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RESEARCH REPORT

Growth Charts for Individuals with Mucopolysaccharidosis VI (Maroteaux–Lamy Syndrome)

Adrian Quartel · Christian J. Hendriksz · Rossella Parini · Sue Graham · Ping Lin · Paul Harmatz

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Abstract *Background*: The skeletal phenotype of mucopolysaccharidosis VI (MPS VI) is characterized by short stature and growth failure.

Objective: The purpose of this study was to construct reference growth curves for MPS VI patients with rapidly and slowly progressive disease.

Methods: We pooled cross-sectional and longitudinal height for age data from galsulfase (Naglazyme®, BioMarin Pharmaceutical Inc.), treatment naïve patients ($n = 269$) who participated in various MPS VI studies, including galsulfase clinical trials and their extension programs, the MPS VI clinical surveillance program (CSP), and the MPS VI survey and resurvey studies, to construct growth charts for the MPS VI population. There were 229 patients included in this study, of which data from 207 patients $\leq$25 years of age with 513 height measurements were used for constructing reference growth curves.

Results: Height for age growth curves for the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles were constructed for patients with rapidly and slowly progressing disease defined by the pre-enzyme replacement therapy (ERT) uGAG levels of $>$ or $\leq$200 μg/mg creatinine. The mean (SD) pre-ERT uGAG levels were 481.0 (218.6) and 97.8 (56.3) μg/mg creatinine for the patients $\leq$25 years of age with rapidly ($n = 131$) and slowly ($n = 76$) progressing MPS VI disease, respectively. The median growth curves for patients with $\leq$ and $>$200 μg/mg creatinine were above and below the median (50th percentile) growth curve for the entire MPS VI population.

Conclusion: MPS VI growth charts have been developed to assist in the clinical management of MPS VI patients.

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Abbreviations

CDC	Centers for Disease Control and Prevention
CSP	Clinical surveillance program
ERT	Enzyme replacement therapy
GAG	Glycosaminoglycans
HSCT	Hematopoietic stem cell transplant
LMS	Lambda Mu Sigma
MPS VI	Mucopolysaccharidosis VI
uGAG	Urinary glycosaminoglycans

Introduction

The skeletal phenotype of mucopolysaccharidosis VI (MPS VI; OMIM: 253200), a rare lysosomal storage disorder, is characterized by short stature, growth failure, and dysostosis multiplex (Neufeld et al. 2013). Dysostosis multiplex involves severe abnormalities in the development of

skeletal cartilage and bone and is a characteristic of all MPS disorders except MPS III (Lachman et al. 2010). Accelerated growth with advanced bone maturation has been reported during the first year of life in MPS VI infants, which is followed by a deceleration and growth failure after the 2nd year of life, and delayed onset and progression of puberty (Heron et al. 2004; Scarpa et al. 2009; Decker et al. 2010). The adult height (range 110–140 cm) of most MPS VI patients is below −2 SD of the reference growth curves from the Centers for Disease Control (CDC) growth charts for normal age-adjusted peers (Neufeld et al. 2013; Kuczmarski et al. 2002). The CDC provides data from the normal population from which height *Z*-scores can be calculated based on age and gender. These CDC curves refer specifically to the normal population. The rationale for generating MPS VI-specific curves is to be able to judge the normality of growth for this unique population. MPS VI patients exhibit severe and continuous deceleration in growth compared to the growth rates exhibited in the normal population as portrayed in the CDC growth charts, thus limiting the value of using the CDC growth charts to readily interpret and follow growth of MPS VI patients over time. Reference growth charts for the MPS VI population are currently not available.

Disease-specific growth charts are valuable tools to clinicians for tracking growth as deviations from what is considered normal for that patient population and can alert them of secondary problems such as malnutrition, endocrine abnormality, or psychosocial deprivation (Ranke 1989). In addition, disease-specific growth charts are valuable for counseling parents in regard to expected growth or developmental milestones (Ranke 1989). While disease-specific growth charts are available for several other syndromes where short stature is a common feature (Hall et al. 2012), these references cannot be used as a guide for MPS VI patients because of dissimilar growth patterns (Horton et al. 1982; Tomatsu et al. 2012). Specific growth charts are needed for MPS VI patients with either rapidly or slowly progressive disease (as defined in Methods section (Swiedler et al. 2005)) to assist clinicians in managing their development.

