1 gene mutation, and the results of this study suggest that these symptoms are common. As in male counterparts, overactive bladder symptoms were most often reported. The results of previous studies evaluating urodynamic findings in patients with AMN (Griffin et al 1977; Tezuka et al 1991; Walther and Cutler 1997; Sakakibara et al 1998) suggest that these symptoms are due to detrusor overactivity. Overactive bladder symptoms are prevalent in the general population (Milsom et al 2001); however, there is reason to believe that symptoms were neurological in origin. Firstly, the severity of overactive bladder symptoms was similar between males and females, with comparable degrees of neurological disability between the two cohorts. The severity of bladder symptoms correlated with disability on the EDSS. Secondly, when symptoms were compared to that reported in a cohort of women with a known spinal cord disorder (MS), where lower urinary tract dysfunction is well described (Fingerman and Finkelstein 2000; Fowler et al 2009), overactive bladder scores were found to be not significantly different. Stress incontinence was reported less often and is likely to be multifactorial in AMN (Chaudhry et al 1996) though this was not further studied. Symptoms of bowel dysfunction were apparent in males and females; however, they were less common than bladder symptoms. Some patients did however experience severe symptoms, and these should therefore be specifically enquired about during the clinical evaluation, as effective treatments are available to address these (Preziosi and Emmanuel 2009).

Bladder dysfunction in the setting of X-linked adrenoleukodystrophy remains a poorly addressed problem. The routine workup of patients with progressive neurological disorders, involving a focussed history and non-invasive tests, is often sufficient to initiate treatment for the neurogenic bladder. The treatment options available for managing overactive bladder symptoms are many, including lifestyle modifications, antimuscarinic medications and intradetrusor injections of botulinum toxin (Fowler et al 2009; Panicker and Fowler 2010).

We present possibly the largest cohort of X-ALD patients reporting bladder and bowel symptoms and use validated questionnaires providing an accurate assessment of symptoms. The study was hospital based, and therefore the prevalence of bladder and bowel symptoms observed might not accurately reflect that of men and women with an ABCD1 mutation in general. Patients with minimal symptoms who might not report to a tertiary centre are likely to be underrepresented. Nevertheless, the study

shows that bladder and bowel complaints are common in patients with X-ALD, notably in female carriers, and have a significant impact on the quality of life yet are under-reported and under-treated. It is therefore important to enquire about these symptoms, as they are eminently treatable with gratifying results. Though having an X-linked pattern of inheritance, female carriers may experience overactive bladder symptoms which are as severe as in male patients and are likely to be neurological in origin.

Acknowledgements This project received a proportion of funding from the NIHR Biomedical Research Centre. Matthew D. Smith received a research bursary from the Wellcome Trust. K I Tudor is a recipient of the EFNS grant named "Department–Department Co-operation Programme".

Take-Home Message (Synopsis)

Bladder and bowel dysfunction is common in both men and women with mutation of the ABCD1 gene for X-linked adrenoleukodystrophy, notably in female carriers, and has a significant impact on the quality of life yet are under-reported and under-treated.

Compliance with Ethical Guidelines

Conflict of Interest

Johann Hofereiter, Matthew D. Smith, Jai Seth, Katarina Ivana Tudor, Zoe Fox, Anton Emmanuel, Elaine Murphy, Robin H. Lachmann and Jalesh Panicker declare that they have no conflict of interest.

Ethics Approval

This was an evaluation of current care provided by a clinical service and, according to the guidance from the National Research Ethics Service of the United Kingdom, would fall under the remit of a service evaluation, which does not require ethics review.

Details of the Contributions of Individual Authors

Johann Hofereiter (first author) was involved in the conception and design of the study, collecting data, analysis and drafting and revising the manuscript.

Matthew D. Smith was involved in collecting data, analysis and drafting and revising the manuscript.

Jai Seth was involved in the design of the study, collecting data and revising the manuscript.

Zoe Fox was involved in data analysis and revising the manuscript.

Anton Emmanuel was involved in the design of the study and revising the manuscript.

Elaine Murphy was involved in the design of the study and revising the manuscript.

Robin H. Lachmann was involved in the design of the study and revising the manuscript.

Jalesh Panicker was involved in the conception and design of the study, monitoring data collection and revising the manuscript. He is the guarantor.

References

Barry MJ, Fowler FJ Jr, O'Leary MP et al (1992) The American Urological Association symptom index for benign prostatic hyperplasia. The measurement committee of the American Urological Association. J Urol 148:1549–1557, discussion 1564

Bonniaud V, Bryant D, Parratte B, Guyatt G (2008) Development and validation of the short form of a urinary quality of life questionnaire: SF-Qualiveen. J Urol 180:2592–2598

Chai TC, Belville WD, McGuire EJ, Nyquist L (1993) Specificity of the American Urological Association voiding symptom index: comparison of unselected and selected samples of both sexes. J Urol 150:1710–1713

Chancellor MB, Rivas DA (1993) American Urological Association symptom index for women with voiding symptoms: lack of index specificity for benign prostate hyperplasia. J Urol 150:1706–1708, discussion 1708–1709

Chaudhry V, Moser HW, Cornblath DR (1996) Nerve conduction studies in adrenomyeloneuropathy. J Neurol Neurosurg Psychiatry 61:181–185

Di Filippo M, Luchetti E, Prontera P et al (2011) Heterozygous X-linked adrenoleukodystrophy-associated myelopathy mimicking primary progressive multiple sclerosis. J Neurol 258:323–324

Dooley JM, Wright BA (1985) Adrenoleukodystrophy mimicking multiple sclerosis. Can J Neurol Sci 12:73–74

Engelen M, BarbierM DIM et al (2014) X-linked adrenoleukodystrophy in women: a cross-sectional cohort study. Brain 137:693–706

Fingerman JS, Finkelstein LH (2000) The overactive bladder in multiple sclerosis. J Am Osteopath Assoc 100:S9–S12

Fowler CJ, Panicker JN, Drake M et al (2009) A UK consensus on the management of the bladder in multiple sclerosis. J Neurol Neurosurg Psychiatry 80:470–477

Griffin JW, Goren E, Schaumburg H, Engel WK, Loriaux L (1977) Adrenomyeloneuropathy: a probable variant of adrenoleukodystrophy. I. Clinical and endocrinologic aspects. Neurology 27:1107–1113

Haab F, Richard F, Amarenco G et al (2008) Comprehensive evaluation of bladder and urethral dysfunction symptoms: development and psychometric validation of the urinary symptom profile (USP) questionnaire. Urology 71:646–656

Jangouk P, Zackowski KM, Naidu S, Raymond GV (2012) Adrenoleukodystrophy in female heterozygotes: underrecognized and undertreated. Mol Genet Metab 105:180–185

Kemp S, Berger J, Aubourg P (2012) X-linked adrenoleukodystrophy: clinical, metabolic, genetic and pathophysiological aspects. Biochim Biophys Acta 1822:1465–1474

Krenn M, Bonelli RM, Niederwieser G, Reisecker F, Koltringer P (2001) Adrenoleukodystrophy mimicking multiple sclerosis. Nervenarzt 72:794–797

Krogh K, Christensen P, Sabroe S, Laurberg S (2006) Neurogenic bowel dysfunction score. Spinal Cord 44:625–631

Kurtzke JF (1983) Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 33:1444–1452

Lepor H, Machi G (1993) Comparison of AUA symptom index in unselected males and females between fifty-five and seventy-nine years of age. Urology 42:36–40, discussion 40–31

Lourenco CM, Simao GN, Santos AC, Marques W Jr (2012) X-linked adrenoleukodystrophy in heterozygous female patients: women are not just carriers. Arq Neuropsiquiatr 70:487–491

Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ (2001) How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. BJU Int 87:760–766

Moser HW, Mahmood A, Raymond GV (2007) X-linked adrenoleukodystrophy. Nat Clin Pract Neurol 3:140–151

O'Neill BP, Marmion LC, Feringa ER (1981) The adrenoleukomyeloneuropathy complex: expression in four generations. Neurology 31:151–156

O'Neill BP, Moser HW, Saxena KM, Marmion LC (1984) Adrenoleukodystrophy: clinical and biochemical manifestations in carriers. Neurology 34:798–801

Panicker JN, Fowler CJ (2010) The bare essentials: uro-neurology. Pract Neurol 10:178–185

Preziosi G, Emmanuel A (2009) Neurogenic bowel dysfunction: pathophysiology, clinical manifestations and treatment. Expert Rev Gastroenterol Hepatol 3:417–423

Sakakibara R, Hattori T, Fukutake T, Mori M, Yamanishi T, Yasuda K (1998) Micturitional disturbance in a patient with adrenomyeloneuropathy (AMN). Neurourol Urodyn 17:207–212

Shinbo H, Kageyama S, Hayami S et al (2001) Voiding dysfunction in a patient with adolescent adrenoleukodystrophy. Int J Urol 8:144–147

Silveri M, De Gennaro M, Gatti C, Bizzarri C, Mosiello G, Cappa M (2004) Voiding dysfunction in x-linked adrenoleukodystrophy: symptom score and urodynamic findings. J Urol 171:2651–2653

Stockler S, Millner M, Molzer B, Ebner F, Korner E, Moser HW (1993) Multiple sclerosis-like syndrome in a woman heterozygous for adrenoleukodystrophy. Eur Neurol 33:390–392

Tezuka H, Tachibana Y, Terashi A (1991) A case of adrenomyeloneuropathy with marked spinal cord atrophy on magnetic resonance imaging. Rinsho Shinkeigaku 31:461–464

Walther MM, Cutler GB (1997) Urodynamic abnormalities in two brothers with adrenomyeloneuropathy. World J Urol 15:262–265

JIMD Reports
DOI 10.1007/8904_2015_415

CASE REPORT

Extreme Contrast of Postprandial Remnant-Like Particles Formed in Abetalipoproteinemia and Homozygous Familial Hypobetalipoproteinemia

Masa-aki Kawashiri • Hayato Tada • Marowa Hashimoto • Matsuo Taniyama • Takamitsu Nakano • Katsuyuki Nakajima • Takeshi Inoue • Mika Mori • Chiaki Nakanishi • Tetsuo Konno • Kenshi Hayashi • Atsushi Nohara • Akihiro Inazu • Junji Koizumi • Hirotaka Ishihara • Junji Kobayashi • Tsutomu Hirano • Hiroshi Mabuchi • Masakazu Yamagishi

Received: 21 October 2014 / Revised: 25 January 2015 / Accepted: 27 January 2015 / Published online: 13 March 2015

Communicated by: Robert Steiner

Competing interests: None declared

M.-a. Kawashiri (✉) · H. Tada · T. Inoue · M. Mori · C. Nakanishi · T. Konno · K. Hayashi · M. Yamagishi
Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8641, Japan
e-mail: mk@med.kanazawa-u.ac.jp

M. Hashimoto · J. Koizumi
General Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8641, Japan

M. Taniyama
Division of Endocrinology and Metabolism, Showa University Fujigaoka Hospital, Tokyo, Japan

T. Nakano · K. Nakajima
School of Health Sciences, Gunma University, Maebashi, Japan

A. Nohara · H. Mabuchi
Department of Lipidology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8641, Japan

A. Inazu
Department of Clinical Science, School of Health Science, Kanazawa University, 5-11-80 Kodatsuno, Kanazawa 920-0942, Japan

H. Ishihara
Ishihara Clinic, Fujisawa, Japan

J. Kobayashi
Department of General Medicine, Kanazawa Medical University, Uchinada, Japan

T. Hirano
Division of Diabetes, Metabolism, and Endocrinology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan

Abstract *Background*: Familial hypobetalipoproteinemia (FHBL) and abetalipoproteinemia (ABL) are rare inherited forms of hypolipidemia. Their differential diagnosis is important for predicting of the prognosis and selecting appropriate therapy.

Materials and Methods: Genetic analysis was performed in two patients with primary hypocholesterolemia born from consanguineous parents. The oral fat tolerance test (OFTT) was performed in one patient with FHBL (apoB-87.77) and one with ABL as well as in four normal control subjects. After overnight fasting, blood samples were drawn. Serum lipoprotein and remnant-like particle (RLP) fractions were determined by HPLC analysis.

Results: Both patients with homozygous FHBL were asymptomatic probably because of preserved levels of fat-soluble vitamins, especially vitamin E. The patients with FHBL were homozygous because of novel apoB-83.52 and apoB-87.77 mutations, and although one of them (apoB-87.77) had fatty liver disease, microscopic findings suggesting nonalcoholic steatohepatitis were absent. Fasting apoB-48 and RLP-triglyceride levels in the patient with homozygous FHBL, which were similar to those in normal control subjects, increased after OFTT both in normal control subjects and the patient with FHBL but not in the patient with ABL, suggesting that the fat load administered was absorbed only in the patient with FHBL.

Conclusion: Although lipid levels in the patients with homozygous FHBL and ABL were comparable, fasting, postoral fat loading of apoB-48, as well as RLP-triglyceride

levels, may help in the differential diagnosis of FHBL and ABL and provide a prompt diagnosis using genetic analysis in the future.

Introduction

Hypobetalipoproteinemia is defined as low-density lipoprotein (LDL) cholesterol and apolipoprotein B (apoB) levels below the fifth percentile for age- and sex-matched general populations that have primary and secondary conditions, such as strict vegetarianism, in a hospital setting, cachexia, malnutrition, severe liver disease, and hyperthyroidism (Burnett et al. 2014a, b). Familial hypobetalipoproteinemia (FHBL) (OMIM 107730) is an autosomal dominant hereditary disorder characterized by low levels of LDL-cholesterol and apoB. FHBL heterozygotes are distributed at approximately one in 500–1,000 people in the general population; they are generally asymptomatic and are rarely accompanied by atherosclerosis, but instead by fatty liver (Burnett et al. 2014a; Hooper and Burnett 2014; Katsuda et al. 2009; Lee and Hegele 2014; Welty 2014). The best known molecular cause of FHBL is a mutation in *APOB* gene. A loss-of-function mutation in proprotein convertase subtilisin/kexin type 9 is also a cause of FHBL (Burnett et al. 2014b; Cohen et al 2005; Hooper and Burnett 2014; Welty 2014). On the other hand, FHBL homozygotes are very rare, and their lipid levels are similar to those of patients with abetalipoproteinemia (ABL) (OMIM 200100) (Burnett et al. 2014a; Hooper and Burnett 2014; Lee and Hegele 2014; Welty 2014).

ABL is an extremely rare recessive disorder characterized by undetectable or very low levels of LDL-cholesterol and apoB, failure to thrive, oral fat intolerance, diarrhea, steatorrhea, and acanthocytosis as well as fatty liver and lipid accumulation in enterocytes (Burnett et al. 2014a; Hooper and Burnett 2014; Lee and Hegele 2014; Welty 2014; Yang et al 1999). Neurological abnormalities such as spinocerebellar ataxia and retinitis pigmentosa are caused by deficiencies in fat-soluble vitamins. Although ABL is treatable by fat-soluble vitamins, especially vitamin E, their effectiveness is sometimes limited because of the lack of carrier lipoproteins (Burnett et al. 2014a). The molecular cause of ABL is genetic mutations of the microsomal triglyceride transfer protein (MTTP), which acts as a chaperone in the lipidation on nascent apoB (Burnett et al. 2014a; Hooper and Burnett 2014; Lee and Hegele 2014; Welty 2014; Wetterau et al 1992). Thus, deficiencies in MTP result in the lack of apoB lipidation and, consequently, a deficiency in all apoB-containing lipoproteins.

The frequencies of homozygous FHBL and ABL are estimated at less than 1 in a million in the general population. Although lipid levels in homozygous FHBL and ABL are similar, clinical manifestations of ABL are more severe than those of homozygous FHBL when the truncation of apoB is longer than apoB-48, probably because of lower levels of fat-soluble vitamins. On the other hand, homozygous FHBL shorter than apoB-48 represents a severe phenotype resembling ABL due to impaired formation of chylomicrons. Little is known about the difference in postprandial lipid levels, which could explain the difference between these two similar disorders. Thus, we performed genetic analysis in two patients with homozygous FHBL and then performed the oral fat tolerance test (OFTT) in one of them and in a patient with ABL in order to elucidate the differences between them with regard to postprandial lipid metabolism.

Methods

Genetic Analysis

Genetic analysis was performed after obtaining written informed consent from both patients in accordance with the guidelines of the Bioethical Committee on Human Genome and Genetic Analysis Study, School of Medicine, Kanazawa University. Genomic DNA was purified from peripheral leukocytes using a genomic DNA purification kit (Qiagen, Venlo, the Netherlands). We designed 63 primer pairs to cover all 29 exons and intronic junctions of the *APOB* gene using Primer 3 online software (http://simgene.com/Primer3). We screened the *APOB* gene by PCR-single-strand conformational polymorphism analysis of all exons. DNA sequencing was performed with fluorescently labeled dideoxy terminators using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Measurement of Lipids, Apolipoproteins, and Fat-Soluble Vitamins

Blood samples were drawn after overnight fasting. Serum total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol levels were measured by standard enzymatic methods; LDL-cholesterol levels were measured by a direct method (Sekisui Medical, Tokyo, Japan); and serum apolipoprotein levels were measured by a turbidimetric immunoassay, except for apoB-48, whose level was measured by ELISA using a monoclonal antibody against the C-terminal decapeptide of apoB-48 as described

previously (Kinoshita et al 2005). Remnant-like particle (RLP) was estimated as the unbound fraction of serum after incubation with an immunoaffinity gel of apoB-100 monoclonal antibody and apoA-I monoclonal antibody (Japan Immunoresearch Laboratories, Takasaki, Japan) as described previously (Nakajima et al. 1993). Serum lipoprotein and RLP-cholesterol distributions were measured using an HPLC system using 0.05-M Tris-buffered acetate (pH 8.0) at a flow rate of 0.7 mL/min (Tosho, Tokyo, Japan) as described elsewhere (Usui et al 2002). Serum levels of vitamin A were measured by HPLC, and those of vitamin E were determined by a fluorimetric method and by RIA (Yang et al 1999), respectively.

Oral Fat Tolerance Test

OFTT was performed in the patients with FHBL and ABL, as well as normal control subjects according to an established method (Tada et al 2012; Inazu et al 2008). Briefly, 50 g per m^2 of body surface of the OFTT cream (Jomo Shokuhin, Takasaki, Japan) was administered after overnight fasting (Tada et al 2012; Inazu et al 2008). The cream, which consists of 33% fat, 74 mg of cholesterol, and 341 kcal per 100 g, is rich in palmitic and oleic acids. Blood was drawn periodically before and 2, 4, and 6 h after fat loading. The normal control subjects were volunteers, and they also served as controls in our previous study (Tada et al 2012).

Case Report

Case 1 (FHBL): A 42-year-old Japanese male patient was referred to Showa University Hospital for further examination of his hypocholesterolemia in 2010. He was born from a first-cousin consanguineous marriage. He suffered from type 2 diabetes mellitus and hypertension since the age of 35 years and was treated with insulin injection therapy and pioglitazone, metformin, and carvedilol. His basal serum lipid and apolipoprotein levels, fat-soluble vitamin levels, and other biochemical parameters are shown in Table 1. His body measurements were as follows: height, 184 cm; body weight, 85.9 kg; and body mass index, 25.4 kg/m^2. He was asymptomatic with no physical or neurological abnormalities, and no retinitis pigmentosa was found. The genetic analysis revealed that he was homozygous for a novel nucleotide duplication (c.11433dupT, p.E3812X) in exon 26 of the *APOB* gene (RefSeqNM_000384.2), resulting in the formation of a truncated apoB of 3,811 amino acids (apoB-83.52) (Fig. 1).

Case 2 (FHBL): A 23-year-old Japanese male patient was referred to Kanazawa University Hospital for further examination of his hypocholesterolemia in 2010. He was also born from a first-cousin consanguineous marriage (Fig. 2). His laboratory data and those of his family members are shown in Table 1. His body measurements were as follows: height, 172 cm; body weight, 58.5 kg; and body mass index, 19.8 kg/m^2. He was also asymptomatic and had no physical or neurological abnormalities. These findings do not suggest that ABL was present. No acanthocytosis was found in the blood smear. No lipid accumulation was found in the upper and lower gastrointestinal tract by gastroscopy and colonoscopy, respectively, but a severe fatty liver was observed by ultrasonography. Severe steatosis with mild inflammatory changes and mild pericellular fibrosis was also found in the liver by histopathology, but no ballooning of hepatocytes or Mallory bodies were detected. These findings do not suggest that nonalcoholic steatohepatitis was present (Fig. 3). The genetic analysis revealed that case 2 was homozygous for a novel nucleotide deletion (c.11928delC, p.L3976fsX31) in exon 28 in the *APOB* gene, resulting in the formation of a downstream stop codon and, thereafter, of a truncated apoB of 4,005 amino acids (apoB-87.77) (Fig. 1).