Methods

Data Source for Integrated Height Analysis

We pooled cross-sectional and longitudinal height for age data from patients who participated in the MPS VI cross-sectional Survey Study done in 2001–2002 (Swiedler et al. 2005); clinical trials and their extension programs, including phase 1/2, phase 2, phase 3 (Harmatz et al. 2004; Harmatz et al. 2005; Harmatz et al. 2006; Harmatz et al. 2008), and a phase 4 study (Harmatz et al. 2013); the MPS VI clinical surveillance program (CSP; ClinicalTrials.gov: NCT00214773); and the Resurvey Study (Giugliani et al. 2014) to construct growth charts for patients with the MPS VI disorder. Patients treated with galsulfase (recombinant human arylsulfatase B; rhASB; Naglazyme®; an enzyme replacement therapy [ERT] for MPS VI) or patients status post hematopoietic stem cell transplant (HSCT) were excluded from this analysis. The number of patients in the above mentioned studies that contributed height data for the development of the growth charts is listed in Table 1. Because patients may have participated in more than one MPS VI study, duplicate entries were identified in order to determine a unique dataset for the patients ($n = 269$). All height measurements included in the integrated dataset were from ERT or HSCT treatment naïve patients.

Definitions of Slowly and Rapidly Progressive Disease

Patients with rapidly or slowly progressive disease were defined by the pre-ERT urinary glycosaminoglycan (uGAG) levels of $>$ or $\leq$200 μg/mg creatinine, respectively (Swiedler et al. 2005). High uGAG levels ($>$200 μg/mg creatinine) have been reported to be associated with age-adjusted short stature, reduced joint range of motion, compromised pulmonary function, and impaired endurance as measured by the 6-minute walk test. Low uGAG levels ($\leq$200 μg/mg creatinine) have been reported to be associated with later onset of typical symptoms with less pronounced growth retardation. The pre-ERT uGAG values were obtained from 2 sources: (1) the uGAG measurements of ERT-naïve patients, such as those in the placebo arm of clinical trials and the cross-sectional Survey Study, and (2) the measurements made prior to the initiation of ERT on ERT-treated patients in all studies. When more than one pre-ERT uGAG measurement was available, an average of all values was used for determination of rapidly or slowly progressive disease.

The height data included all height measurements for age for ERT-naïve patients as well as measurements from ERT patients prior to or within one month of the first ERT infusion.

Laboratory Assessments

Urinary GAG levels were determined by spectrophotometric detection of metachromatic change in the 1,9-dimethylmethylene blue (DMMB) dye upon GAG binding (Whitley et al. 1989) (performed at BioMarin Pharmaceutical Inc. labs). Urinary GAG levels were measured on the first morning voided samples and normalized for urine creatinine levels.

Table 1 Baseline characteristics of MPS VI patients included in the study[a,b,c]