Case 3 (ABL): A 49-year-old Japanese male patient was admitted to Kanazawa University Hospital for regular evaluation of multiple organ complications due to ABL. He was born from a non-consanguineous marriage and was diagnosed with ABL caused by maternal uniparental disomy of intron 9 splice donor site mutation (c.1237-1G>A, p.Q413_K448del) of the *MTTP* gene (RefSeqNM_000253.1), which was previously reported (Yang et al 1999). His serum lipid and apolipoprotein levels, fat-soluble vitamin levels, and other biochemical parameters under vitamin E supplementation therapy are shown in Table 1. His body measurements were as follows: height, 152 cm; body weight, 51 kg; and body mass index, 22.0 kg/m^2. He showed typical clinical manifestations of ABL, such as acanthocytosis, lipid accumulation in enterocytes, spinocerebellar ataxia, and retinitis pigmentosa (Yang et al 1999).

Although all three patients presented with an undetectable amount of apoB and extremely low levels of LDL-cholesterol, the patients with FHBL showed relatively high levels of HDL-cholesterol. Of note, the fasting serum apoB-48 level was almost undetectable in the patient with ABL, whereas in the patients with FHBL, the level was similar to that in normal control subjects. To elucidate the difference in lipoprotein metabolism after the postprandial state, we performed the OFTT in case 2 (FHBL; apoB-87.77), case 3 (ABL), and normal control subjects ($n = 4$).

As shown in Fig. 4, serum fasting triglyceride levels in the patients with FHBL (apoB-87.77) and ABL were lower than those in normal control subjects. After oral fat loading, although the peak time points were different, serum

Table 1 Fasting serum levels of lipids, apolipoproteins, fat-soluble vitamins, and the other biochemical parameters of the study subjects

Case	Case 1	Case 2							Case 3	Control ($n = 4$)		
		IV-1	III-1	III-2	III-3	III-4	III-5	III-6				
Sex	M	M	M	M	F	F	F	F	M	M		
Gene mutation	ApoB-83.52/83.52	ApoB-87.77/87.77	ApoB-87.77/100	ApoB-87.77/100	ApoB-87.77/100	ApoB-100/100	ApoB-100/100	ApoB-87.77/100	c.1237-1G>A	Normal		
Total cholesterol (mg/dL)	55	63	181	150	180	208	280	161	39	173	±	44
LDL-cholesterol (mg/dL)	5	1	96	80	96	110	197	85	2	88	±	6
HDL-cholesterol (mg/dL)	43	51	74	55	64	77	39	67	24	66	±	21
Triglyceride (mg/dL)	37	13	54	73	99	104	219	47	9	79	±	45
ApoA-I (mg/dL)	119	117	160	150	156	164	114	153	54	156	±	26
ApoA-II (mg/dL)	22	25.9	29.1	28.9	30.4	33.1	27.7	33.9	10.2	32.3	±	4.2
ApoB (mg/dL)	u.d.	u.d.	56	52	63	79	142	54	u.d.	80	±	13
ApoB-48 (μg/mL)	3.30	2.03	n.d	n.d	n.d	n.d	n.d	n.d	0.03	2.3	±	1
ApoC-II (mg/dL)	1.5	0	3.1	1.7	3.1	3.5	8	2.6	0.1	4.7	±	1.3
ApoC-III (mg/dL)	3.2	2.2	7.7	7.3	7.7	9.3	11.8	5.4	1.7	11.1	±	3.1
ApoE (mg/dL)	1.4	2.3	4.1	3.1	3.9	4.2	6.1	3	5.6	4.3	±	0.8
AST (IU/L)	44	72	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	96		n.d.	
ALT (IU/L)	66	158	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	90		n.d.	
GGTP (IU/L)	82	49	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	124		n.d.	
PG (mg/dL)	116	101	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	84		n.d.	
HbA1c (%)	9.2	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.4		n.d.	
Vit A (IU/dL) (97–316)	n.d.	163	154	184	169	158	136	170	85		n.d.	
Vit E (mg/dL) (0.75–1.41)	n.d.	0.41	1.05	0.84	1.00	1.13	1.79	0.89	0.37		n.d.	

n.d. not determined, *u.d.* undetectable

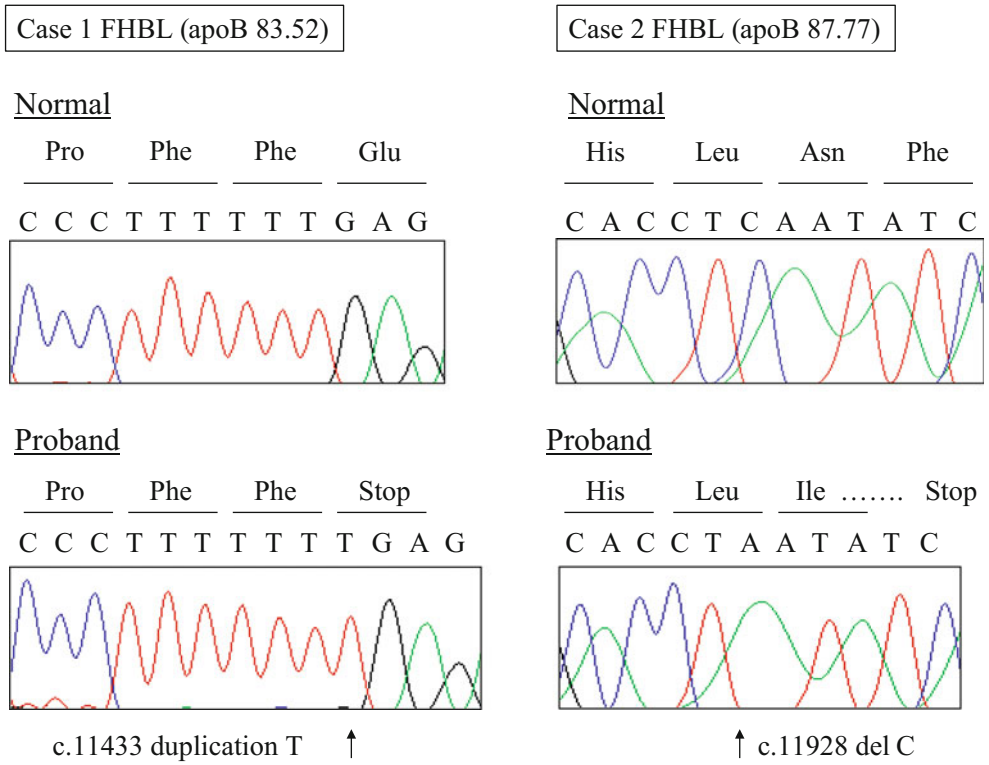


Fig. 1 Genetic analysis of homozygous familial hypobetalipoproteinemia (FHBL) (**a**) apolipoprotein B (apoB)-83.52 and (**b**) apoB-87.77. Case 1 was homozygous for a novel nucleotide duplication (c.11433dupT, p.E3812X), resulting in the formation of a stop codon in exon 26 of the *APOB* gene and a truncated apoB of 3,811 amino acids (apoB-83.52). Case 2 was homozygous for a novel nucleotide deletion (c.11928delC, p.L3976fsX31) in exon 28 of the *APOB* gene, resulting in the formation of a stop codon at nucleotide 31 downstream and, thereafter, in a truncated apoB of 4,005 amino acids (apoB-87.77)

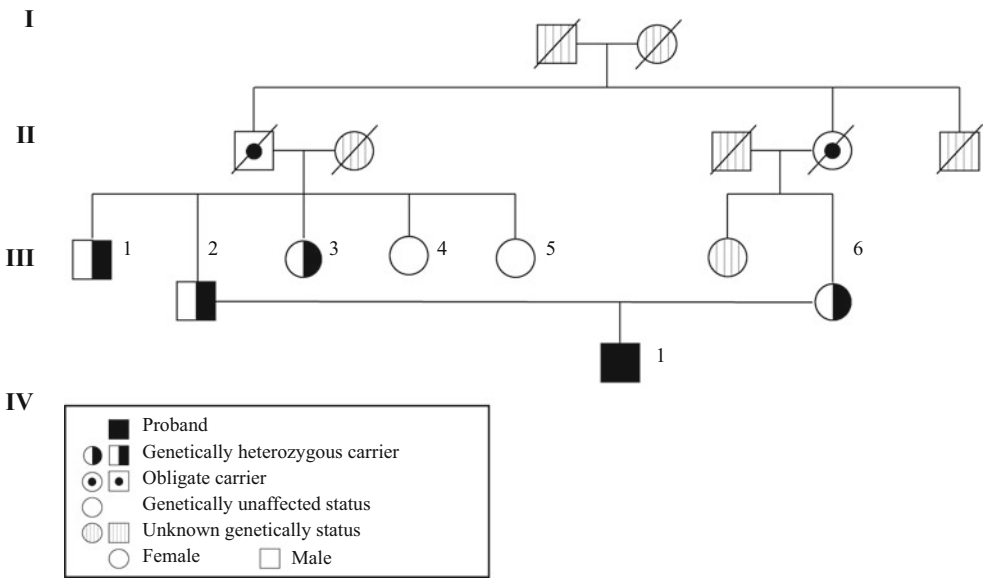


Fig. 2 Pedigree of the patient with familial hypobetalipoproteinemia (FHBL) [apolipoprotein B (apoB-87.77)] (Case 2). *Squares* indicate males and circles indicate females. *Closed and semi-closed* symbols indicate homozygosity and heterozygosity for apoB-87.77, respectively. The paternal grandfather and maternal grandmother are siblings, indicating that the proband was born from a first-cousin consanguineous marriage

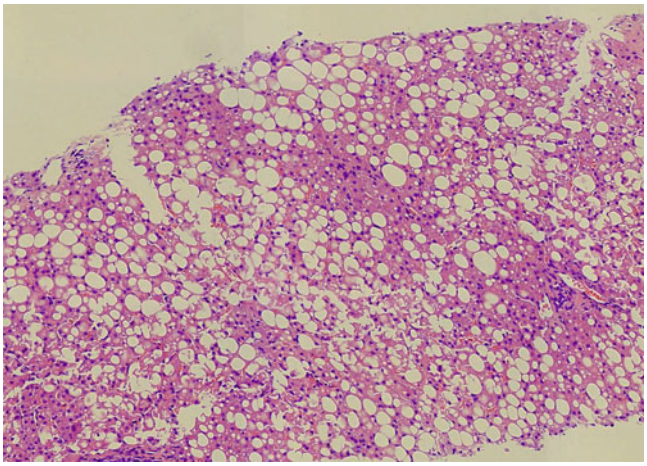
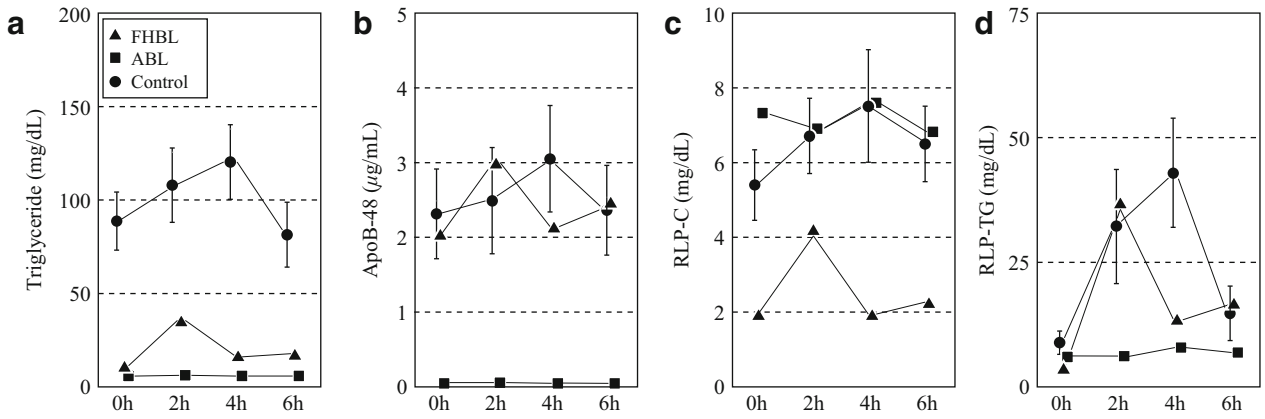


Fig. 3 Liver biopsy of a patient with homozygous familial hypobetalipoproteinemia (FHBL) [apolipoprotein B (apoB-87.77)] (Case 2). Because severe fatty liver was observed by ultrasonography, a liver biopsy was performed. Severe steatosis with mild inflammatory changes and mild pericellular fibrosis was observed, but there was no ballooning of hepatocytes or Mallory bodies. These findings do not suggest the presence of nonalcoholic steatohepatitis

triglyceride levels increased in both normal control subjects and the patient with FHBL (apoB-87.77) but not in the patient with ABL. As a result, the incremental areas under the curve of serum triglyceride of the patients with FHBL and ABL were lower than those of control subjects. Similar tendencies after oral fat loading were observed for serum apoB-48, a specific carrier protein of chylomicrons. Similarly, RLP-triglyceride levels after oral fat loading were increased in the patient with FHBL (apoB-87.77) as well as in normal control subjects but not in the patient with ABL. Interestingly, the incremental areas under the curve of serum apoB-48 and RLP-triglyceride in the patients with FHBL and normal control subjects were similar, and those in patient with ABL were small. These observations suggest that no fat was absorbed in the patient with ABL, whereas fat was absorbed in the patient with FHBL (apoB-87.77). On the other hand, increases in the serum RLP-cholesterol level after oral fat loading were almost the same in normal control subjects and the patient with FHBL (apoB-87.77), despite the baseline level in the latter (apoB-87.77) being lower than that in the former. Interestingly, the RLP-cholesterol level in the patient with ABL was almost the same as that in normal control subjects before and after oral fat loading.

To elucidate the more detailed changes in the lipoprotein subfraction before and after oral fat loading, HPLC analyses of cholesterol and RLP fractions were performed (Fig. 5). The HPLC method can separate lipoproteins according to their particle size, with the larger-sized lipoproteins being eluted earlier. In normal control subjects, a small but significant RLP-cholesterol peak eluted at around 15 min 2–6 h after oral fat loading, indicating the presence of chylomicrons, and two large peaks eluted at around 21 and 25 min, indicating the presence of the LDL



Incremental AUC	(mg・hr./dL)	(μg・hr./mL)	(mg・hr./dL)	(mg・hr./dL)
FHBL	141.0	14.64	16.3	133.6
ABL	45.1	0.20	43.1	49.0
Control	627.7	16.00	40.1	179.7

Fig. 4 Chronological changes in serum lipid and apolipoprotein levels after oral fat loading. Serum levels of (**a**) triglyceride, (**b**) apolipoprotein B-48 (apoB-48), (**c**) remnant-like particle (RLP)-cholesterol, and (**d**) RLP-triglyceride were measured periodically after oral fat loading. *Triangles*, *squares*, *circles*, and *bars* indicate familial hypobetalipoproteinemia (FHBL), abetalipoproteinemia (ABL), control, and 1SD, respectively. The incremental area under the curve (AUC) is indicated below the figures. Fasting serum levels of triglyceride in the patients with FHBL (apoB-87.77) and ABL were lower than those in normal control subjects. The incremental AUC of apoB-48 in the patient with FHBL (apoB-87.77) and normal control subjects was similar. On the other hand, in the patient with ABL, it was extremely low. This indicated that the patient with FHBL (apoB-87.77) absorbed fat to the same degree as normal control subjects and the patient with ABL absorbed no fat

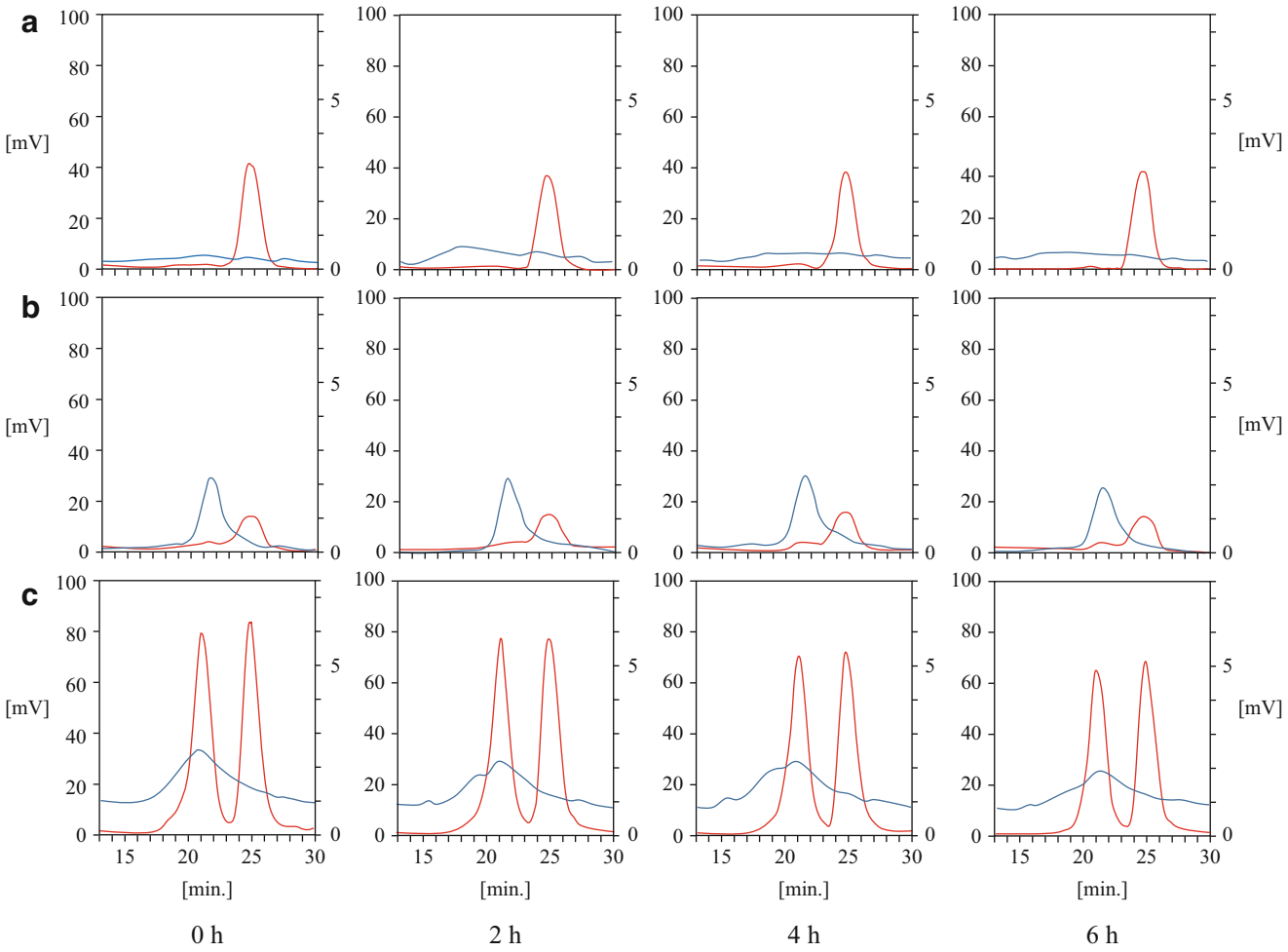


Fig. 5 HPLC analysis of serum from homozygous familial hypobetalipoproteinemia (FHBL) [apolipoprotein B (apoB-87.77)], abetalipoproteinemia (ABL), and representative normal control subject. The HPLC method can separate lipoproteins according to their particle size, with the larger-sized lipoproteins being eluted earlier. In the normal control subjects, a small but significant remnant-like particle (RLP)-cholesterol peak eluted at around 15 min 2–6 h after oral fat loading, indicating the presence of chylomicrons, and two large peaks eluted at around 21 and 25 min, indicating the low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions, respectively. The HPLC analyses of serum cholesterol levels and RLP fractions are shown as red and blue lines, respectively. (**a**) FHBL, (**b**) ABL, and (**c**) normal control

and HDL fractions, respectively (Fig. 5c). As expected from the lack of increase in apoB-48 after oral fat loading in the patient with ABL (Fig. 4b), no RLP-cholesterol peak was eluted at around 15 min, suggesting the absence of chylomicron formation (Fig. 5b). Moreover, there was an RLP-cholesterol peak between the original LDL and HDL peaks, suggesting the presence of apoE-rich large HDL particles (Fig. 5b). In contrast, a relatively wide slope of RLP-cholesterol was observed in the patient with FHBL (apoB-87.77), suggesting the presence of unusually small chylomicron particle (Fig. 5a).

Serum levels of vitamins A and E were measured in some patients and their family members. Although the patient with ABL was supplemented with weekly 1,200-mg vitamin E drip intravenous injections, his serum vitamin A and vitamin E levels were lower than the normal range. The serum vitamin A level of the patient with FHBL (apoB-87.77) was within the normal limit and was almost the same as that of his family members, which included a patient with heterozygous FHBL and normal subjects. On the other hand, his serum vitamin E level was lower than the normal range and that of all his family members, but it was higher than that of the patient with ABL who was supplemented with vitamin E.

In summary, although lipid levels in the patients with homozygous FHBL and ABL were comparable, the former patients (whose apoB was longer than apoB-48) were asymptomatic. The fasting, postoral fat loading of apoB-48,

as well as RLP-triglyceride levels of homozygous FHBL, was higher than those of the patient with ABL.

Discussion

There were three main findings of this study. First, we identified novel *APOB* gene mutations (apoB-83.52 and apoB-87.77) in two Japanese patients with homozygous FHBL. Second, these two patients were almost asymptomatic, although the former suffered from type 2 diabetes mellitus and developed complications of fatty liver. Third, for the first time, OFTTs were performed in a patient with ABL and with homozygous FHBL (apoB-87.77), revealing clear, clinically noteworthy differences in lipoprotein metabolism after oral fat loading.

Since the cloning of the *APOB* gene, more than 60 different gene mutations have been identified as the cause of FHBL (Tarugi et al 2007). Most of them result in truncated proteins of various lengths ranging from apoB-2 to apoB-89 due to nonsense or frameshift mutations. Previously, we detected two novel *APOB* gene mutations in 14 Japanese patients with FHBL (apoB-13.7 and apoB-82) and demonstrated that such a gene mutation is not rare in patients with Japanese primary hypocholesterolemia (Katsuda et al 2009). In the present study, we screened and found *APOB* gene mutations in two patients who resembled ABL in terms of extremely low LDL-cholesterol levels and birth from consanguineous parents. As a result, we identified two novel mutations in the *APOB* gene: apoB-83.52 and apoB-87.77.

Although some cases of homozygous or heterozygous FHBL with type 2 diabetes mellitus (Groenewegen et al 1994; Ohashi et al 1998; Pulai et al 1998; Turk et al 2012) have been reported, no predominant causality has been proposed. Ohashi et al. (1998) reported an unusual Japanese patient with homozygous FHBL (apoB-38.7) presenting with retinitis pigmentosa and some atypical neurological abnormalities despite a normal plasma level of vitamin E, such as paresthesia in both hands, glove-/stocking-type hypesthesia, the absence of deep tendon reflexes in the lower extremities, and a positive Romberg's sign. Moreover, unlike in ABL, this specific patient presented with normal pyramidal, cerebellar, and posterior column functions. In general, most complications associated with ABL or FHBL can be explained by deficiencies in fat-soluble vitamins, especially vitamin E (Lee and Hegele 2014; Tarugi et al 2007). Thus, the absence of physical abnormalities in the two patients with homozygous FHBL in the present study, in contrast to the patient with ABL, may be explained by the fact that truncation of apoB is longer than apoB-48 and by the resultant existence of lower-than-normal plasma fat-soluble vitamin levels.

The metabolism of apoB in heterozygous FHBL due to several truncated apoBs has been studied using a stable isotope technique (Parhofer and Barrett 2006). These results indicated that apoB-containing lipoproteins showed both decreased production rates and increased clearance rates. Truncations that retain the LDL receptor-binding domain, such as apoB-89, apoB-87, and apoB-75, have increased fractional catabolic rates, whereas every 1% of apoB truncation results in an approximately 1.4% reduction of VLDL-apoB secretion (Parhofer et al 1996).

OFTT is easy to perform and enables the detection of postprandial lipoprotein metabolism. Averna et al. (1993) performed OFTT in a patient with heterozygous FHBL caused by an apoB truncation. They compared fat absorption between patients with heterozygous FHBL with apoB <48 and apoB >48 and found no difference between them suggesting that one allele of intestinal apoB-48 is sufficient for normal fat absorption. Hooper et al. (2007) performed OFTT and detected postprandial apoB-48 kinetics in patients with heterozygous FHBL (apoB-6.9, apoB-25.8, and apoB-40.3). As a result, these patients with heterozygous FHBL, caused by truncations shorter than apoB-48, showed a lower production and normal clearance of triglyceride-rich lipoproteins.

In the present study, for the first time, we directly compared lipid and apolipoprotein levels after OFTT in a patient with homozygous FHBL (apoB-87.77) and ABL (Figs. 4 and 5). The serum triglyceride level in the former patient was similar to that in the latter patient at fasting, and it increased 2 h after OFTT only in the former patient. This result suggests that the loaded fat was not absorbed in the patient with ABL. This concept was confirmed by measuring apoB-48 levels, a specific carrier protein of chylomicrons (Fig. 4) and performing an HPLC analysis (Fig. 5) over time. The HPLC analysis revealed small but significant peaks of RLP-cholesterol that eluted at around 15 min, indicating the presence of chylomicrons in the patient with homozygous FHBL (apoB-88.29) at 2, 4, and 6 h after oral fat loading, but not in the patient with ABL. Because the truncated length of apoB was longer than apoB-48 in the patient with homozygous FHBL, it was speculated that the loaded fat was absorbed to the same degree as that in normal control subjects. Although lipid levels in the patients with homozygous FHBL and ABL are similar, fasting or postoral fat loading apoB-48 levels as well as RLP-triglyceride levels may help in the differential diagnosis of these two rare disorders and provide an opportunity to promptly diagnose them using genetic analysis in the future.

The present study has several limitations. First, a relatively small number of subjects were included in the current study because of the extreme rarity of the disorder. The results from a single patient are not suitable for

statistical analysis. However, the differences in OFTT-derived FHBL and ABL data are quite distinct. Second, the truncation length of apoB in both the patients with FHBL exceeded apoB-48, making it impossible to compare the OFTT data between apoB >48 and apoB <48.

Acknowledgment We express sincere gratitude to Kazuko Honda (staff of Kanazawa University) for her outstanding technical assistance. The authors would like to thank Enago (www.enago.jp) for the English language review.

Take Home Message

Measuring apolipoprotein B-48 and remnant-like particle triglyceride levels before and after the oral fat tolerance test may help in the differential diagnosis of homozygous familial hypobetalipoproteinemia and abetalipoproteinemia.

Compliance with Ethics Guidelines

Conflict of Interest

We hereby certify that this paper, which consists of unpublished original observations, is not under consideration for publication elsewhere. All coauthors have been involved in drafting and revising the article, and finally, this manuscript has been read and approved by all coauthors. Atsushi Nohara and Hiroshi Mabuchi have received research grants from MSD K.K., Sanofi K.K., Shionogi & Co., Ltd., Kowa Co., Ltd., Astellas Pharma Inc., AstraZeneca K.K., Keiaikai Medical Corp., and Biopharm of Japan Co. Masakazu Yamagishi has received research grants from Astellas Pharma Inc., Daiichi Sankyo Co., Ltd., and Ono Pharmaceutical Co., Ltd. Masa-aki Kawashiri, Hayato Tada, Marowa Hashimoto, Matsuo Taniyama, Takamitsu Nakano, Katsuyuki Nakajima, Takeshi Inoue, Mika Mori, Chiaki Nakanishi, Tetsuo Konno, Kenshi Hayashi, Akihiro Inazu, Junji Koizumi, Hirotaka Ishihara, Junji Kobayashi, and Tsutomu Hirano have no financial or other relations that could lead to a conflict of interest. All coauthors agreed to submit this article to the *Journal of Inherited Metabolic Disease*.

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

Author Contributions

Masa-aki Kawashiri designed and performed all functions throughout the research and wrote the paper. Hayato Tada collected clinical data and performed the genetic analysis and fat-loading test. Marowa Hashimoto performed the genetic analysis. Takamitsu Nakano and Katsuyuki Nakajima performed the biochemical assay. Takeshi Inoue, Mika Mori, Chiaki Nakanishi, Tetsuo Konno, Kenshi Hayashi, and Atsushi Nohara collected the clinical data and performed the genetic analysis. Akihiro Inazu performed and supervised the genetic and biochemical analyses. Junji Koizumi collected the clinical data and supervised the genetic analysis. Hirotaka Ishihara collected the clinical data. Junji Kobayashi collected and supervised the clinical data and wrote the paper. Tsutomu Hirano collected and supervised the clinical data. Hiroshi Mabuchi and Masakazu Yamagishi supervised all aspects through the research period and wrote the paper.

References

Averna M, Seip RL, Mankowitz K, Schonfeld G (1993) Postprandial lipemia in subjects with hypobetalipoproteinemia and a single intestinal allele for apoB-48. J Lipid Res 34:1957–1967

Burnett JR, Bell DA, Hooper AJ, Hegele RA (2014a) Clinical utility gene card for: abetalipoproteinaemia - Update 2014. Eur J Hum Genet. doi:10.1038/ejhg.2014.224

Burnett JR, Bell DA, Hooper AJ, Hegele RA (2014b) Clinical utility gene card for: familial hypobetalipoproteinaemia (APOB) - Update 2014. Eur J Hum Genet. doi:10.1038/ejhg.2014.225

Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH (2005) Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nat Genet 37:161–165

Groenewegen WA, Averna MR, Pulai J, Krul ES, Schonfeld G (1994) Apolipoprotein B-38.9 does not associate with apo[a] and forms two distinct HDL density particle populations that are larger than HDL. J Lipid Res 35:1012–1025

Hooper AJ, Burnett JR (2014) Update on primary hypobetalipoproteinemia. Curr Atheroscler Rep 16:423

Hooper AJ, Robertson K, Barrett PH, Parhofer KG, van Bockxmeer FM, Burnett JR (2007) Postprandial lipoprotein metabolism in familial hypobetalipoproteinemia. J Clin Endocrinol Metab 92:1474–1478

Inazu A, Nakajima K, Nakano T et al (2008) Decreased post-prandial triglyceride response and diminished remnant lipoprotein formation in cholesteryl ester transfer protein (CETP) deficiency. Atherosclerosis 196:953–957

Katsuda S, Kawashiri MA, Inazu A et al (2009) Apolipoprotein B gene mutations and fatty liver in Japanese hypobetalipoproteinemia. Clin Chim Acta 399:64–68

Kinoshita M, Kojima M, Matsushima T, Teramoto T (2005) Determination of apolipoprotein B-48 in serum by a sandwich ELISA. Clin Chim Acta 351:115–120

Lee J, Hegele RA (2014) Abetalipoproteinemia and homozygous hypobetalipoproteinemia: a framework for diagnosis and management. J Inherit Metab Dis 37:333–339

Nakajima K, Saito T, Tamura A et al (1993) Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apo B-100 and anti apo A-I immunoaffinity mixed gels. Clin Chim Acta 223:53–71

Ohashi K, Ishibashi S, Yamamoto M, Osuga J, Yazaki Y, Yukawa S, Yamada N (1998) A truncated species of apolipoprotein B (B-38.7) in a patient with homozygous hypobetalipoproteinemia associated with diabetes mellitus. Arterioscler Thromb Vasc Biol 18:1330–1334

Parhofer KG, Barrett PH (2006) Thematic review series: patient-oriented research. What we have learned about VLDL and LDL metabolism from human kinetics studies. J Lipid Res 47:1620–1630

Parhofer KG, Barrett PH, Aguilar-Salinas CA, Schonfeld G (1996) Positive linear correlation between the length of truncated apolipoprotein B and its secretion rate: in vivo studies in human apoB-89, apoB-75, apoB-54.8, and apoB-31 heterozygotes. J Lipid Res 37:844–852

Pulai JI, Latour MA, Kwok PY, Schonfeld G (1998) Diabetes mellitus in a new kindred with familial hypobetalipoproteinemia and an apolipoprotein B truncation (apoB-55). Atherosclerosis 136:289–295

Tada H, Kawashiri MA, Tanaka A et al (2012) Post-prandial remnant lipoprotein metabolism in autosomal recessive hypercholesterolaemia. Eur J Clin Invest 42:1094–1099

Tarugi P, Averna M, Di Leo E et al (2007) Molecular diagnosis of hypobetalipoproteinemia: an ENID review. Atherosclerosis 195: e19–e27

Turk U, Basol G, Barutcuoglu B, Sahin F, Habif S, Tarugi P, Bayindir O (2012) A 54-year-old diabetic man with low serum cholesterol. Clin Chem 58:826–829

Usui S, Hara Y, Hosaki S, Okazaki M (2002) A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. J Lipid Res 43:805–814

Welty FK (2014) Hypobetalipoproteinemia and abetalipoproteinemia. Curr Opin Lipidol 25:161–168

Wetterau JR, Aggerbeck LP, Bouma ME et al (1992) Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 258:999–1001

Yang XP, Inazu A, Yagi K, Kajinami K, Koizumi J, Mabuchi H (1999) Abetalipoproteinemia caused by maternal isodisomy of chromosome 4q containing an intron 9 splice acceptor mutation in the microsomal triglyceride transfer protein gene. Arteioscler Thromb Vasc Biol 19:1950–1955

JIMD Reports
DOI 10.1007/8904_2015_416

RESEARCH REPORT

Girls with Seizures Due to the c.320A>G Variant in *ALG13* Do Not Show Abnormal Glycosylation Pattern on Standard Testing

Bethanny Smith-Packard · Scott M. Myers · Marc S. Williams

Received: 30 October 2014 / Revised: 05 January 2015 / Accepted: 28 January 2015 / Published online: 03 March 2015

Abstract A girl with early onset severe epilepsy, developmental delay, intellectual disability, visual maturation delays, and feeding problems was without a diagnosis despite an extensive genetic and metabolic evaluation. She initially manifested infantile spasms which responded to high-dose ACTH. Seizures seemed to resolve, but then at age 5, she developed complex partial seizures resistant to antiepileptics that responded to a ketogenic diet. Additional features included visual impairment, hypotonia, reflux, and severe feeding problems requiring a G-tube. She was referred to the Geisinger Health System whole-genome sequencing clinical research program. A variant in the X-linked gene *ALG13* (c.320A->G p. 107 N->S) was identified. Four additional girls from three published exome sequencing studies were found to have the identical c.320A>G variant in *ALG13*. All presented with early onset severe epilepsy and intellectual disability. Three of the five exhibited visual impairment and possible developmental regression. A boy with a variant in *ALG13* presented with a severe congenital disorder of glycosylation type Is. Glycosylation studies in the case reported here were normal; none of the other girls reported in the literature have had glycosylation studies. X-inactivation studies have not been done. The N107 residue and the surrounding region – MNNHQ – are highly conserved across species and are found in a presumed functional domain of this glycotransferase superfamily. The consistent clinical presentation of a severe phenotype in girls coupled with identical variants in an X-linked gene strongly suggests a critical position effect. Negative glycosylation studies in one individual suggest the possibility of a new mechanism requiring investigation.

Communicated by: Robert Steiner

Competing interests: None declared

B. Smith-Packard · M.S. Williams (✉)
Genomic Medicine Institute, Geisinger Health System, 100 N Academy Dr. Mail Stop 26-20, Danville, PA 17822-2620, USA
e-mail: mswilliams1@geisinger.edu

S.M. Myers
Autism & Developmental Medicine Institute, Geisinger Health System, 120 Hamm Drive, Lewisburg, PA 17837, USA

Introduction

Over 100 congenital disorders of glycosylation (CDG) have been described (Freeze et al. 2014). These are generally grouped into disorders of *N*-glycosylation, disorders of *O*-glycosylation, and disorders of the Glycosylphosphatidylinositol-Anchor Pathway (Freeze et al. 2014). Timal et al. (2012) reported a boy with refractory epilepsy with polymorphic seizures, hepatomegaly, horizontal nystagmus, optic nerve atrophy, susceptibility to infections, a bleeding diathesis, swelling of the eyelids and distal extremities, and extrapyramidal and pyramidal signs. He died at 1 year of age. Biochemical analysis identified a CDG with a metabolic signature consistent with a defect in *N*-glycosylation type Is (aka CDG-ALG13). Exome sequencing identified a 280A>G transition in the *ALG13* gene, resulting in a lys 94-to-glu (K94E) substitution at a highly conserved residue in the C-terminal glycosyltransferase domain thought to be causal for the CDG. Cell studies demonstrated only 17% residual enzyme activity of UDP-GlcNAc transferase. The variant was inherited from his unaffected mother. *ALG13* is located on the X-chromosome and encodes the protein ALG13 which forms the UDP-GlcNAc transferase with ALG14 and catalyzes a key step in endoplasmic reticulum N-linked glycosylation (Averbeck

et al. 2007). To date, this is the only CDG reported due to a variant in *ALG13*. Bissar-Tadmouri et al. (2014) identified a novel missense variant, 3221A>G in *ALG13* (p.Y1074C), that segregated with a non-syndromic X-linked intellectual disability disorder family with four affected boys. No additional clinical information was provided in the report. The mother was heterozygous for the variant and was normal. X-inactivation studies in the mother were normal. Glycosylation studies were not performed for these boys.

We report a case of a girl with a severe epileptic encephalopathy identified with a variant in *ALG13* and review the literature relative to this variant.

Case Report and Literature Review

A 7-year-old girl was referred to the Geisinger Health System IRB-approved whole-genome sequencing clinical research project focused on children with undiagnosed intellectual disability. Parents underwent genetic counseling and were consented for participation including return of primary and secondary findings and reporting of deleterious variants.

This child was the product of a pregnancy complicated by cholecystectomy at 13 weeks gestation, several urinary tract infections, and dehydration. During the pregnancy, her mother took multiple medications at various times (albuterol, budesonide, fluticasone propionate + salmeterol, fexofenadine [after first trimester], promethazine HCl, oxycodone HCl, acetaminophen, propofol, fentanyl, midazolam, labetalol ondansetron HCl, atropine, neostigmine, meperidine, trimethoprim and sulfamethoxazole, melatonin, cephalexin, misoprostol). Newborn screening was normal. She was hospitalized at 2 weeks of age with RSV infection, gastroesophageal reflux, and apnea. At 5 months of age, she developed repetitive eye rolling and was initially diagnosed with delayed visual maturation. On multiple occasions, eyes were normal on examination, and she was ultimately diagnosed with cortical visual impairment. At 6 months, the parents were concerned about regression (lost cooing, responsive smile, and laugh).

Infantile spasms were diagnosed at 8 months by EEG (hypsarrhythmia was present). She was treated with ACTH with a good response. She had no clinically apparent seizures until 5 years of age although EEGs were not normal (findings included excessive, superimposed, theta frequency background activity; rare, irregular bursts of higher amplitude and somewhat sharply contoured activity seen asymmetrically over the left hemisphere; and diffuse excessive slowing) and all visual evoked response tests showed greater than the expected latency. At 5 years old, she developed complex partial seizures resistant to antiepileptics. EEG was abnormal with irregular generalized polyspike and wave activity, as well as left and right paroxysmal fast wave activity over the temporal regions suggesting generalized, multifocal seizures arising from right and left hemispheres. She responded well to a ketogenic diet and achieved better seizure control. She continues to experience severe reflux and significant feeding problems which required placement of a G-tube. She exhibits pica. She has severe cognitive impairment with limited expressive language; IQ is estimated to be in the range of 20–25.