Group	Parameter		All ages	Age 2–18 years	Age ≤25 years
All	*n* Patients		229	168	207
	Males	*n* (%)	112 (49)	79 (47)	100 (48)
	Females	*n* (%)	117 (51)	89 (53)	107 (52)
	Age (years)	Mean (SD)	12.7 (9.6)	9.5 (4.2)	10.4 (5.9)
		Median (Q1, Q3)	10.2 (6.1, 16.5)	8.9 (6.0, 13.2)	9.2 (5.7, 15.0)
		Range	0.0, 56.2	2.0, 17.8	0.0, 25.0
	uGAG	Mean (SD)	313.2 (257.9)	347.1 (216.8)	340.3 (256.1)
		Median (Q1, Q3)	289.5 (94.0, 466.7)	343.7 (172.4, 485.7)	323.6 (131.0, 488.5)
		Range	0.0, 1491.3	0.0, 1292.4	0.0, 1491.3
	n Height Measurements[d]		558	396	513
	Height (cm)	Mean (SD)	112.1 (26.6)	105.9 (19.8)	107.9 (24.0)
		Median (Q1, Q3)	103.0 (93.3, 132.4)	99.5 (93.0, 112.1)	100.0 (92.2, 122.0)
		Range	49.8, 182.9	80.0, 182.9	49.8, 182.9
Low uGAG	*n* Patients		97	51	76
	uGAG	Mean (SD)	87.4 (56.5)	107.1 (55.6)	97.8 (56.3)
		Median (Q1, Q3)	68.6 (42.0, 134.7)	102.1 (65.0, 154.1)	92.7 (51.7, 144.4)
		Range	0.0, 199.3	0.0, 199.3	0.0, 199.3
	Age (years)	Mean (SD)	17.7 (11.7)	10.2 (4.7)	12.9 (6.7)
		Median (Q1, Q3)	15.7 (8.7, 23.4)	10.2 (5.8, 14.7)	14.2 (6.1, 18.8)
		Range	0.0, 56.2	2.7, 17.2	0.0, 25.0
	Height (cm)	Mean (SD)	132.3 (27.0)	121.6 (25.6)	126.2 (27.2)
		Median (Q1, Q3)	140.5 (113.5, 152.6)	119.8 (99.5, 142.6)	127.5 (106.5, 150.0)
		Range	57.9, 182.9	81.5, 182.9	57.9, 182.9
High uGAG	*n* Patients		132	117	131
	uGAG	Mean (SD)	479.1 (218.9)	451.7 (173.1)	481.0 (218.6)
		Median (Q1, Q3)	437.9 (338.9, 547.8)	419.2 (336.8, 531.4)	438.7 (340.9, 549.3)
		Range	202.0, 1491.3	202.0, 1,292.4	202.0, 1,491.3
	Age (years)	Mean (SD)	9.0 (5.2)	9.3 (4.0)	8.9 (4.9)
		Median (Q1, Q3)	8.3 (5.4, 12.0)	8.5 (6.3, 12.0)	8.2 (5.4, 12.0)
		Range	0.1, 29.8	2.0, 17.8	0.1, 22.4
	Height (cm)	Mean (SD)	97.4 (13.3)	99.0, 11.3	97.3 (13.4)
		Median (Q1, Q3)	97.3 (90.0, 103.7)	98.0 (92.2, 104.2)	97.0 (90.0, 103.8)
		Range	49.8, 159.0	80.0, 159.0	49.8, 159.0

[a] The baseline characteristics were calculated from data collected at study baseline, ie, first baseline. All patients had both pre-ERT uGAG levels and pre-ERT (or within 1 month of ERT) height measurements
[b] Low and high uGAG refers to pre-ERT uGAG levels of ≤ or >200 μg/mg creatinine, respectively
[c] Height in cm refers to height at enrollment
[d] Total number of height measurements include all pre-ERT visits from patients who also had pre-ERT uGAG values available

Statistical Methods

All analyses were conducted with PC-SAS 9.3 (TS Level 1 M1) software (SAS Institute, Cary, NC) and Lambda Mu Sigma (LMS) Chartmaker Pro, Version 2.54 (the Institute of Child Health, London). Demographic information was obtained on patients' pre-ERT and summarized using descriptive statistics, including the following parameters: age, gender, pre-ERT uGAG, height, and the associated characteristic collected with that measurement (e.g., height by pre-ERT uGAG group). Height by age was plotted by uGAG categories, ≤200 and >200 μg/mg creatinine.

Height measurements were taken as standing height as per the center's procedure for height measurement. Height for age data from patients who provided both pre-ERT uGAG measurement and pre-ERT (or within 1 month of ERT) height measurement(s) was included in the analyses for construction of MPS VI reference growth curves using

the LMS method (Cole and Green 1992). The distribution of height measurements as a function of age (covariate) is summarized by 3 curves representing the following parameters: skewness (Lambda, L), median (Mu, M), and the generalized coefficient of variation (Sigma, S). For the LMS procedure, the method of maximum penalized likelihood was used to generate reference percentile curves for MPS VI. Schwarz Bayesian Criteria (SBC) was used for the model selection. Many patients provided multiple height measurements; however, the LMS method allowed for statistical assignment of equal weight per patient regardless of the number of measurements leading to robust calculations of reference growth curves. The final L, M, and S parameters and percentile data are shown in Supplement Tables 1–3. These age-specific L, M, and S parameters can be used to calculate *z*-score height of an individual child at any given age using a simple formula (height of child /M)L − 1(L/S)) (Cole 1989).

Reference height for age curves for the 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles was constructed for patients with rapidly and slowly progressive disease as defined by the pre-ERT uGAG level. Data were plotted for comparison purposes with the normative 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentiles using the CDC's national growth data. The LMS *Z*-scores graphs were generated for patients with rapidly and slowly progressive disease, respectively.

The growth curves in this report represent ages 2–18 years; however, height data from patients ≤25 years of age was used in the growth modeling to attenuate flaring "right edge effect" of data truncation on statistical smoothing as previously described (Tarquinio et al. 2010). The growth curves in this report exclude 0–2 years of age due to a small sample size for this age range (Supplement Table 4) and the limitations of the methodology used for this age group.

Results

Demographics

Of the unique set of 269 patients identified across various MPS VI studies, pre-ERT uGAG and pre-ERT (or within 1 month of ERT) height measurements were available for 229 patients with 558 height measurements (Table 1). Both male and female patients were nearly equally represented in this dataset. The mean (SD) age of all patients was 12.7 (9.6) years and the mean (SD) height was 112.1 (26.6) cm (Table 1). Of the 229 patients, height for age data from patients ≤25 years of age ($n = 207$) (total height measurements = 513) was included for the construction of the MPS VI growth charts. The number of height measurements per patient ranged from 1 to 41 (median: 1) with a mean (SD) of 1.5 (1.4) to 2.7 (6.9) data points across various age ranges (Supplement Table 4).

MPS VI Growth Curves

Height data for ages 0–25 years were used to generate growth curves; however, the growth curves are displayed for the age range of 2–18 years as discussed in methods. Figure 1 shows the height for age of patients 2–18 years of age. The estimated median height of the overall population showed a significant increase from 81.4 cm at age 2 to 130.5 cm at age 18 (Fig. 1).The heights of MPS VI patients as shown in Fig. 1 scatterplot were heterogeneous; therefore, we used the LMS procedure to transform the height for age data into normal distribution and smooth the resultant curves as described in the methods.

There were limited differences in the height for age curves for male and female MPS VI individuals (Supplement Fig. 1). Therefore, we combined data from the male and female patients to create more robust reference growth charts as shown in Figs. 1, 2, and 3.

Growth of Rapidly and Slowly Progressing MPS VI Patients

The patients were further stratified as rapidly and slowly progressive patients based on the pre-ERT uGAG levels as defined in the methods. The mean (SD) pre-ERT uGAG levels were 481.0 (218.6) μg/mg creatinine for the rapidly progressive ($n = 131$) and 97.8 (56.3) μg/mg creatinine for the slowly progressive ($n = 76$) patients ≤25 years of age (Table I).

The slowly progressing ≤25 years of age patient group had higher age-adjusted mean (SD) heights compared to the rapidly progressing group (126.2 [27.2] vs. 97.3 [13.4] cm; Table 1 and Fig. 1). The difference between the two groups progressively increased after age 4–5 years (Fig. 1). The estimated median height of the slowly progressing group continued to increase during the teenage years (estimated median height at age 18 = 144.1 cm; Fig. 1). On the other hand, the estimated median height of the rapidly progressing patients showed a much slower but gradual increase in height past age 10–12 years, increasing to 109.3 cm by age 18 years (Fig. 1).

MPS VI Growth Charts and *Z*-Scores for Rapidly and Slowly Progressing MPS VI Patients

Figures 2 and 3 show reference growth curves for rapidly and slowly progressive patients, respectively, for 7 percentiles (5th, 10th, 25th, 50th, 75th, 90th, and 95th) along with the CDC reference percentiles for normal peers. The growth