She has undergone an extensive diagnostic evaluation including high-resolution chromosomes, chromosomal microarray, extensive metabolic evaluation (plasma and urine amino acids, urine organic acids, lysosomal studies, lactate, pyruvate, ammonia, very long-chain fatty acids, creatine kinase, carnitines), CSF protein, multiple MRIs, thyroid studies, hearing evaluations, and *FOXG1* sequencing, all of which were normal. A PET scan showed changes consistent with a seizure focus. CDG was not suspected clinically and no diagnostic studies were performed.

Whole-genome sequencing was performed using an Illumina platform. The sequence was analyzed using the SimulConsult Genome-Phenome Analyzer (Segal et al. 2014). Multiple variants were identified and assessed. A de novo variant in *ALG13* (c.320A->G) was initially given a low priority given that this is an X-linked gene that would not be expected to cause disease in a female heterozygote. However, a review of the literature identified four additional girls that were part of exome sequencing studies with the identical de novo c.320A->G *ALG13* variant.

The first girl was reported by de Ligt et al. (2012) from a large cohort of subjects undergoing diagnostic exome sequencing for severe intellectual disability, although the authors did not assign causality based on the available information. Subsequently, three additional girls were identified, two from the Epi4k (2013) epileptic encephalopathy exome sequencing project and one from a genetic study of infantile spasms (Michaud et al. 2014). All four girls from the three studies presented with severe early onset seizures and severely delayed psychomotor development. Hypotonia and feeding problems were noted in all but the Michaud patient (limited clinical information presented). Three of the girls (Epi4k et al. 2013; Michaud et al. 2014) had hypsarrhythmia on EEG. Both of the girls from the Epi4k study were reported to have normal or mildly delayed development prior to the onset of the seizures, while the de Ligt et al. patient was noted to be delayed from birth. One of the Epi4k patients responded well to high-dose ACTH but seizures returned after tapering. This girl was also reported to have no visual tracking. The de Ligt et al. patient was noted to have no visual fixation until 18 months of age. None of the four previously reported cases had CDG studies performed,

although personal communication regarding the case reported by de Ligt et al. indicated the clinical presentation "...would fit in the N107S 'epilepsy phenotype' as reported by Michaud and in the Epi4K paper" [personal communication]. Since our patient has been evaluated at multiple institutions, we confirmed that she had not been included in any of the referenced sequencing projects.

Including our patient, all five girls share features of severe early onset seizures, severe psychomotor delay and intellectual disability, and feeding problems and demonstrated hypsarrhythmia on EEG. Four of the five had possible developmental regression and abnormalities of visual development that seem to be central in nature. All five have the identical variant in *ALG13*. The authors of the Epi4k project estimated that the probability of their two patients having the same variant by chance was $p = 7.8 \times 10^{-12}$, a number that is large compared to the chance of this occurring in five unrelated patients with strikingly similar presentations.

Since CDG studies had not been performed in the reported patients, we sent a sample for transferrin isoform analysis and isoelectric focusing with affinity chromatography and mass spectrometry to a laboratory with extensive experience in diagnostic testing for CDG (tests included the mono-oligosaccharide/di-oligosaccharide transferrin ratio, the a-oligosaccharide/di-oligosaccharide transferrin ratio, the tri-sialo/di-oligosaccharide transferrin ratio, the apolipoprotein CIII-1/apolipoprotein CIII-2 ratio, and the apolipoprotein CIII-0/apolipoprotein CIII-2 ratio). These studies were normal. Specifically, no abnormalities were seen that were suggestive of a disorder of *N*-glycosylation (Table 1).

Discussion

The c.320A>G variant in *ALG13* results in a substitution of serine for asparagine at position 107 in the ALG13 protein (N107S). The N107 residue and the surrounding region – MNNHQ – are highly conserved across species (from mammals to bird to yeast) and are found in a presumed functional domain of this glycotransferase superfamily. There are numerous human splice variants of ALG13: all but one splice variant included the N107 residue at a variable distance from the C-terminus (up to 100 bp from the C-terminus).

The evidence presented strongly suggests that this variant causes the severe phenotype seen in these girls; however, the mechanism by which the variant is acting is not apparent. Potential explanations could include skewed X-inactivation or a dominant negative variant resulting in reduced ALG13 activity. However, one would expect to see evidence of the *N*-glycosylation defect as observed in the one affected boy that underwent testing unless the skewed X-inactivation was limited to the central nervous system. Haplo-insufficiency by itself does not seem to be sufficient to explain the severe phenotype as the two heterozygous mothers reported in the literature were said to be normal although only one had been tested. However, differential expression or enzymatic requirements in different tissues could result in a tissue-specific phenotype, such as an encephalopathy as seen in these girls. Enzyme activity studies have not been performed at this time. It is possible that the variant causes a form of CDG that escapes detection by the usual laboratory evaluation. It is also possible that this variant may act through an as yet unknown mechanism.

The variant appears to be on a conserved loop deep inside the protein. This suggests it may be important for the catalytic activity of the protein, although, as previously noted, ALG13 does not appear to have any activity unless combined with ALG14. If this variant does affect the catalytic domain, it could create novel catalytic activity leading to the severe neurocognitive phenotype. Finally, there is only one splice variant that does not include this residue, so it is possible that an overabundance or imbalance of the protein product that does not include this residue could have a deleterious effect on brain development.

It is of interest that three of the five girls were reported to have normal or mildly delayed development in the first months of life prior to onset of clinical seizures, suggesting that the impact of the variant does not manifest until after birth. The defect also seems to have a significant impact on visual development without evidence of retinal or optic nerve abnormalities. Given the severity of the phenotype in the girls reported thus far and that it has not been identified in any males in published exome projects, one might presume that this variant causes embryonic lethality in males. Additional studies including CSF protein glycosylation studies, enzyme activity, neuronal cell culture, and model organism knockouts or knockdowns will be needed to elucidate the mechanism of this variant.

In conclusion, while the mechanism of action is unknown, the c.320A>G variant in *ALG13* should be sought in girls presenting with severe early onset epileptic encephalopathy and possible developmental regression, particularly if associated with delayed visual development.

Summary Sentence

Girls with the c.320A>G variant in *ALG13* do not have laboratory evidence of congenital disorder of glycosylation type Is on standard testing suggesting a different mechanism of action which causes severe epileptic encephalopathy

Table 1 Summary of patients with *ALG13* variants

Patient(s)	Sex	*ALG13* variant	Protein change	Glycosylation studies	Clinical findings
This report	F	c.320A>G	N107S	Normal	ID, DR IS, HA, SZ, DVM, FP
de Ligt et al. (2012)	F	c.320A>G	N107S	Not reported	ID, SZ, HYP, DVM, FP
Epi4k #1 (2013)	F	c.320A>G	N107S	Not reported	ID, DR, SZ, HA, DVM
Epi4k #2 (2013)	F	c.320A>G	N107S	Not reported	ID, DR, SZ, HA,
Michaud et al. (2014)	F	c.320A>G	N107S	Not reported	ID, IS, SZ, HA
Timal et al. (2012)	M	c.280A>G	K94E	*N*-glycosylation defect type Is	SZ, MC, HM, died first year of life
Timal et al. (2012)	F mother	c.280A>G	K94E	Normal	Normal
Bissar-Tadmouri et al. (2014)	M ($n = 4$) brothers	c.3221A>G	Y1074C	Not tested	ID
Bissar-Tadmouri et al. (2014)	F mother	c.3221A>G (heterozygous)	Y1074C	Not tested	Normal

Key: *ID* intellectual disability, *DR* developmental regression, *IS* infantile spasms, *HA* hypsarrhythmia, *SZ* seizures other than IS, *DVM* delayed visual maturation, *FP* feeding problems, *HYP* hypotonia, *MC* microcephaly, *HM* hepatomegaly

with visual impairment and possible developmental regression.

Compliance with Ethics Guidelines

Informed Consent

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

Conflict of Interest

Bethanny Smith-Packard declares no conflict of interest.
Scott M. Myers declares no conflict of interest.
Marc S. Williams declares no conflict of interest.

Author Contributions

Marc S. Williams: Conception and design, drafting article, coordination of revisions, guarantor

Bethanny Smith-Packard: Provided data, critical revision, contribution of intellectual content

Scott M. Myers: Provided data, critical revision, contribution of intellectual content

References

Averbeck N, Keppler-Ross S, Dean N (2007) Membrane topology of the Alg14 endoplasmic reticulum UDP-GlcNAc transferase subunit. J Biol Chem 282:29081–29088

Bissar-Tadmouri N, Donahue WL, Al-Gazali L, Nelson SF, Bayrak-Toydemir P, Kantarci S (2014) X chromosome exome sequencing reveals a novel ALG13 mutation in a nonsyndromic intellectual disability family with multiple affected male siblings. Am J Med Genet A 164A:164–169

de Ligt J, Willemsen MH, van Bon BW et al (2012) Diagnostic exome sequencing in persons with severe intellectual disability. N Engl J Med 367:1921–1929

Epi4K and EPGP Investigators (2013) De novo mutations in epileptic encephalopathies. Nature 501:217–221

Freeze HH, Chong JX, Bamshad MJ, Ng BG (2014) Solving glycosylation disorders: fundamental approaches reveal complicated pathways. Am J Hum Genet 94:161–175

Michaud JL, Lachance M, Hamdan FF et al (2014) The genetic landscape of infantile spasms. Hum Mol Genet 23:4846–4858

Segal MM, Abdellateef M, El-Hattab AW, Hilbush BS, De La Vega FM, Tromp G, Williams MS, Betensky RA, Gleeson J (2014) Clinical pertinence metric enables hypothesis-independent genome-phenome analysis for neurologic diagnosis. J Child Neurol. doi:10.1177/0883073814545884

Timal S, Hoischen A, Lehle L et al (2012) Gene identification in the congenital disorders of glycosylation type I by whole-exome sequencing. Hum Mol Genet 21:4151–4161

JIMD Reports
DOI 10.1007/8904_2015_417

RESEARCH REPORT

Monitoring of Therapy for Mucopolysaccharidosis Type I Using Dysmorphometric Facial Phenotypic Signatures

Stefanie Kung • Mark Walters • Peter Claes • Peter LeSouef • Jack Goldblatt • Andrew Martin • Shanti Balasubramaniam • Gareth Baynam

Received: 24 November 2014 / Revised: 29 January 2015 / Accepted: 30 January 2015 / Published online: 03 March 2015

Abstract There is a pattern of progressive facial dysmorphology in mucopolysaccharidosis type I (MPS I). Advances in 3D facial imaging have facilitated the development of tools, including dysmorphometrics, to objectively and precisely detect these facial phenotypes. Therefore, we investigated the application of dysmorphometrics as a noninvasive therapy-monitoring tool, by longitudinally scoring facial dysmorphology in a child with MPS I receiving enzyme replacement therapy (ERT) and bone marrow transplantation (BMT). Both dysmorphometric measures showed a decreasing trend, and the greatest differences were found in the severity of facial discordance (Z-RMSE), displaying scores >3 SD higher than the mean at their peak, in comparison to Z-RSD scores that mostly fell within the normative range (maximum; 1.5 SD from the mean). In addition to the general trend of reduced facial dysmorphology with treatment, initial fluctuations were also evident that may have related to transient subcutaneous facial fluctuations, in the context of conditioning for bone marrow transplant. These findings support the potential of our approach as a sensitive, noninvasive, and rapid means of assessing treatment response or failure in clinical trials, and for established therapies, and would be applicable for other inherited disorders of metabolism.

Communicated by: Frits Wijburg, MD, PhD

Competing interests: None declared

S. Kung (✉) · P. LeSouef · J. Goldblatt · A. Martin · G. Baynam
School of Paediatrics and Child Health, University of Western Australia, GPO Box D184, Perth, WA 6840, Australia
e-mail: Stefanie.Kung@health.wa.gov.au

M. Walters
Cranio-maxillo-facial Unit, Princess Margaret Hospital for Children, Perth, WA, Australia

P. Claes
Department of Electrical Engineering, ESAT/PSA, KU Leuven, Medical Image Computing, Leuven, Belgium

P. Claes
UZ Leuven, Medical Imaging Research Center, Leuven, Belgium

P. Claes
iMinds, KU Leuven, Medical IT Department, Leuven, Belgium

J. Goldblatt · G. Baynam (✉)
Genetic Services of Western Australia, King Edward Memorial & Princess Margaret Hospitals, 4th Floor Agnes Walsh House, 374 Bagot Road, Subiaco, WA 6008, Australia
e-mail: Gareth.Baynam@health.wa.gov.au

J. Goldblatt · G. Baynam
Office of Population Health Genomics, Western Australian Department of Health, Perth, WA, Australia

S. Balasubramaniam
Storage Disorders Clinic, Princess Margaret Hospital for Children, Perth, WA, Australia

G. Baynam
Institute for Immunology and Infectious Diseases, Murdoch University, Murdoch, WA, Australia

Abbreviations

3D	Three dimensional
AM	Anthropometric mask
BMT	Bone marrow transplant
ERT	Enzyme replacement therapy
GvHD	Graft-versus-host disease
HSCT	Hematopoietic stem cell transplantation
LSD	Lysosomal storage disorder
MPS IH	Mucopolysaccharidosis type I – Hurler syndrome
NE	Normal equivalent
PCA	Principal component analysis
PCs	Principal components
RMSE	Root mean squared error

RSD Relative significant discordance
SD Standard deviation

Introduction

Lysosomal storage disorders (LSD), which include mucopolysaccharidosis type I (MPS I), are a heterogeneous group of genetic disorders caused by a deficiency of one or more degradation enzymes essential for normal cell metabolism (Muhlstein et al. 2013). The lack of α-L-iduronidase leads to multisystemic accumulation of its substrates within the lysosomes. This causes a variably expressed systemic disorder, which can be clinically classified into severe or attenuated forms; this distinction influences therapeutic options (Clarke and Heppner 2011).

The development of MPS I-targeted treatments, including enzyme replacement therapy (ERT) and allogeneic hematopoietic stem cell transplantation (HSCT), has dramatically changed prognosis. However, limitations of current treatments and cost of therapy are motivating the development of novel approaches (van Gelder et al. 2012). This requires the means to objectively and recurrently assess treatment response. These assessments will preferentially be noninvasive, deeply precise, relatively inexpensive, and portable. Existing monitoring modalities are limited in their ability to objectively document responses to therapy following short-term clinical trials due to the variability of the phenotypes, the irreversibility of some complications, and the invasive nature of some investigations. Assessing response to therapy for MPS I has generally included physical/mobility tests to examine joint function, the 6-min walking test (6MWT), lung function, changes in hepatosplenomegaly, and biochemical assays of glycosaminoglycan (GAG) substrate (Church et al. 2007).

MPS I has a pattern of progressive facial dysmorphology, particularly in untreated cases. Advances in 3D facial imaging have facilitated the development of anthropometric tools, including dysmorphometrics, to objectively detect these facial phenotypes (Claes et al. 2012a, b, 2013; Hammond and Suttie 2012). Previously, dysmorphometrics was used to cross-sectionally detect and localize MPS I-associated facial dysmorphologies and, thereby, establish an objective facial phenotype. These individual signatures were attributed severity scores to discriminate between individuals clinically diagnosed with MPS I subtypes (Kung et al. 2012). In this study, we investigate the application of dysmorphometrics as a noninvasive treatment-monitoring tool, by longitudinally scoring facial dysmorphology in a treated MPS I-affected child.

Methods

Participants

A normative reference cohort of approximately 1,000 individuals was obtained from the Perth Face-Space Project, aged 1 month to 25 years and of self-reported ancestry. Participants completed a questionnaire on relevant medical history, and those with prior craniofacial surgery or a suspected syndromic condition with craniofacial manifestations were excluded from this cohort. This reference cohort was imperative for the construction of patient-specific controls. Ethics approvals (PMHEC: 1801/EP, 1443/EP, and 1488/EP) were granted by Princess Margaret Hospital for Children Ethics Committee in Perth, Western Australia.

The child with severe MPS I was recruited from the Princess Margaret Hospital for Children in Perth, Western Australia. He initially presented to the Emergency Department at 6 months of age with a cough, and a chest X-ray revealed paddle-shaped ribs, suggestive of a mucopolysaccharidosis. Weekly ERT infusions began at 10 months of age, followed by a bone marrow transplantation at 12 months of age, with a further 3 months of ERT post-BMT. 3D facial scans were ascertained at eight time points.

3D Image Acquisition, Data Preparation

3D facial scans were captured using a 3dMDFacial™ stereophotogrammetric camera system, and facial shape was expressed as a point cloud of approximately 200,000 points in a 3D coordinate space, the reliability and precision of which have been validated (Aldridge et al. 2005). These facial scans or point cloud data were brought into closer alignment by manual indication of 12 anatomical landmarks (right/left exocanthion, right/left endocanthion, pronasale, lateral nasal ala corners, right/left cheilion, upper lip tubercle, vermillion border, and chin point), in preparation for the facial mapping process. This decreases computational time and provides a basis for surface registration during facial mapping.

Anthropometric Masks and Facial Mapping

A spatially dense indicated set of 10,000 quasi-landmarks was obtained using a nonrigid surface registration (mapping) technique of a predefined facial template (anthropometric mask) (Claes et al. 2012b). This fully automated facial mapping process was performed across all faces in the dataset and required re-sampling of the raw point cloud data into a more comparable format. A reference scan, known as an anthropometric mask and representing the standard of connectivity for all scans, was

then created through an iterative "bootstrapping" method, as described by Claes (2007). Once facial scans were mapped, dense anatomical correspondence was achieved and allowed for biologically valid comparisons to be performed.

Reference Face Space

To define the statistical limits of typical facial variation in a normative reference population, a statistical face space was constructed from 1,000 individuals in our reference range cohort. A generalized Procrustes fit rotated, translated, and scaled the quasi-landmark configurations into the same coordinate space, where shape variation was described by Procrustes distance residuals. This statistical face space, via principal component analysis (PCA), describes variations in facial form and elucidates complex harmonic interrelationships between these variations.

Dysmorphometrics

Using the dysmorphometric approach (Claes et al. 2012a), normative references were encoded within the face space, and outliers in comparison to this normative reference reflected discordancy in the facial form (i.e., facial dysmorphology). This process involves the robust superimposition of the reference face space onto the patient's facial scan, where each of the 10,000 quasi-landmarks is assigned a confidence value against a *p*-value of 0.05. A value closer to 1 reflects the tendency of that point being harmonic (inlier), while a value closer to 0 reflects its tendency of being discordant (outlier). Through this superimposition, patient-specific and population-based matched references called "normal equivalents" (NE) were generated. The NE (Claes et al. 2013) describes any given face in terms of harmonious facial variation (i.e., patient's facial scan without the dysmorphology), and its construction was performed without any a priori knowledge of the condition itself. NE facial scans were generated at the eight assessment time points during the treatment course.

Normal Equivalent Facial Assessments

To analyze the facial discordancy at each time point, each NE was superimposed onto its chronologically corresponding patient scan. The differences between corresponding landmarks of each NE patient scan pair provided the means to measure both the magnitude and vectors (direction) of the observed facial discordances. Global scores RSD (relative significant discordance; %) and RMSE (root mean squared error; mm) provide an overall measure of discordant facial proportions and discordance severity, respectively. The NE assessment also outputs two dysmorphograms that enable visualization of discordances on a facial manifold, namely, (1) distance and (2) outlier facial maps. Distance facial maps highlight localized regions of RMSE, which take into account both variance and possible bias, as an error in millimeters (mm). Outlier (confidence) maps highlight localized regions of RSD on the facial surface, while vector maps provide directional information on the observed facial discordance. Collectively, these distance, outlier, and vector maps provide an individualized dysmorphometric signature. The method workflow presented in this study is summarized in the Fig. 1. Normalized Z-scores (Z-RSD, Z-RMSE) were also generated from reference summary NE statistics obtained from the normative population, RSD (mean 10.6 and SD 1.8) and RMSE (mean 0.91 and SD 0.22).

Results

The objective measures showed similar trends for both global RSD and RMSE discordance scores over the treatment course; their regional differences are highlighted in facial outlier and distance maps, respectively (Fig. 2). The greatest amount of change was seen at the lower two-thirds of the face; in particular, facial discordance at the nasal, perioral/labial, cheek, and mandibular regions diminished over the treatment course. The fullness of the upper lip, though reduced, was relatively persistent.