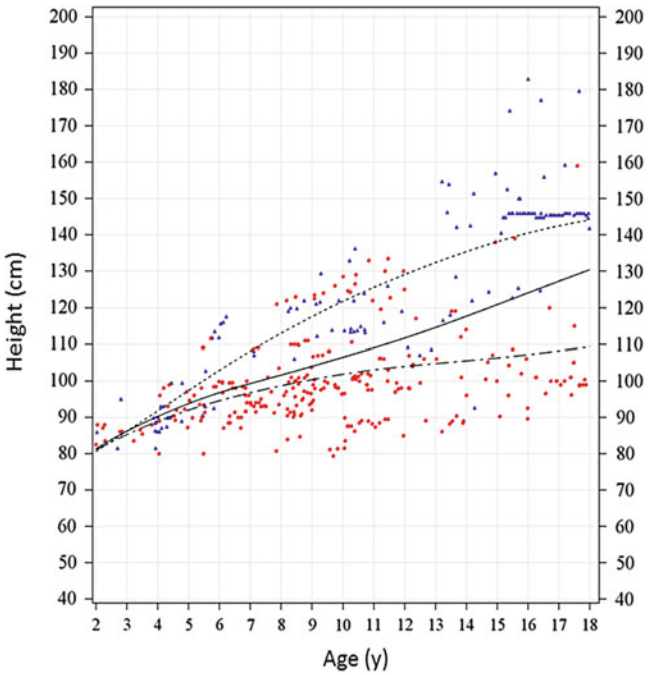

Fig. 1 Height for age scatterplot for MPS VI patients 2–18 years of age. Height for age individual measurements for patients 2–18 years of age is plotted using color-coded symbols per pre-ERT uGAG levels (*red circles* for high and *blue triangles* for low pre-ERT uGAG levels). The LMS-generated smoothened growth curves for rapidly progressing (*dotted-dash*), slowly progressing (*dotted*), and whole population (*solid line*) are shown. The L, M, and S parameters for generating these smoothened curves are shown in Supplement Tables 1–3. Many patients provided multiple height measurements as shown in this figure; however, LMS method allowed for statistical assignment of equal weight per patient regardless of the number of measurements leading to robust calculations of reference growth curves. The growth curve for the slowly progressing population continued on a positive trajectory between ages 18 - 25 years likely due to distribution of patients with taller heights in this subgroup containing largely cross-sectional data (not shown). The positive slope of the growth curve for slowly progressing patients (ages 18–25 years) also skewed the overall growth curve for MPS VI (not shown). Thus, in the absence of a larger dataset (including longitudinal data) for this age range, the growth curves were truncated at age 18 years. Further, heights of MPS VI patients increase by <1 cm after age 18 years (Giugliani et al. 2014)

of rapidly progressing patients followed a slower but continuous trajectory during the teen years (Fig. 2), whereas the 50th percentile of slowly progressing patients followed a growth trajectory below and nearly parallel to the 5th percentile of the normative CDC curve (Fig. 3). The 90th percentile of slowly progressing patients had heights comparable to the 25th percentile of the normative CDC curves by late teen years.

The LMS method also allowed for *Z*-score calculation. Based on the growth charts developed for rapidly and slowly progressing patients, the *Z*-scores were distributed between +2 SD and −2 SD for the respective groups (Supplement Figs. 2 and 3).

Discussion

We collected pre-ERT (or within 1 month of ERT) height measurements from the largest cohort of MPS VI patients, to date, to construct growth charts for the MPS VI population. The 207 patients ≤25 years of age with available pre-ERT (or within 1 month of ERT) height measurements represent approximately 19% of the estimated worldwide MPS VI population (Swiedler et al. 2005); however, these numbers are an underestimation of the true prevalence as the referenced numbers are based on the estimates from the USA, Europe, and Brazil. The patient population in this dataset included patients from many countries and, thus, supports wide applicability of these MPS VI growth charts. We further developed growth charts specific for patients with rapidly or slowly progressive MPS VI disease given their different progressions of growth.

The pre-ERT heights of patients plotted on a scatterplot were heterogeneous suggesting a possibility of a nonlinear relationship. Therefore, we used the LMS procedure to smooth the height for age scatterplots as described in the methods.