Both discordance outcomes showed some fluctuations over the first few months, before a steady decline. This pattern is visually most notable in the graphs of normalized Z-scores presented in Fig. 3 (Z-RSD) and Fig. 4 (Z-RMSE), respectively. Within the first month (T1–2), ERT had a greater apparent impact on the severity of the facial discordance, compared to the proportion of discordance. Z-RSD and Z-RMSE scores both increased rapidly after BMT conditioning (T3–5) and peaked at around 4 months after BMT (T5, T6), during the BMT/ERT/cyclosporin phase. Progressive lessening of facial severity and discordance becomes apparent after 4–5 months (T5, T6), which corresponded to the BMT/ERT/cyclosporin treatment phase. This reduction continued until the final assessment time point. Overall, both Z-scores showed a decreasing trend, which was more pronounced in the Z-RMSE scores; Z-RMSE displayed larger scores (>3 SD higher than the mean) at its peak, in comparison to the Z-RSD scores that mostly fell within the normative range (max point: 1.5 SD from the mean).

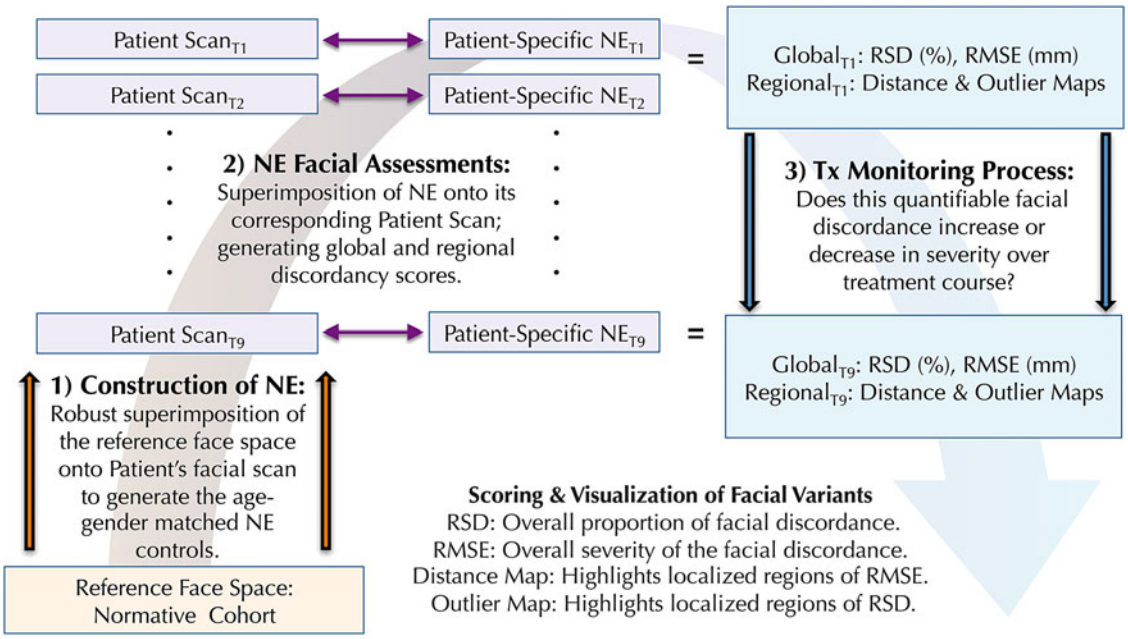


Fig. 1 Method workflow used for the MPS I treatment-monitoring process

Discussion

For the first time we objectively, noninvasively, and dynamically assessed the changing 3-dimensional facial dysmorphology in a child undergoing disorder-specific treatment for a systemic metabolic disorder, namely, MPS I. This deeply precise dysmorphometric assessment demonstrated a reduction in facial dysmorphology which was in accordance with expectations from clinical experience and with that based on an exploratory cross-sectional study, demonstrating the correlation of the severity of facial and clinical MPS I phenotype (Kung et al. 2012). Additionally, it builds upon previous 3D facial analysis-based treatment monitoring of a localized facial pathology (Baynam et al. 2013) and of a dysmorphic non-metabolic disorder (de Souza et al. 2013). This study extends the findings of the aforementioned investigations, and they support that deep facial phenotyping may have applications for the development of treatment response biomarkers.

The greatest differences were found in the severity of facial discordance (root mean square error, RMSE). Additionally, overall, the longitudinal pattern of changes in the proportion of facial discordance (relative significant discordance; RSD) was in accordance with the pattern of the RMSE. This supports that approaches that provide multiple facial outcome measures may act to (1) corroborate each other, (2) provide complementary approaches to summarize variations in facial form, and (3) provide contrasting windows through which to consider the implications of found facial variation.

As treatments, including ERT and BMT, may ameliorate, but not reverse or prevent all MPS I manifestations (Wynn 2011), the expectation is of an initial period of improvement followed by a period of stabilization. Therefore, it is possible that there may be some persistence of residual facial dysmorphology and/or its partial recurrence. Our study's findings are in accordance with anticipated improvement/stabilization; however, it is possible that there may be persisting residual/recurrent facial dysmorphology over time.

The fluctuations in the global discordance scores detected during the first few months of therapy are notable. There was an initial reduction in facial severity during the initiation of ERT. Subsequently there was an overall ballooning facial effect, which may be related to transient subcutaneous facial fluctuations that were the result of the effects of conditioning and events in the initial post-BMT period. Should the documented variations be correlating with acute treatment events, this might then indicate our approach could be both a sensitive and rapid means of assessing treatment response or failure for established therapies, in drug development and clinical trials.

It is important to note that the measures (RSD, RMSE) used in this study are, by definition, relative scores. The purpose of the NE is to remove confounding factors like within-population variances (e.g., age, gender, ethnicity, and body mass index), which provides a more individualized assessment. It is the harmonic regions of the patient facial scan that drives the construction of the corresponding NE facial scan. Longitudinal changes, such as ageing, alter the patient's facial configuration at each time point, which

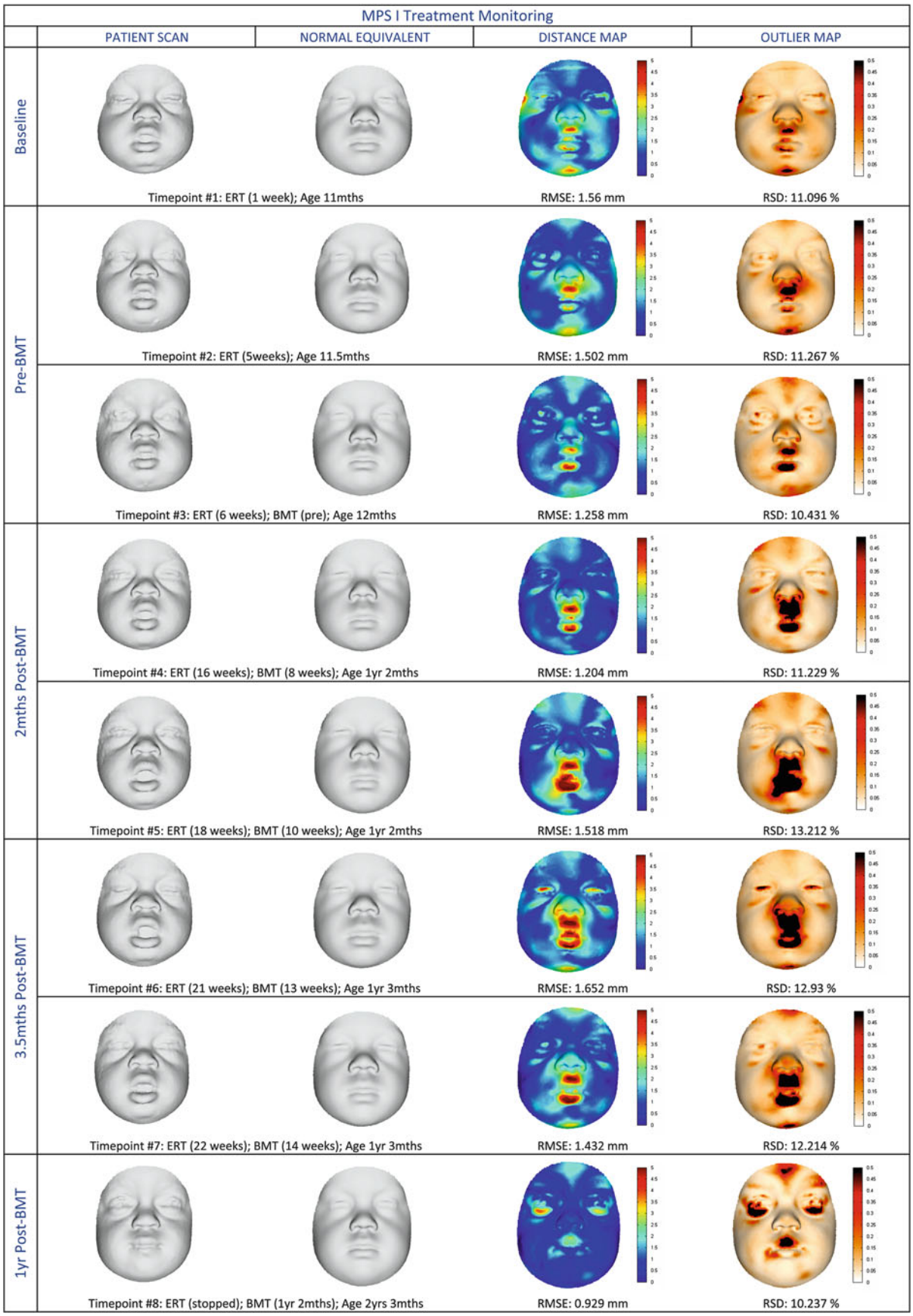


Fig. 2 MPS I longitudinal treatment monitoring over eight time points

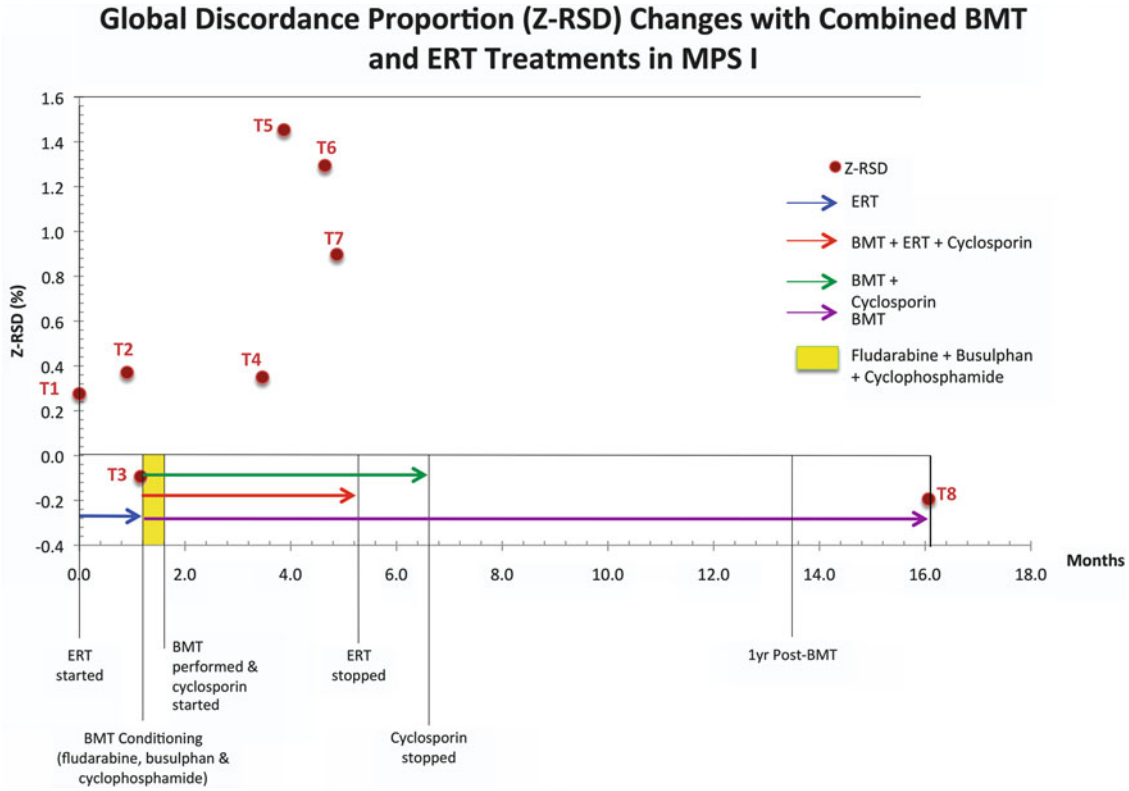


Fig. 3 Progression of normalized facial discordance proportions along the MPS I treatment course. Z-relative significant discordance (Z-RSD; %; *red points*) scores were computed to enable standardization against the normative reference population

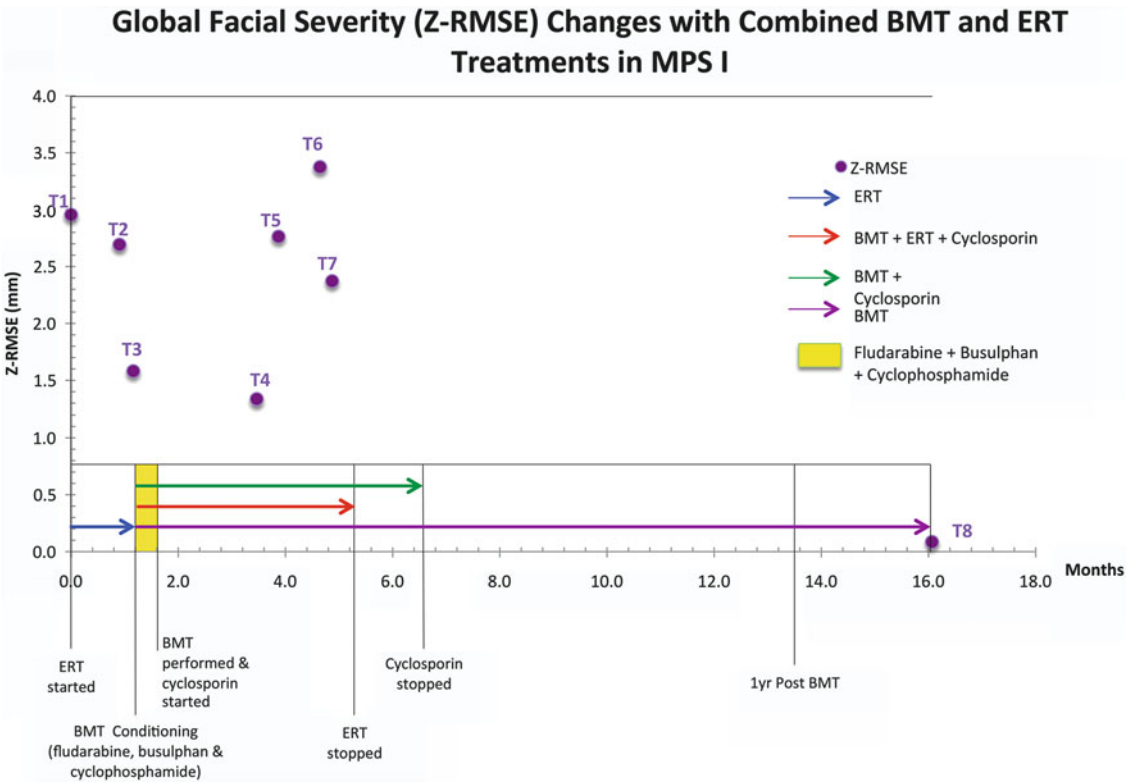


Fig. 4 Progression of normalized facial severity scores along the MPS I treatment course. Z-root mean squared error (Z-RMSE; mm; *purple points*) scores were computed to enable standardization against the normative reference range

then alters the harmonic interrelationships within that patient scan. This, in turn, changes the NE with each new time point. Hence, in this study we see eight similar yet different NE facial scans of the same patient. Therefore, direct comparisons of normal equivalents between time points require consideration within this context. For instance, the fullness of the labial region, on a background of the typical fuller face of infancy, might be more "harmonic" and therefore less discordant when compared to labial fullness in an older child. Interestingly, this labial fullness is seen in the 14-year-old MPS IH individual reported by Kung et al. (2012), where lip prominence was detected as a discordant feature 13 years after BMT. This unmasking of present, but challenging to discern without objective support, and age-dependent dysmorphology could partly explain challenges in unaided clinical facial assessment. However, they may be identified with objective and precise approaches such as the one described herein.

This study was limited to the assessment of one individual, and other treatment endpoints were not available for comparison. Further studies will be required to investigate additional individuals and importantly to relate their facial phenotype to existing outcome measures. Given the individual rarity of these conditions, this will require multi-institutional assessments, e.g., urinary glycosaminoglycans and measures of respiratory function and endurance. Fortunately, the wide geographic dispersion of currently available robust and precise imaging equipment can facilitate this in the immediate term. Also, rapid advances in the development of increasingly portable and less costly 3D imaging equipment will make this increasingly feasible in the intermediate term including for point of care or ultimately in-home image capture.

This approach could be expanded to other metabolic and non-metabolic disorders with known (or as yet unappreciated) facial phenotypes with current, and emerging, disorder-specific therapies.

Conclusion

This longitudinal study demonstrated objective and deeply precise changes in facial morphology in a child with treated MPS I. If corroborated by further studies, including correlation with other objective treatment outcomes, this supports the use of dysmorphometric 3D facial analysis as a noninvasive and relatively inexpensive treatment biomarker to rapidly assess therapeutic response in this condition and possibly other disorders with facial dysmorphology. Additionally, our longitudinal findings in a young child, particularly when coupled with previous cross-sectional studies (Kung et al. 2012), suggest that this approach could aid early clinical classification.

Acknowledgments We are indebted to the child with MPS I and his family for participation in this study and permission for image use. We would like to thank Genzyme Australia for providing an unrestricted educational grant. The Princess Margaret Hospital Foundation in Perth, Western Australia, also provided support for this study. RD-Connect, the associated National Health and Medical Research Council of Australia, and the Raine Clinician Research Fellowship supported GB's contribution.

Synopsis

Longitudinal quantification of objective and deeply precise changes in MPS I facial morphology during treatment demonstrates the potential of dysmorphometric 3D facial analysis as a noninvasive treatment response biomarker.

Compliance with Ethics Guidelines

Conflict of Interests

Stefanie Kung (SK), Mark Walters (MW), Peter Claes (PC), Peter LeSouef (PLS), Jack Goldblatt (JB), Andrew Martin (AM), Shanti Balasubramaniam (SB), and Gareth Baynam (GB) declare that they have no conflict of interests. Genzyme provided an unrestricted educational grant and had no role in the interpretation/analysis of the data or the decision to submit the manuscript for publication.

Informed Consent

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

Author Contributions

SK drafted the initial manuscript with subsequent revisions and input from **MW**, **GB**, **JG,** and **PC. GB** and **JG** conceived the study. **SK** acquired the longitudinal facial data and implemented the facial assessments. **PC** performed the facial mapping of the data and generated normative reference discordancy statistics. All authors were involved in the interpretation of the results. **SB**, **AM**, **JG,** and **PLS**

provided valuable clinical insight into the patient's treatment regime, and the investigated syndrome.

Guarantor

GB accepts full responsibility for this work and conduct of this study as guarantor for this article. He has had access to the research data and controls the decision to publish.

References

Aldridge K, Boyadjiev SA, Capone GT, DeLeon VB, Richtsmeier JT (2005) Precision and error of three-dimensional phenotypic measures acquired from 3dMD photogrammetric images. Am J Med Genet A 138A(3):247–253

Baynam GS, Walters M, Dawkins H, Bellgard M, Halbert AR, Claes P (2013) Objective monitoring of mTOR inhibitor therapy by three-dimensional facial analysis. Twin Res Hum Genet 16 (4):840–844

Church H, Tylee K, Cooper A et al (2007) Biochemical monitoring after haemopoietic stem cell transplant for Hurler syndrome (MPSIH): implications for functional outcome after transplant in metabolic disease. Bone Marrow Transplant 39(4):207–210

Claes P (2007) A robust statistical surface registration framework using implicit function representations: application in craniofacial reconstruction. KU Leuven, Leuven

Claes P, Daniel K, Walters M, Clement JG, Vandermeulen D, Suetens P (2012a) Dysmorphometrics: the modelling of morphological anomalies. Theor Biol Med Model 9(5):1–39

Claes P, Walters M, Clement JG (2012b) Improved facial outcome assessment using a 3D anthropometric mask. Int J Oral Maxillofac Surg 41:324–330

Claes P, Walters M, Gillett D, Vandermeulen D, Clement JG, Suetens P (2013) The normal-equivalent: a patient-specific assessment of facial harmony. Int J Oral Maxillofac Surg 42(9):1150–1158

Clarke LA, Heppner J (2011) Mucopolysaccharidosis type I. University of Washington, Seattle

de Souza MA, McAllister C, Suttie M et al (2013) Growth hormone, gender and face shape in prader-willi syndrome. Am J Med Genet A 161(10):2453–2463

Hammond P, Suttie M (2012) Large-scale objective phenotyping of 3D facial morphology. Hum Mutat 33(5):817–825

Kung S, Walters M, Claes P, Goldblatt J, Le Souef P, Baynam G (2012) A dysmorphometric analysis to investigate facial phenotypic signatures as a foundation for non-invasive monitoring of lysosomal storage disorders. Springer, Berlin/Heidelberg, pp 1–9

Muhlstein A, Gelperina S, Kreuter J (2013) Development of nanoparticle-bound arylsulfatase B for enzyme replacement therapy of mucopolysaccharidosis VI. Pharmazie 68(7):549–554

van Gelder CM, Vollebregt AA, Plug I, van der Ploeg AT, Reuser AJ (2012) Treatment options for lysosomal storage disorders: developing insights. Expert Opin Pharmacother 13 (16):2281–2299

Wynn R (2011) Stem cell transplantation in inherited metabolic disorders. Hematology 2011:285–291

JIMD Reports
DOI 10.1007/8904_2015_418

RESEARCH REPORT

Age at First Cardiac Symptoms in Fabry Disease: Association with a Chinese Hotspot Fabry Mutation (IVS4+919G>A), Classical Fabry Mutations, and Sex in a Taiwanese Population from the Fabry Outcome Survey (FOS)

Hao-Chuan Liu · Amandine Perrin · Ting-Rong Hsu · Chia-Feng Yang · Hsiang-Yu Lin · Wen-Chung Yu · Dau-Ming Niu

Received: 14 August 2014 / Revised: 30 January 2015 / Accepted: 02 February 2015 / Published online: 12 March 2015

Abstract This is a descriptive analysis of a cohort of 59 Taiwanese patients with Fabry disease and either classical Fabry or cardiac variant IVS4+919G>A (IVS4) mutations from a disease registry, the Fabry Outcome Survey (FOS; sponsored by Shire). Most of our classical Fabry patients were symptomatic and were identified upon seeking medical advice at our clinics, whereas most of our IVS4 patients attended our clinics after newborn screening identified this mutation in their grandsons. The objective was to determine differences in cardiac manifestations between patients with classical Fabry or IVS4 mutations by comparing age at onset of selected cardiac symptoms. Data were extracted in August 2013 and analyzed retrospectively. Fifty-nine Taiwanese patients (median age at extract 60.7 years [range 15.0–86.9]; $n = 36$ [61%] male) with proven IVS4 ($n = 41$ [69%]) or classical Fabry mutations ($n = 18$ [31%]) had available data on cardiac symptoms. Of 55 (93%) patients with reported left ventricular hypertrophy (LVH), mean [SD] age (years) at first symptom was lower in classical Fabry males (30.0 [15.1]; $n = 4$) than classical Fabry females (49.6 [8.9]; $n = 11$; $p < 0.05$), but not in IVS4 females (57.4 [13.7]; $n = 10$) compared with IVS4 males (55.9 [11.3]; $n = 30$). Mean age at first LVH diagnosis was significantly lower in classical Fabry males versus IVS4 males ($p < 0.05$). No significant difference in age at onset of arrhythmia or conductive abnormality, chest pain, or palpitations or cardiac syncope was found between the groups. The most noteworthy finding of this study is the lack of a significant gender sex difference in age at onset of cardiac symptoms in IVS4 patients.

Communicated by: Robert J Desnick, PhD, MD

Competing interests: None declared

H.-C. Liu
Department of Pediatrics, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan

A. Perrin
Shire, Eysins, Switzerland

T.-R. Hsu · C.-F. Yang · D.-M. Niu
Department of Pediatrics, Taipei Veterans General Hospital, Taipei, Taiwan

T.-R. Hsu · D.-M. Niu (✉)
Institute of Clinical Medicine, National Yang-Ming University, School of Medicine, Taipei, Taiwan
e-mail: dmniu1111@yahoo.com.tw

H.-Y. Lin
Department of Pediatrics, Mackay Memorial Hospital, Taipei, Taiwan

H.-Y. Lin
Department of Medicine, Mackay Medical College, Taipei, Taiwan

W.-C. Yu (✉)
Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan
e-mail: wcyu@vghtpe.gov.tw

W.-C. Yu
National Yang-Ming University, School of Medicine, Taipei, Taiwan

D.-M. Niu
Genetic Consultant Center, Taipei Veterans General Hospital, Taipei, Taiwan

Introduction

Fabry disease is caused by the deficiency or absence of alpha-galactosidase A (α-Gal A) activity, leading to progressive deposition of glycosphingolipids, mainly

globotriaosylceramide (Gb_3), in the lysosomes of multiple tissues and organs. Originally thought to be less severe in females (Desnick et al. 2001), more recent evidence indicates that symptoms of this X-linked disorder can manifest as severely in females as in males (Mehta et al. 2004; Wilcox et al. 2008), although they generally occur later in life and show greater variation in severity among female patients (Deegan et al. 2006). The frequency of classic Fabry disease has been estimated as one in 40,000, and its symptoms typically manifest during childhood, including acroparesthesias, angiokeratoma, corneal opacities, and anhidrosis (Desnick et al. 2001; Ries et al. 2005). Atypical, late-onset phenotypes have been reported that lack these classic symptoms but instead present with cardiac (Nakao et al. 1995), renal (Nakao et al. 2003), or cerebrovascular disease (Brouns et al. 2010). The frequency of atypical Fabry disease is unknown, but it has been suggested to be more common than previously believed (Nakao et al. 1995). In Taiwan, our team first revealed a surprisingly high incidence (approximately one in 1,600 males) of a cardiac variant GLA splicing mutation, IVS4 +919G>A, in our newborn population (Chong et al. 2008) and subsequently identified this mutation in a number of Taiwan Chinese adult patients with idiopathic hypertrophic cardiomyopathy (Lin et al. 2009, 2010, 2013). Thereafter, another newborn screening center in Taiwan also revealed a very similar incidence (one in 1,460 males) of this mutation in their study (Hwu et al. 2009). In addition to Taiwan, this mutation has also been found in Japan (Ishii et al. 2002), China, and in Han populations from Singapore, Malaysia, the Philippines, and Vietnam by our team (Niu, unpublished data).

Our previous study (Lin et al. 2010) showed that a high proportion of adults (>40 years of age) carrying the IVS4 +919G>A mutation experienced microalbuminuria and retinal vessel tortuosity, but symptoms involving these organs were very mild and did not cause significant morbidity. However, a high frequency of severe cardiac symptoms causing significant morbidity was also found among these adults. More recently, DNA-based newborn screening for this mutation revealed a higher incidence (one in 875 males and one in 399 females) than our previous enzyme-based Fabry newborn screening in Taiwan (Chien et al. 2012).

Although the hotspot IVS4+919G>A mutation is now being observed with greater frequency, understanding of the natural course of cardiac variant Fabry disease with this specific mutation remains limited. The objective of this study was to determine differences in cardiac manifestations between patients with the IVS4 and classical Fabry mutations by comparing age at first manifestation of selected cardiac symptoms in Taiwanese patients with data recorded in the Fabry Outcome Survey (FOS). The FOS is an international registry, sponsored by Shire, for the long-term collection of data on the natural history of Fabry disease in patients who are either untreated or treated with agalsidase alfa enzyme replacement therapy (ERT).

Patients and Methods

Entry of data from Taiwanese patients into the FOS database began in July 2012. All Taiwanese patients with the IVS4+919G>A mutation (IVS4 patients) or classical mutations (classical Fabry patients; Table 1) are eligible for inclusion in FOS, whether or not they have received ERT with agalsidase alfa. Fabry disease diagnosis in all patients was confirmed by enzyme assay measuring α-Gal A activity (males) and/or α-Gal A gene mutation analysis (males and females). All of the procedures undertaken in this study were approved by the institution review board, and all patients gave written, informed consent prior to data entry.

Anonymous data for analysis are submitted electronically to the central FOS database. Each patient's medical history is documented by a physician or nurse specialist, including the year of Fabry disease diagnosis, signs and symptoms of the disease, treatment, demographic details, and family history.

All measurements routinely performed in clinical practice are entered into the database. Echocardiographic data are collected in accordance with pre-specified guidelines contained within FOS. Measurements were performed according to the American Society of Echocardiography recommendations (Nagueh et al. 2009). Left ventricular parameters including diastolic interventricular septal thickness (IVSd), systolic and diastolic left ventricular internal

Table 1 Classical Fabry mutations

Classical Fabry mutation
• c.274G>T(D92Y)
• c.319C.T(p.Q107X)
• c.394G>A(p.G132R)
• c.612G>A(p.W204X)
• c.901C>T(p.R301X)
• c.1034C>G(p.5345X)
• c.1066C>G(p.R356W)
• c.1081G>C(p.G361X)
• c.1087C>T(p.R363C)
• c.1095delT(p.Y365X)
• c.1194delA(p.E398DfsX6)
• c.1228A>G(p.T410A)

diameter (LVIDd and LVIDs), and diastolic left ventricular posterior wall thickness (LVPWd) were measured by two-dimensional guided M-Mode echocardiography. Left ventricular mass (LVM) was calculated according to the formula published by the American Society of Echocardiography (Lang et al. 2005): $LVM\ (g) = 0.8 \times (1.04 \times [(LVIDd) + (IVSd) + (LVPWd)]^3 - [LVIDs]^3) + 0.6$. Left ventricular mass was normalized to height in meters$^{2.7}$ ($LVMI = LVM/height^{2.7}$). Left ventricular hypertrophy (LVH) is defined as left ventricular mass indexed to height$^{2.7}$ (g/m$^{2.7}$) of >51 g/m$^{2.7}$ in males and >48 g/m$^{2.7}$ in females (de Simone et al. 1992).

In this study, we examine the baseline data of cardiac manifestations in Taiwanese patients with IVS4+919G>A or classical Fabry mutations recorded in the FOS database. Age, sex, genetic mutation, LVMI, and age at first symptoms/signs, including arrhythmia or conductive abnormality, chest pain, left ventricular hypertrophy, and palpitations or cardiac syncope, were analyzed. Data for this study were extracted from the FOS database in August 2013. Data extraction and analysis in FOS are supported by Shire.

Statistical Analyses

Descriptive statistics were calculated, and statistical significance to compare age at onset of the selected cardiac symptoms by mutation group (IVS4 or classical Fabry) and sex was evaluated using the full interaction analysis of variance (ANOVA) model methodology. Fisher's exact test was used to compare the prevalence of cardiac symptoms between the IVS4 and classical mutation groups. Statistical significance was set at 5%, and SAS version 9.2 software was used to perform the tests.

Results

By August 2013, from a total of 71 Taiwanese patients registered in the FOS database, there were 67 Taiwanese patients with proven IVS4+919G>A mutation or classical Fabry mutations. Fifty-nine had cardiac symptoms recorded and were included in this analysis (Fig. 1). The median age at the time of data extraction was 60.7 years (range 15.0–86.9). Two of the patients with classical Fabry mutations were younger than 18 years. Thirty-six patients (61%) were male, and 23 patients (39%) were female. A total of 41 patients (69%) had IVS4+919G>A mutations, which is twice as many as had classical Fabry mutations (18 patients, 31%). Of the IVS4 patients, there were more males (30 patients, 73%) than females (11 patients, 27%). The ratio of male to female patients with the IVS4 +919G>A mutation was 2.73:1, compared with 1:2 with classical Fabry mutations (six male patients, 33%; 12 female patients, 67%). The demographic characteristics of the study population are shown in Table 2.

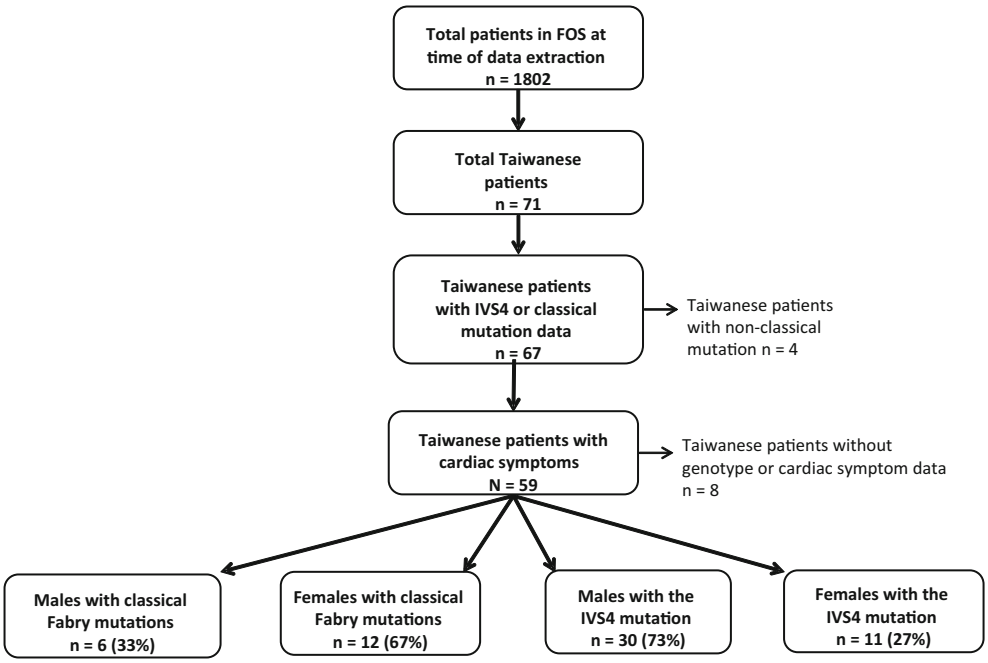


Fig. 1 Flow diagram of patients included in this analysis

Table 2 Demographic characteristics and sex and mutation status of Taiwanese patients in FOS with cardiac symptoms and IVS4 or classical Fabry mutations ($n = 59$)

Characteristic	$N = 59$
Median (range) age at extract, years	60.7 (15.0–86.9)
Aged <18 years at extract, *n* (%)	2 (3)
Male, *n* (%)	36 (61)
Female, *n* (%)	23 (39)
Residual α-Gal A activity, % of midpoint of normal range, mean (SD)	
IVS4 overall ($n = 41$)	21.6 (21.0)
IVS4 male ($n = 30$)	10.4 (4.5)
IVS4 female ($n = 11$)	52.2 (17.4)
Classical Fabry overall ($n = 16$)[a]	31.4 (25.0)
Classical male ($n = 4$)[a]	2.9 (3.5)
Classical female ($n = 12$)	41.0 (21.2)
Sex and Fabry mutation status, *n* (%)	
IVS4 overall	41 (69)[b]
IVS4 male	30 (73)
IVS4 female	11 (27)
Classical overall	18 (31)[b]
Classical male	6 (33)
Classical female	12 (67)

[a] $n = 2$ missing
[b] Prevalence, IVS4 overall vs. classical overall; $p < 0.05$

The prevalence, mean, and median age at first cardiac symptoms in Taiwanese patients in FOS are shown in Table 3. Left ventricular hypertrophy was the most common sign of cardiac manifestation in both IVS4 and classical Fabry patients. Fifty-five (93%) patients, including 40 (97.6%) with the IVS4+919G>A mutation (100% in males and 90.9% in females) and 15 (83.3%) with classical Fabry mutations (66.7% in males and 91.7% in females), were found to have LVH. The mean [SD] age at first diagnosis of LVH was significantly lower in classical Fabry males (30 years [15.1]; $n = 4$) than in classical Fabry females (49.6 years [8.9]; $n = 11$; $p < 0.05$), but in the IVS4 patients there was no difference between IVS4 males (55.9 years [11.3]; $n = 30$) and IVS4 females (57.4 years [13.7]; $n = 10$). Also, a significantly lower age at first LVH diagnosis was found for classical Fabry males compared with IVS4 males ($p < 0.05$); however, no significant difference was found in mean age at first LVH diagnosis between classical Fabry females and IVS4 females. Furthermore, age at LVH diagnosis was significantly lower in classical Fabry males compared with classical Fabry females and IVS4 males and females combined ($p < 0.01$).

Regarding arrhythmia or conductive abnormality, none of our classical Fabry males had these signs; however, more than half of the IVS4 females (54.5%), classical Fabry females (58.3%), and most of the IVS4 males (83.3%) did. Interestingly, no significant difference in age at onset of arrhythmia or conductive abnormality was found between these three groups. Regarding chest pain, 30% of IVS4 males, 45.5% of IVS4 females, 16.7% of classical Fabry males, and 66.7% of classical Fabry females had this symptom. No significant difference in age at onset of this cardiac symptom was found among these four groups. Similarly, there was no significant difference between these four groups in age at onset of palpitations or cardiac syncope, which occurred in 33.3% IVS4 males, 45.5% IVS4 females, 16.7% classical Fabry males, and 50% classical Fabry females.

Discussion

The most noteworthy finding in this study is that no significant difference in age at onset of cardiac manifestations was found between IVS4 males and females. This is quite different to the situation in classical Fabry patients, where males show a trend toward a more severe clinical course and earlier disease onset age than females (Mehta et al. 2009). It is generally thought that cardiac manifestations of Fabry disease, such as arrhythmia, angina, and LVH, are caused by Gb_3 accumulation in sinus nodes, the

Table 3 Prevalence (%) of cardiac symptoms and mean (SD) and median (range) age at onset in Taiwanese patients in FOS

	IVS4 mutation		Classical Fabry mutation	
Age at first symptom	Male ($N = 30$)	Female ($N = 11$)	Male ($N = 6$)	Female ($N = 12$)
LVH[a]	$n = 30$ (100%)	$n = 10$ (90.9%)	$n = 4$ (66.7%)	$n = 11$ (91.7%)
Mean (SD)	55.9 (11.3)[b]	57.4 (13.7)	30.0 (15.1)[b,c]	49.6 (8.9)[c]
Median (range)	58.9 (19.9–68.3)	57.8 (34.7–86.0)	28.1 (14.3–49.4)	51.7 (32.6–59.7)
Chest pain	$n = 9$ (30%)	$n = 5$ (45.5%)	$n = 1$ (16.7%)	$n = 8$ (66.7%)
Mean (SD)	48.6 (12.7)	40.9 (10.8)	43.5	47.8 (13.6)
Median (range)	47.4 (27.1–66.6)	43.2 (24.4–52.1)	43.5 (43.5–43.5)	48.8 (29.2–68.2)
Arrhythmia or conductive abnormality	$n = 25$ (83.3%)[d]	$n = 6$ (54.5%)	$n = 0$ (0%)[d]	$n = 7$ (58.3%)
Mean (SD)	58.0 (7.3)	63.3 (19.6)	–	49.2 (11.5)
Median (range)	60.3 (39.8–67.5)	60.5 (37.1–86.0)	–	51.2 (32.7–65.7)
Palpitations or syncope	$n = 10$ (33.3%)	$n = 5$ (45.5%)	$n = 1$ (16.7%)	$n = 6$ (50%)
Mean (SD)	54.7 (10.2)	42.4 (11.9)	43.5	51.6 (13.2)
Median (range)	58.8 (39.8–66.6)	47.0 (24.4–54.8)	43.5 (43.5–43.5)	52.5 (29.2–68.2)

[a] Age at first diagnosis of LVH
[b] Age, IVS4 male vs. classical Fabry male; $p < 0.05$
[c] Age, classical Fabry male vs. classical Fabry female; $p < 0.05$
[d] Prevalence, IVS4 male vs. classical Fabry male; $p < 0.001$

conduction system, vascular endothelium, and myocardiocytes. Therefore, the more severe the α-Gal A defect, the greater the accumulation of Gb_3 and the more severe the clinical manifestations and earlier the disease onset will be. Fabry disease is believed to be an X-linked disease, where female patients usually have two X chromosomes containing two α-Gal A genes. After random X-chromosome inactivation, a heterozygote Fabry female becomes mosaic, with two cell populations, one of which expresses the normal α-Gal A gene and the other the abnormal α-Gal A gene. As a consequence, except in rare cases of skewed X-chromosome inactivation, random X-chromosome inactivation usually leads to greater residual enzyme activity, lower levels of Gb_3 accumulation, a milder clinical and biochemical phenotype, and a later Fabry disease onset age in females compared with males, especially when they have the same α-Gal A gene mutation. Since this phenomenon can be observed in classical Fabry patients, we found the lack of a significant difference in age at onset of cardiac manifestations between IVS4 males and females in the current study surprising. Interestingly, we also found that females with classical Fabry mutations appeared to have a similar age at onset of the selected cardiac symptoms as male and female IVS4 patients. Currently, we do not have a good explanation for this finding. It could mean that the amount of Gb_3 accumulation is not the main factor inducing cardiac manifestations in heterozygous females and patients with a milder form of Fabry disease. Indeed, there is some evidence to suggest that increased levels of urinary and plasma Gb_3 are not correlated with clinical Fabry symptoms (Vedder et al. 2007). Perhaps there are other contributing pathogenic factors, such as degree of the inflammatory reaction (Biancini et al. 2012; De Francesco et al. 2013), the vulnerability of cardiomyocytes (modified by some cardiomyopathic genes or other unknown genes) (Desnick and Doheny 2014), or cell non-autonomous phenomena.