Pre-ERT height and uGAG measurements from patients ≤25 years of age were chosen for calculations to construct MPS VI growth charts. Even though data from 18 to 25 years of age were used for developing the growth curves,

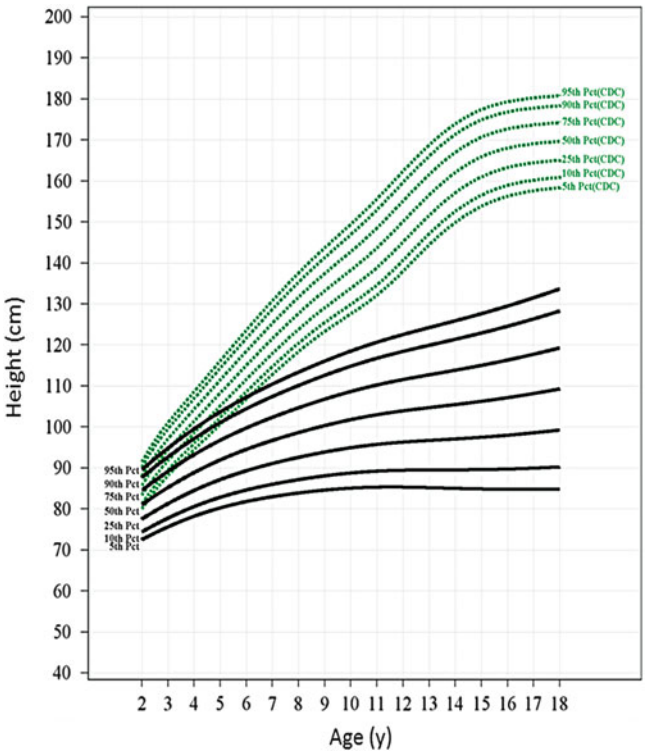

Fig. 2 Growth charts for the rapidly progressing MPS VI population. The 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentile reference growth curves for the rapidly progressing population (*black*) ages 2–18 years are shown along with the 5th to 95th CDC reference curves for normal population (*green*). CDC curves were recreated from data from the CDC website using mean heights of males and females at each age point. The L, M, and S parameters used for generating reference curves for rapidly progressing patients and the percentile values are shown in Supplement Table 2

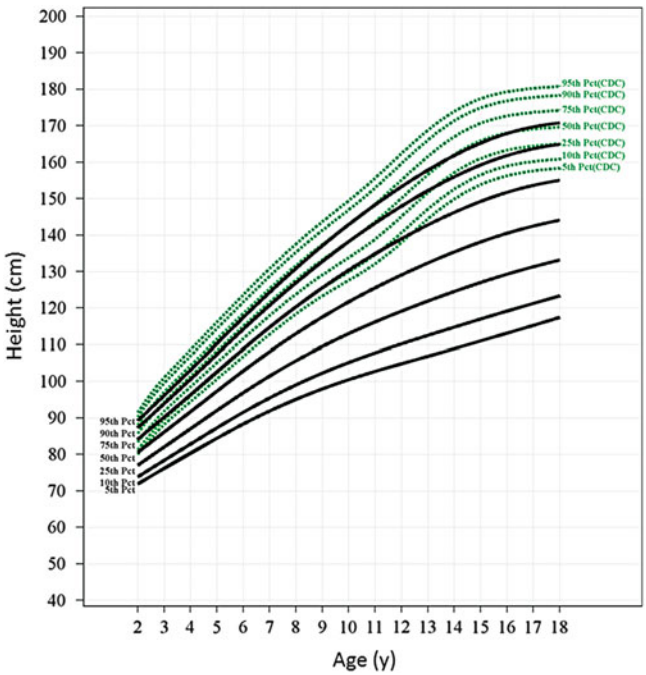

Fig. 3 Growth charts for the slowly progressing MPS VI population. The 5th, 10th, 25th, 50th, 75th, 90th, and 95th percentile reference growth curves for the slowly progressing population (*black*) aging 2–18 years are shown along with the 5th to 95th CDC reference curves for normal population (*green*). The L, M, and S parameters used for generating reference curves for rapidly progressing patients and the percentile values are shown in Supplement Table 3

patients >18 years of age were not displayed due to the changing ratio between slowly and rapidly progressive disease in this age cohort limiting the dataset available for more severely affected patients because of early mortality, as well as to attenuate the flaring "right edge effect" of data truncation on statistical smoothing. Similarly, MPS VI reference growth curves in this report do not extend to the <2 years age group due to limited availability of data and the limitations of the methodology used for this age group.