Recently, a new hypothesis of cross-induction by globotriaosylsphingosine (lyso-Gb_3) was proposed in order to explain damage to cells containing active non-mutated X chromosomes in heterozygous Fabry females (Pinto et al. 2010). This hypothesis is based on increased levels of plasma lyso-Gb_3, but not Gb_3, found in most symptomatic female heterozygotes, which appears to be positively correlated with the severity of the clinical picture (Aerts et al. 2008). This widely diffusible lyso-Gb_3 might have the capacity to induce cell damage via some mechanism that we do not yet understand well. Interestingly, in a previous study on our IVS4 patients, we also found only lyso-Gb_3 levels, not Gb_3, elevated in our symptomatic male and female patients, and these lyso-Gb_3 levels also appeared to positively correlate with the severity of cardiac manifestations (Liao et al. 2013). Since we found that serum lyso-Gb_3 can be elevated in both male and female infants, does this mean that cardiac manifestations are induced after a long and insidious course of elevated lyso-Gb_3?

We believe that it is too soon to propose any alternative hypotheses regarding the pathogenesis of cardiac manifestations in Fabry disease, especially with such inconclusive evidence. Although this study is the first and largest analysis comparing age at onset of cardiac manifestations between male and female IVS4 cardiac variant and classical

Fabry disease patients, the sample size is not large enough to reach any definitive conclusions. However, we hope that this report will encourage investigators in the field of Fabry disease to collect more data to help clarify whether or not a significant difference exists in age at onset of cardiac manifestations between cardiac variant Fabry male and female patients or even among classical Fabry female patients. If there is, it will be interesting to investigate the possible alternative pathogenesis of cardiac manifestations in these patients.

There are some limitations in this study. This analysis is retrospective in nature and uses data from FOS (a physician-driven registry for patient data in real-world healthcare settings); thus, the criteria for patient selection were not as stringent as in a clinical trial. As previously noted, the sample size was small (zero in one of the cardiac symptom groups); this is not unusual in rare disease registries, but for this analysis some of the potentially eligible patients could not be included due to incomplete database entries at data extraction. However, of the 71 Taiwanese patients registered in FOS at the time of data extraction, the majority ($n = 59$; 83.1%) met the inclusion criteria. Due to the small sample size, only descriptive statistics were used, and the possibility of a confounding effect, or that some of these analyses may have lacked the statistical power to detect a significant effect, cannot be ruled out without further follow-up to confirm the trends observed. There is also the possibility of selection bias in this analysis, since most of our classical Fabry patients came to our clinics owing to their symptoms/signs of Fabry disease (acroparesthesia, renal insufficiency, or hypertrophic cardiomyopathy). In contrast, most of our IVS4 patients attended our clinics as a result of newborn screening identifying the IVS4+919G>A mutation in their grandsons. Some of these IVS4 grandparents did not have significant clinical manifestations of the heart, but they were found to have hypertrophic cardiomyopathy; thus, the prevalence of hypertrophic cardiomyopathy was high in these patients. Furthermore, the patients of the IVS4 group tended to be older than those of the classical Fabry group, and we do not know how long LVH had been present in these patients; therefore, the analysis of age at onset of symptoms such as chest pain and palpitations might be subject to recall bias.

Conclusions

This retrospective study on Taiwanese patients with Fabry disease found no significant difference in onset age of cardiac manifestations between males and females with the cardiac variant IVS4+919G>A mutation.

Acknowledgments This analysis was funded by Shire. The authors would like to thank Dr HF Chen, Dr TH Chu, YH Wang, WT Su, and CC Yeh for their contributions to data collection. Medical writing support was provided by Tina Rose of Excel Scientific Solutions, which was funded by Shire.

Compliance with Ethics Guidelines

Conflict of Interest

Dau-Ming Niu has received research support, reimbursement for travel, and speaker honoraria from Shire and Genzyme.

Wen-Chung Yu has received travel grants and speaker honoraria from Shire and Genzyme.

Hao-Chuan Liu, Ting-Rong Hsu, Chia-Feng Yang, and Hsiang-Yu Lin declare that they have no conflicts of interest.

Amandine Perrin is an employee of Shire.

Informed Consent

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

Details of the Contributions of Individual Authors

All authors contributed to the planning and conduct of the study. Amandine Perrin performed the statistical analyses. All authors drafted the manuscript and approved the final version.

References

Aerts JM, Groener JE, Kuiper S et al (2008) Elevated globotriaosylsphingosine is a hallmark of Fabry disease. Proc Natl Acad Sci U S A 105(8):2812–2817

Biancini GB, Vanzin CS, Rodrigues DB et al (2012) Globotriaosylceramide is correlated with oxidative stress and inflammation in Fabry patients treated with enzyme replacement therapy. Biochim Biophys Acta 1822(2):226–232

Brouns R, Thijs V, Eyskens F et al (2010) Belgian Fabry study: prevalence of Fabry disease in a cohort of 1000 young patients with cerebrovascular disease. Stroke 41(5):863–868

Chien YH, Lee NC, Chiang SC, Desnick RJ, Hwu WL (2012) Fabry disease: incidence of the common later-onset alpha-galactosidase A IVS4+919G->A mutation in Taiwanese newborns–superiority of DNA-based to enzyme-based newborn screening for common mutations. Mol Med 18:780–784

Chong KW, Lu YH, Hsu JH, Lo MY, Hsiao CY, Niu DM (2008) High incidence of cardiac variant of Fabry disease in Taiwanese revealed by newborn screening Conference High incidence of cardiac variant of Fabry disease in Taiwanese revealed by newborn screening, Hualien, Taiwan, 2008, pp 92–98

De Francesco PN, Mucci JM, Ceci R, Fossati CA, Rozenfeld PA (2013) Fabry disease peripheral blood immune cells release inflammatory cytokines: role of globotriaosylceramide. Mol Genet Metab 109(1):93–99

de Simone G, Daniels SR, Devereux RB et al (1992) Left ventricular mass and body size in normotensive children and adults: assessment of allometric relations and impact of overweight. J Am Coll Cardiol 20(5):1251–1260

Deegan PB, Baehner AF, Barba Romero MA, Hughes DA, Kampmann C, Beck M (2006) Natural history of Fabry disease in females in the Fabry Outcome Survey. J Med Genet 43 (4):347–352

Desnick RJ, Doheny DO (2014) Targeted sequencing of over 4000 hypertrophic cardiomyopathy (HCM) patients for mutations causing HCM and Fabry disease: HCM mutations frequent in patients with GLA later-onset mutations, polymorphisms, and variants. Mol Genet Metab 111(2):S36–S37

Desnick RJ, Ioannou YA, Eng CM (2001) α-Galactosidase A deficiency: Fabry disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 3733–3774

Hwu WL, Chien YH, Lee NC et al (2009) Newborn screening for Fabry disease in Taiwan reveals a high incidence of the later-onset GLA mutation c.936+919G>A (IVS4+919G>A). Hum Mutat 30(10):1397–1405

Ishii S, Nakao S, Minamikawa-Tachino R, Desnick RJ, Fan JQ (2002) Alternative splicing in the alpha-galactosidase A gene: increased exon inclusion results in the Fabry cardiac phenotype. Am J Hum Genet 70(4):994–1002

Lang RM, Bierig M, Devereux RB et al (2005) Recommendations for chamber quantification: a report from the american society of Echocardiography's guidelines and standards committee and the chamber quantification writing group, developed in conjunction with the European association of echocardiography, a branch of the European society of cardiology. J Am Soc Echocardiogr 18 (12):1440–1463

Liao HC, Huang YH, Chen YJ et al (2013) Plasma globotriaosylsphingosine (lysoGb3) could be a biomarker for Fabry disease with a Chinese hotspot late-onset mutation (IVS4+919G>A). Clin Chim Acta 426:114–120

Lin HY, Chong KW, Hsu JH et al (2009) High incidence of the cardiac variant of Fabry disease revealed by newborn screening in the Taiwan Chinese population. Circ Cardiovasc Genet 2 (5):450–456

Lin HY, Huang CH, Yu HC et al (2010) Enzyme assay and clinical assessment in subjects with a Chinese hotspot late-onset Fabry mutation (IVS4+919G->A). J Inherit Metab Dis 33(5):619–624

Lin HY, Liu HC, Huang YH et al (2013) Effects of enzyme replacement therapy for cardiac-type Fabry patients with a Chinese hotspot late-onset Fabry mutation (IVS4+919G>A). BMJ Open 3(7)

Mehta A, Ricci R, Widmer U et al (2004) Fabry disease defined: baseline clinical manifestations of 366 patients in the Fabry Outcome Survey. Eur J Clin Invest 34(3):236–242

Mehta A, Clarke JT, Giugliani R et al (2009) Natural course of Fabry disease: changing pattern of causes of death in FOS - Fabry Outcome Survey. J Med Genet 46(8):548–552

Nagueh SF, Appleton CP, Gillebert TC et al (2009) Recommendations for the evaluation of left ventricular diastolic function by echocardiography. J Am Soc Echocardiogr 22(2):107–133

Nakao S, Takenaka T, Maeda M et al (1995) An atypical variant of Fabry's disease in men with left ventricular hypertrophy. N Engl J Med 333(5):288–293

Nakao S, Kodama C, Takenaka T et al (2003) Fabry disease: detection of undiagnosed hemodialysis patients and identification of a "renal variant" phenotype. Kidney Int 64(3):801–807

Pinto LL, Vieira TA, Giugliani R, Schwartz IV (2010) Expression of the disease on female carriers of X-linked lysosomal disorders: a brief review. Orphanet J Rare Dis 5:14

Ries M, Gupta S, Moore DF et al (2005) Pediatric Fabry disease. Pediatrics 115(3):e344–e355

Vedder AC, Linthorst GE, van Breemen MJ et al (2007) The Dutch Fabry cohort: diversity of clinical manifestations and Gb3 levels. J Inherit Metab Dis 30(1):68–78

Wilcox WR, Oliveira JP, Hopkin RJ et al (2008) Females with Fabry disease frequently have major organ involvement: lessons from the Fabry Registry. Mol Genet Metab 93(2):112–128

JIMD Reports
DOI 10.1007/8904_2015_419

CASE REPORT

Mitochondrial Complex III Deficiency Caused by *TTC19* Defects: Report of a Novel Mutation and Review of Literature

Anna Ardissone · Tiziana Granata · Andrea Legati · Daria Diodato · Laura Melchionda · Eleonora Lamantea · Barbara Garavaglia · Daniele Ghezzi · Isabella Moroni

Received: 13 November 2014 / Revised: 20 January 2015 / Accepted: 05 February 2015 / Published online: 13 March 2015

Abstract We report about a patient with infantile-onset neurodegenerative disease associated with isolated mitochondrial respiratory chain complex III (cIII) deficiency. The boy, now 13 years old, presented with language regression and ataxia at 4 years of age and then showed a progressive course resulting in the loss of autonomous gait and speaking during the following 2 years. Brain MRI disclosed bilateral striatal necrosis. Sequencing of a panel containing nuclear genes associated with cIII deficiency revealed a previously undescribed homozygous rearrangement (c.782_786delinsGAAAAG) in *TTC19* gene, which results in a frameshift with premature termination (p. Glu261Glyfs*8). TTC19 protein was absent in patient's fibroblasts.

TTC19 encodes tetratricopeptide 19, a putative assembly factor for cIII. To date *TTC19* mutations have been reported only in few cases, invariably associated with cIII deficiency, but presenting heterogeneous clinical phenotypes. We reviewed the genetic, biochemical, clinical and neuroradiological features of *TTC19* mutant patients described to date.

Communicated by: Wolfgang Sperl, MD, PhD

Competing interests: None declared

A. Ardissone (✉) · T. Granata · I. Moroni
Unit of Child Neurology, The Foundation "Carlo Besta" Neurological Institute (IRCCS), Via Celoria 11, 20133 Milan, Italy
e-mail: anna.ardissone@istituto-besta.it

A. Legati · L. Melchionda · E. Lamantea · B. Garavaglia · D. Ghezzi
Unit of Molecular Neurogenetics, The Foundation "Carlo Besta" Neurological Institute (IRCCS), Via Temolo 4, 20126 Milan, Italy

D. Diodato
Neuromuscular and Neurodegenerative Disease Unit, Children Hospital Bambino Gesù, Rome, Italy

Introduction

Isolated deficiency of mitochondrial respiratory chain (MRC) complex III (cIII) (MIM#124000) is a rare cause of mitochondrial disorder. Excluding mutations in *MTCYB*, the mitochondrial DNA (mtDNA) gene encoding cytochrome b, mutations in nuclear genes encoding other structural cIII subunits are extremely uncommon (Ghezzi and Zeviani 2012; Miyake et al. 2013). Contrariwise, several genetic defects have been described in cIII assembly factor genes, including *BCS1L* (De Lonlay et al. 2001), *TTC19* (Ghezzi et al. 2011), and *LYRM7* (Invernizzi et al. 2013). *TTC19* mutations have been reported in few patients with heterogeneous phenotypes ranging from early onset neurodegenerative disorders (Ghezzi et al. 2011; Atwal 2013; Balasubramaniam et al. 2012) to adult forms with psychiatric manifestations and cerebellar ataxia (Nogueira et al. 2013; Morino et al. 2014). An overall evaluation of known *TTC19* mutant cases including genotype/phenotype correlations has not been performed so far.

Bilateral striatal necrosis (BSN) includes a group of syndromes, usually with onset in infancy, characterized by bilateral and symmetrical degeneration of caudate and putamen nuclei (neostriatum). BSN results from either toxic exposure, infections and metabolic or neurodegenerative disorders (Zevit et al. 2007), including some mitochondrial diseases.

We report a novel deleterious mutation in *TTC19*, identified in a patient presenting with BSN and isolated

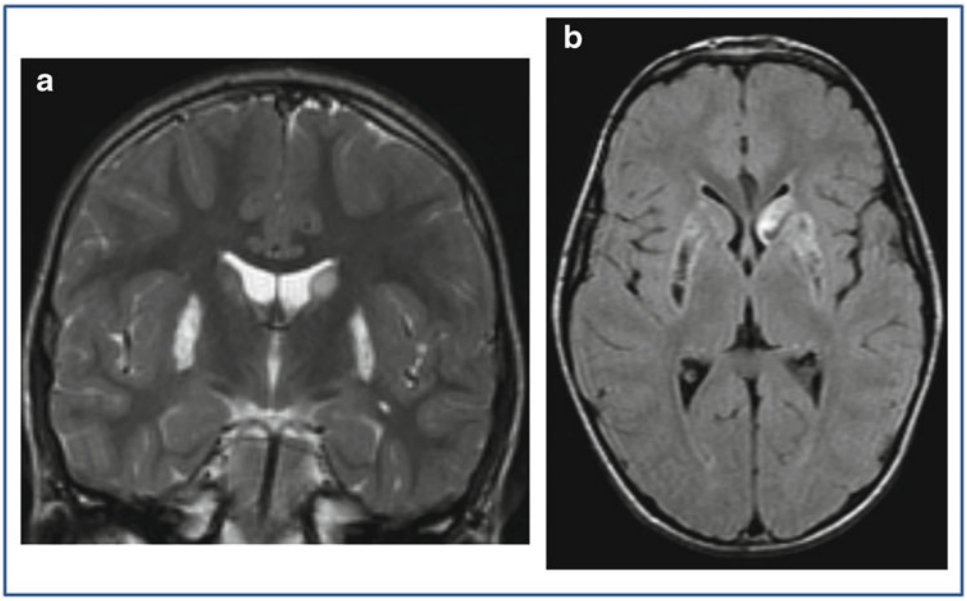


Fig. 1 Bilateral hyperintensities of the putamen and caudate bodies (more evident in left side) in coronal T2-weighted image (**a**), with a cavitated appearance on transverse FLAIR sequence (**b**)

mitochondrial cIII deficiency. In addition we report a review of previously published *TTC19* patients.

Case Report

Our proband is a boy, second child of healthy related (first cousins) parents of Moroccan origin. Two younger sisters are in good health; another sister died 1 week after birth for unknown causes. He was born at term after uneventful pregnancy; psychomotor development was normal, but delay in language skills with possible deafness was referred. Since the age of 4 years, he presented with walking impairment and language regression that progressively worsened during the following 2 years leading to loss of autonomous gait and speaking. He was first examined at 9 years of age when he established in Italy. He showed low body weight and height (≤3rd percentile), diffuse muscle wasting, severe spastic tetraparesis with marked dystonic postures involving both upper and lower limbs and severe cognitive impairment. No epilepsy was reported. Blood routine exams, including copper and ceruloplasmin levels and urinary organic acids, were normal. Elevated levels of lactate in plasma (4,269 umol/L; n.v. 580–2,100) and CSF (3,806 umol/L; n.v. 800–2,100) and of pyruvate in plasma (208 umol/L; n.v. 55–145) and CSF (180 umol/L; n.v. 45–135) were detected.

Fundus oculi, motor and sensory nerve conduction velocities, and electroretinogram resulted normal. Visual and brainstem auditory evoked potentials showed bilateral increased latency and decreased amplitude. Electroencephalography showed normal background activity and few diffuse paroxysmal abnormalities during sleep. Brain MRI showed bilateral hyperintensities of putamen and caudate bodies (Fig. 1); no calcifications were present at CT.

Histological analysis of patient's muscle biopsy showed few hypotrophic fibers, with normal lipids and glycogen content. An isolated cIII deficiency (50%) in muscle homogenate was documented, whereas all MRC complex activities were normal in fibroblasts.

Treatment with coenzymeQ_{10}, thiamine, and riboflavin was started, associated with symptomatic therapy with baclofen. The patient is now 13 years old, and his clinical condition appears stable.

Targeted resequencing of a panel containing nuclear genes associated with cIII deficiency (*BCS1L*, *TTC19*, *LYRM7*, *UQCRB*, *UQCRQ*) revealed the presence of a homozygous variant in *TTC19*, a novel rearrangement (c.782_786delinsGAAAAG) resulting in a frameshift with a predicted premature termination (p.Glu261Glyfs*8) (Fig. 2a, b). The mutation was found to be heterozygous in both parents and absent in two healthy siblings. Quantitative PCR revealed a marked reduction of the *TTC19* transcript in patient's fibroblasts (Fig. 2c), and Western blot analysis showed the absence of TTC19 protein (Fig. 2d).