Published reports suggest that MPS VI infants and toddlers have normal to accelerated growth velocity and often with above-average heights during the first year of life followed by decreased growth rates during the 2nd year (Heron et al. 2004; Scarpa et al. 2009). Although our dataset had limited height for age data for patients <2 years of age the trajectory of the MPS VI reference growth curves described in this report is consistent with growth deceleration during or after the 2nd year of life particularly in the rapidly progressing patients. As expected, the estimated median height of MPS VI patients after age 3 years was lower than the 5th percentile for height of the age-adjusted peers in the normal population (CDC).

The heights of slowly and rapidly progressing patients continue to increase during the teenage years with median heights measuring 144.1 and 109.3 cm by age 18 years, respectively. Unlike the slowly progressing patients, the median height of the rapidly progressing patients increases at a much slower rate reaching a relative plateau by approximately 10 years of age. Thus, it is not only important to use syndrome-specific growth charts for these genetic disorders but also to tailor the use of growth charts according to the severity of the disease. The CDC growth charts do not capture the differences in growth velocities or the variation in growth trajectory seen in MPS VI patients with rapidly or slowly progressive disease. Furthermore, appearance or worsening of joint contractures or scoliosis limits the ability for accurate height or length measurements in this population.

Several MPS and other disorders characterized by growth failure show a correlation of height for age with phenotypic severity as assessed by genotype or surrogate biomarkers (Decker et al. 2010; Tarquinio et al. 2010; Tylki-Szymanska et al. 2010; de Ruijter et al. 2013; Hendriksz et al. 2013; Jurecka et al. 2013). In the MPS VI population, height for age was previously reported to correspond with the pre-ERT uGAG levels (Swiedler et al. 2005; Decker et al. 2010). However, uGAG levels are naturally high in unaffected individuals during infancy (Gallegos-Arreola et al. 2000; Gray et al. 2007), and pretreatment uGAG levels in infants with MPS VI do not correlate closely with disease severity. In addition, uGAG levels decrease post-ERT (Harmatz et al. 2006; Harmatz et al. 2008), again limiting the utility of uGAG in categorizing disease after ERT has been started. Nevertheless, patients 4 - 18 years of age with rapidly progressing disease have pre-ERT uGAG levels in excess of 3 times the upper limit of normal (Swiedler et al. 2005). Although age-specific disease severity-defined uGAG levels have not been established, uGAG levels >200 μg/mg creatinine correlate with the rapidly progressive phenotype in patients <16 years of age (Hendriksz et al. 2013). Untreated patients with rapidly progressive disease usually do not survive beyond their late teens (Swiedler et al. 2005), and therefore, the >16 years age group represented in this analysis are more heavily represented by those with slowly progressive disease. This was supported in a publication on the CSP cohort where the untreated patients ≥16 years of age were found to have uGAG levels ≤200 μg/mg creatinine and reported to have slowly progressive disease (Hendriksz et al. 2013). Although a GAG-based biomarker may not be a reliable predictor of disease severity or in very young (first year of life) MPS patients before or after treatment with galsulfase, mutation analysis or determination of height using the MPS VI growth charts may provide an additional for defining rapidly progressing disease regardless of age or ERT status. Genotype-phenotype correlation studies of 105 arylsulfatase B (ARSB) gene mutations indicate that ARSB active-site mutations and nonsense or deletion mutations resulting in truncated proteins generally correlate with early onset of severe clinical phenotype and high pre-ERT uGAG levels (Karageorgos et al. 2007). Further, some MPS VI mutations have been correlated with severe clinical phenotype and lower height *z*-scores (Jurecka et al. 2013; Kantaputra et al. 2014).