Discussion and Conclusion

Mutations in *TTC19* have been rarely described in patients with mitochondrial diseases. Eleven individuals, presenting

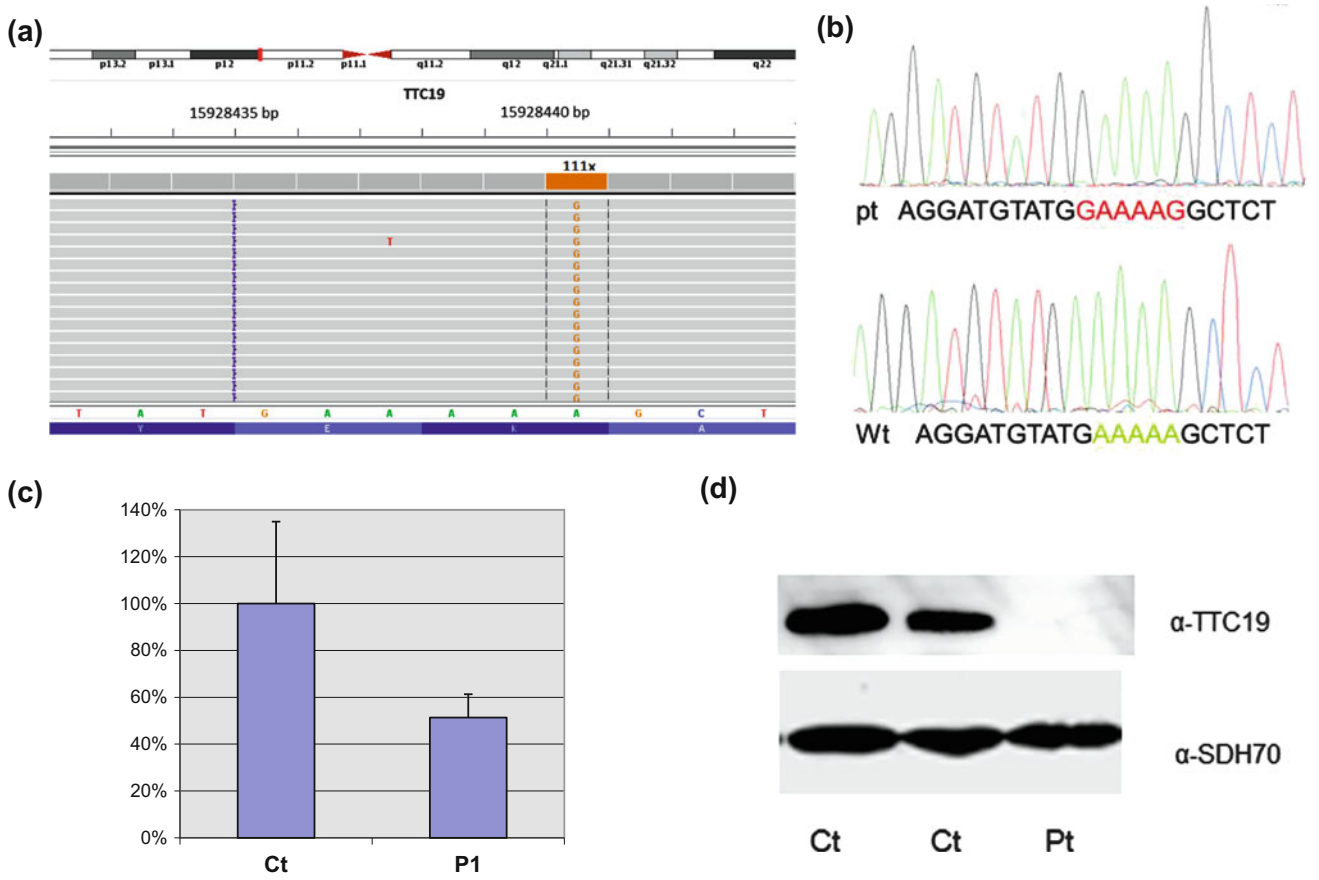


Fig. 2 (**a**) Snapshot from IGV software of the mutation identified in the patient. A customized gene panel (TSCA, Illumina) was sequenced using a MiSeq system. (**b**) Electropherograms of *TTC19* gene showing the homozygous rearrangement (c.782_786delinsGAAAAG) found in the patient (pt) resulting in a frameshift with premature termination (p.Glu261Glyfs*8); wild-type (wt) sequence in a control subject is also shown. (**c**) Quantitative real-time PCR of *TTC19* mRNA in control (Ct) and patient's (Pt) fibroblasts. (d) Immunoblot analysis of lysates from control and patient's fibroblasts using α-TTC19 and α-SDH70 antibodies

with infantile or juvenile-adult onset, and eight different *TTC19* mutations have been reported so far (Table 1).

Ghezzi et al. first reported in 2011 the presence of mutations in *TTC19* in four Italian patients. Three of these cases (P1–P3) carried a homozygous nucleotide change (c.656T>C; p.Leu219*) predicting the synthesis of a truncated protein and presented with infantile onset of cognitive impairment and ataxia. Two patients (P2, P3) showed a rapidly progressive course characterized by severe cognitive regression, cerebellar and extrapyramidal signs, hearing loss and severe axonal neuropathy. They became bedridden, presented a fluctuating comatose state requiring assisted ventilation and percutaneous endoscopic gastrostomy. Brain MRI of P3 showed progressive necrotic lesions in the brainstem and thalami and cerebellar atrophy. P1, sister of P2 and carrying the same mutation, presented a slowly progression of cerebellar and pyramidal signs and developed axonal motor neuropathy, becoming wheelchair bound since the age of 24 years. Brain MRI showed multifocal involvement of the deep gray matter, severe cerebellar atrophy and leukodystrophy. The fourth patient (P4) carried another homozygous nonsense mutation (c.517C>T; p.Gln173*). He presented with an adulthood subacute onset and rapidly progressive multisystemic neurological disease resulting in death 3 years later. Brain MRI showed diffuse cortical atrophy and right caudate and bilateral putamina involvement. Blood lactate was normal in P1, increased in P3 and not investigated in the others. A marked reduction of cIII activity in muscle homogenate was revealed in all patients. Decreased *TTC19* mRNA and virtual absence of the protein in muscle tissue and/or fibroblasts were also detected.

Nogueira et al. 2013 reported on a consanguineous family with four affected siblings (P5–P8) carrying a novel mutation (c.600_604delTGGC) predicting a frameshift and the synthesis of a truncated TTC19 protein (p.Ala200Alafs*8).

Table 1 Clinical, instrumental, and laboratory findings in patients carrying *TTC19* mutations

Patients	P1[a]	P2[a]	P3[a]	P4[a]	P5[b]	P6[b]	P7[b]	P8[b]	P9[c]	P10[d]	P11[e]	P12[f]
Age of onset/ gender	5 year/f	10 year/m	5 year/f	42 year/m	27 year/m	Adolescence/f	Adolescence/m	34 year/f	13 month/ m	31 year/f	?/f	4 year/m
Clinical presentation at onset	Cognitive impairment ataxia	Cognitive impairment ataxia	Cognitive impairment (language regression, lack of interest), ataxia	Subacute muscle weakness	Psychiatric symptoms, ataxia	Psychiatric symptoms	Psychiatric symptoms	Psychiatric symptoms	Development delay, language regression	Dysarthria	?	Cognitive impairment (language regression) ataxia
Evolution	Slowly progressive	Rapidly progressive	Progressive and rapid worsened by cardiorespiratory arrest	Rapidly progressive	Rapidly progressive	Rapidly progressive	Rapidly progressive	Rapidly progressive	Slowly progressive	Rapidly progressive	Progressive	Rapidly progressive
Clinical presentation at diagnosis time	Cerebellar signs, pyramidal signs (right side)	Cerebellar signs, focal dystonia, bilateral hearing loss, severe cognitive deterioration	Cerebellar signs, focal dystonia, severe cognitive deterioration	Gait apraxia, dysarthria bradykinesia, dystonia, paraparesis, psychiatric symptoms	Ataxia, pyramidal signs, psychiatric symptoms	Ataxia, pyramidal signs, muscle atrophy, psychiatric symptoms	Mild ataxia, mild pyramidal and extrapyramidal signs, psychiatric symptoms	Mild ataxia, mild pyramidal and extrapyramidal signs, psychiatric symptoms	Development delay	Ataxia, cognitive impairment, pes cavus	Encephalo-myopathy, failure to thrive	Spastic tetraparesis dystonic posture, severe cognitive impairment
Outcome (last observation)	Wheelchair, upper limb ataxia (37 years)	Bedridden, fluctuating comatose status, PEG-tracheostomy (26 years)	Bedridden, fluctuating comatose status, PEG-assisted ventilation	Deceased (45 years)	Deceased for respiratory insufficiency (49 years)	Deceased for respiratory insufficiency (33 years)	Deceased for cardiac arrest (30 years)	Stable (38 years)	Stable (4 years)	Wheelchair (34 years)	Encephalo-myopathy, failure to thrive (8 years)	Stable
MRI features	CA, L, IO, SN, PG, both C, left P	n.a.	IO, PG, T, CA	CoA, right C, both P	OPCA, BSN, MO, D CoA	OPCA, BSN, MO, D CoA	OPCA, BSN, MO, D	OPCA, BSN, MO, D	BSN, involvement of brainstem	CA, IO	Bilateral P, Pa	BSN
TTC19 mutations	c.656T>G/ c.656 T>G	c.656T>G/ c.656T>G	c.656T>G/ c.656T>G	c.517C>T/ c.517C>T	c.963_ 966delTGGC/ c.963_ 966delTGGC	c.963_ 966delTGGC/ c.963_ 966delTGGC	c.963_ 966delTGGC/ c.963_ 966delTGGC	c.963_ 966delTGGC/ c.963_ 966delTGGC	c.577G>A/ c.964_ 967delGGCT	c.829C>T/ c.829C>T	c.937C>T/ c.829C>T	c.782_ 786delinsGAAAAG/ c.782_ 786delinsGAAAAG
ENG	Axonal motor neuropathy	n.a.	Axonal motor neuropathy	Axonal motor neuropathy	Axonal motor neuropathy	n.a.	Axonal motor neuropathy	n.a	n.a.	n.a.	n.a.	Normal
Lactate level	Normal (plasma)	n.a.	High	n.a.	High	n.a.	n.a	n.a	n.a.	Normal/high 6 y after onset	Normal	High
Complex III deficit in muscle	19%	14%	17%	8%	30%	31%	33%	39%	I + III 79%, II 52%, II + III 36%, IV 46%	n.a.	n.a.	50%

CA cerebellar atrophy, *L* leukodystrophy, *OPCA* olivopontocerebellar atrophy, *CoA* cerebral cortical atrophy, Profound gray matter involvement/necrosis in IO inferior olives, *SN* substantia nigra, *PG* periaqueductal gray matter, *C* caudate nuclei, *P* putamina, *Pa* pallidus nuclei, *T* thalami, *P* pons, *D* dentate nuclei, *MO* medullary olives, *BSN* bilateral striatal necrosis

[a] Ghezzi et al. (2011)

[b] Nogueira et al. (2013)

[c] Atwal (2013)

[d] Morino et al. (2014)

[e] Balasubramaniam et al. (2012)

[f] This report

The clinical phenotype was characterized by late onset (ranging from adolescence to adulthood) with psychiatric symptoms; subsequently patients displayed cerebellar signs and mild pyramidal and extrapyramidal signs. In two siblings (P5, P7) an axonal motor neuropathy was also present. Three patients (P5–P7) showed a progressive course resulting in death about 20 years after onset; the only alive patient (P8) resulted stable 4 years after the disease onset. MRI showed a severe olivopontocerebellar atrophy associated with necrosis in the caudate, putamina and medullary olives. High lactate levels are reported only in one patient (P5), while a marked isolated cIII deficiency was documented in all muscle samples.

An additional case (P9), compound heterozygous for the c.600_603delTGGC and a novel nonsense mutation (c.195G>A; p.Trp65*), was reported by Atwal. He presented with psychomotor delay, followed by language regression at 13 months, and subsequent stable clinical course until the last observation (4 years of age). Brain MRI disclosed necrosis in the putamina, caudate and brainstem. Muscle biopsy showed decreased activity of MRC complexes I + III, II, II + III and IV.

More recently, Morino at al. described a woman (P10) with a spinocerebellar phenotype. She had an adulthood onset with dysarthria at 31 years of age and rapidly developed cognitive impairment, ataxia, and peripheral signs (pes cavus) leading to loss of autonomous gait 3 years later. MRI showed involvement of inferior olives and cerebellar atrophy. Lactate level was normal at onset, but resulted elevated in subsequent measurements. Muscle biopsy was not performed. Exome sequencing revealed a novel homozygous nonsense mutation (c.829C>T; p.Gln277*) in *TTC19*.

Finally, Balasubramaniam et al. reported the case of a girl (P11) affected with a slowly progressive encephalomyopathy and MRI abnormalities in lentiform nuclei associated with a novel homozygous nonsense mutation (c.574C>T; p.Gln192*). Plasma and CSF lactate levels were reported normal.

Our patient (P12 in Table 1) is the fifth infantile case associated with *TTC19* mutation. The clinical onset and the rapidly progressive course leading to spastic tetraparesis, dystonic postures, and marked cognitive impairment were similar to other infantile cases. Both our patient and previous cases had no seizures. Axonal motor neuropathy, described in some *TTC19* mutant individuals, was not present in P12.

The biochemical profile with marked cIII deficiency was consistent in all reported cases, although in P9 reduction of other MRC complex activities were found.

P12 brain MRI showed BSN. In 10/11 cases reported to date, brain MRI was available; the most common alterations were basal ganglia necrosis and subtentorial involvement, including deep gray matter and cerebellar atrophy. MRI alterations, always severe, were similar in infantile and adult cases, in spite of a different clinical phenotype. Our patient and P11 were the only cases without subtentorial involvement.

BSN has been already reported as a typical MRI feature in mitochondrial diseases caused by mutations in either mitochondrial genes (*ATP6*, *ND1*, and *ND6* encoding subunits of respiratory chain complex V and I, respectively) (Lal et al. 2013; Campos et al. 2013; Solano et al. 2003) or in nuclear genes (*NDUFV1* and *NDUFS4* encoding complex I subunits) (Lal et al. 2013; Budde et al. 2003); *AIFM1* encoding apoptosis-inducing factor, mitochondrial 1 (Ghezzi et al. 2010); and *SLC25A19* encoding a mitochondrial thiamine pyrophosphate transporter (Spiegel et al. 2009). Thus, *TTC19* must be added to the list of genes associated with infantile mitochondrial disorders and bilateral striatal necrosis.

Our report and the review of previous cases carrying *TTC19* mutations demonstrate that ataxia and impairment of cortical functions leading to language or cognitive regression are the clinical hallmarks of infantile-onset forms, whereas psychiatric symptoms are typical of juvenile-adult forms. Axonal motor neuropathy is frequent but not always present. MRI pattern, characterized by both supra- or subtentorial gray matter involvement and cerebellar atrophy, shows some common features in spite of different age of onset.

Decreased cIII activity was present in all patients reported to date. Nevertheless, lactate levels may be normal and lactic acidosis is not a reliable biomarker.

The review of published *TTC19* mutant cases does not suggest a strict and univocal genotype/phenotype correlation, but underlines that *TTC19*-related diseases can be severely disabling. Since almost all known *TTC19* mutations were associated with or are predicted to result in the absence of the protein, the differences in disease severity could not be easily ascribed to diverse deleterious effects of different mutations. A larger set of *TTC19* mutant patients should be examined, and the use of unbiased approaches for genetic analysis, such as targeted resequencing of broad gene panel or exome sequencing, would allow to better define the clinical spectrum of diseases caused by *TTC19* mutations.

Acknowledgments This work was supported by Fondazione Pierfranco e Luisa Mariani (CM23), Fondazione Telethon (grant GGP11011), Cell line and DNA Bank of Genetic Movement Disorders and Mitochondrial Disesases of Telethon Network of Genetics Biobanks (grant GTB12001), Italian Ministry of Health (GR 2010–2316392) and the Italian Association of Mitochondrial Disease Patients and Families (Mitocon ONLUS)

Synopsis

We describe a novel deleterious mutation in *TTC19* associated with early onset severe and progressive neurological deterioration. The review of previous cases does not suggest a strict and univocal genotype/phenotype correlation, but demonstrates that some clinical hallmarks and common MRI pattern are available in spite of different age of onset. Biochemical (decreased cIII activity) and molecular data (all known TTC19 mutations were associated with or are predicted to result in the absence of the protein) are common in spite of different phenotypes.

Compliance with Ethics Guidelines

Conflict of Interest

Dr. Ardissone reports no conflict of interest.

Dr. Granata reports no conflict of interest.

Dr. Legati reports no conflict of interest.

Dr. Diodato reports no conflict of interest.

Dr. Melchionda reports no conflict of interest.

Dr. Lamantea reports no conflict of interest.

Dr. Garavaglia received funding and grants for research from Fondazione Mariani (CM23), Telethon Network of Genetics Biobank (GTB12001).

Dr. Ghezzi received funding and grants for research from the Italian Ministry of Health, Telethon Italy (GGP11011), and CARIPLO foundation.

Dr. Moroni received funding and grants for research from Telethon Italy (GUP11001; GUP13006).

Informed Consent

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

Animal Rights

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

Details of the Contributions of Individual Authors

AA, TG, and IM evaluated the patient and wrote the case report. LM and DD performed genetic analyses; AL analyzed targeted resequencing data. EL performed biochemical analyses under the supervision of BG. DG monitored genetic/protein analyses. AA and DG wrote the manuscript; IM critically revised the manuscript for important intellectual content. All authors read and approved the manuscript.

References

Atwal PS (2013) Mutations in the complex III assembly factor tetratricopeptide 19 gene TTC19 are a rare cause of Leigh syndrome. JIMD Rep 25:43–45

Balasubramaniam et al. (2012) Human Genetics Society of Australasia Meeting 2012

Budde SM, van den Heuvel LP, Smeets RJ et al (2003) Clinical heterogeneity in patients with mutations in the NDUFS4 gene of mitochondrial complex I. J Inherit Metab Dis 200326 (8):813–815

Campos Y, Martín MA, Rubio JC, Gutiérrez del Olmo MC, Cabello A, Arenas J (2013) Bilateral striatal necrosis and MELAS associated with a new T3308C mutation in the mitochondrial *ND1* gene. Neurogenetics 14(1):85–87

De Lonlay P, Valnot I, Barrientos A et al (2001) A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure. Nat Genet 29(1):57–60

Ghezzi D, Arzuffi P, Zordan M et al (2011) Mutations in *TTC19* cause mitochondrial complex III deficiency and neurological impairment in humans and flies. Nat Genet 43(3):259–263

Ghezzi D, Sevrioukova I, Invernizzi F et al (2010) Severe X-linked mitochondrial encephalomyopathy associated with a mutation in apoptosis-inducing factor. Am J Hum Genet 86(4):639–649

Ghezzi D, Zeviani M (2012) Assembly factors of human mitochondrial respiratory chain complexes: physiology and pathophysiology. Adv Exp Med Biol 748:65–106

Invernizzi F, Tigano M, Dallabona C et al (2013) A homozygous mutation in LYRM7/MZM1L associated with early onset encephalopathy, lactic acidosis, and severe reduction of mitochondrial complex III activity. Hum Mutat 34(12):1619–1622

Lal D, Becker K, Motameny S et al (2013) Homozygous missense mutation of NDUFV1 as the cause of infantile bilateral striatal necrosis. Neurogenetics 14(1):85–87

Miyake N, Yano S, Sakai C et al (2013) Mitochondrial complex III deficiency caused by a homozygous UQCRC2 mutation presenting with neonatal-onset recurrent metabolic decompensation. Hum Mutat 34:446–452

Morino H, Miyamoto R, Ohnishi S, Maruyama H, Kawakami H (2014) Exome sequencing reveals a novel TTC19 mutation in an autosomal recessive spinocerebellar ataxia patient. BMC Neurol 14:5

Nogueira C, Barros J, SÄ MJ et al (2013) Novel *TTC19* mutation in a family with severe psychiatric manifestations and complex III deficiency. Neurogenetics 14(2):153–160

Solano A, Roig M, Vives-Bauza C, Hernandez-Peña J et al (2003) Bilateral striatal necrosis associated with a novel mutation in the mitochondrial *ND6* gene. Ann Neurol 54(4):527–530

Spiegel R, Shaag A, Edvardson S et al (2009) *SLC25A19* mutation as a cause of neuropathy and bilateral striatal necrosis. Ann Neurol 66(3):419–424

Zevit N, Steinmetz A, Kornreich L, Straussberg R (2007) Acute infantile bilateral striatal necrosis: single-photon emission computed tomography (SPECT) imaging and review. J Child Neurol 22(10):1222–1226