The pathophysiology of short stature in MPS VI is not well elucidated. A combination of early bone maturation, growth failure, bone growth plate disorganization, joint contractures, and endocrine abnormalities has been proposed for short stature of patients with various MPS disorders (Polgreen et al. 2008; Decker et al. 2010; White 2011). Growth hormone (GH)/insulin-like growth factor (IGF-1) deficiency or resistance has been reported in some patients with MPS IH (Hurler syndrome), MPS II, and MPS IIIA and in a rare case of MPS VI (Buyukgebiz et al. 1995; Polgreen et al. 2008; Gardner et al. 2011). However, others have not observed GH/IGF-1, thyroid hormone, or pituitary-hypothalamic insufficiency (in spite of a report of empty pituitary sella) as cause(s) of growth retardation in MPS VI (Von Muhlendahl and Bradac 1975; Borges et al. 2003; Decker et al. 2010). Growth plate abnormalities and disorganization secondary to GAG accumulation most likely contribute to growth failure in MPS disorders. Disruption of chondrocyte proliferative and hypertrophic zones in the growth plate secondary to GAG accumulation, cytokine/inflammatory response, and cellular dysfunction

may lead to early bone maturation and impaired growth velocity (Abreu et al. 1995; Alliston 2010; Simonaro et al. 2010).

We did not observe increased acceleration in growth during preteen or early-teen years in the MPS VI patients with either slowly or rapidly progressive disease. Since most of the data presented was collected in a cross-sectional fashion through a variety of data sources, individual change in growth spurts during adolescence, if any, may not have been evident in the growth curves presented here. Further, no evidence of a growth spurt was apparent when growth curves for males and female patients were plotted separately (Supplement Fig. 1). Bone ages were not available to assess potential delays in growth and puberty. However, limited published data does not indicate differences between bone and chronological ages in MPS VI (Polgreen et al. 2014). Tanner staging was not collected in this study. However, a sub-analysis of patients who participated in previous clinical trials ($n = 56$) showed that when excluding prepubertal patients who were appropriate Tanner stage for age ($n = 32$), a large percentage (42%,10/24) of the remaining patients had delayed onset or delayed progression of puberty (Decker et al. 2010). The exact etiology of possible endocrine failure responsible for delayed puberty and possible growth failure in MPS VI patients remains unknown.

The impact of early initiation and long-term galsulfase ERT on growth is under investigation. In the galsulfase clinical trials program, increased growth rates by 1–2 cm/year were demonstrated in patients (mean age: 12 years) receiving up to 96 weeks of galsulfase ERT as compared to growth rates in MPS VI patients treated with placebo (Decker et al. 2010). There have been few studies on the impact of very early initiation of galsulfase ERT in MPS VI patients. Growth data from a retrospective study ($n = 34$) with follow-up data available from 32 children who initiated galsulfase ERT at mean age 2.9 years showed that 12 (37.5%) patients remained on the same growth percentiles, 3 (9.4%) moved to higher percentiles, and 17 (53%) fell below their baseline percentiles after ERT mean duration of 2.06 years (Horovitz et al. 2013). A Japanese sibling study reported that a patient who started ERT at age 5.6 years showed catch-up growth after 3 years of ERT (Furujo et al. 2011). Further investigations are needed to study if early diagnosis coupled with ERT initiation and long-term treatment may help avoid or mitigate the growth failure seen in MPS VI patients. The challenge is early recognition, and ultimately early diagnosis will depend on the development and initiation of newborn screening programs (Giugliani et al. 2007). MPS VI-specific growth charts will be an important tool to monitor growth outcomes in these ERT-treated patients.

In conclusion, we have developed reference growth charts for the rapidly and slowly progressive MPS VI patients. We encourage the use of these charts in clinical practice to help provide clinically meaningful information to physicians, patients, and parent/caregivers on expected outcomes of normal growth in this MPS VI population; provide an estimate of disease severity based on height; and assess the impact of therapeutic interventions. These charts are provided as Supplement Figs. 4–6 with this report.

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Synopsis

Growth charts for rapidly and slowly progressing MPS VI patients.

Compliance with Ethics Guidelines

Conflict of Interest

Christian J. Hendriksz, Rossella Parini, and Paul Harmatz have worked as consultants and study investigators for BioMarin Pharmaceutical Inc., received research grants, participated in BioMarin advisory board meetings, and received speaker honoraria and travel support from BioMarin Pharmaceutical Inc. Adrian Quartel, Sue Graham, and Ping Lin are employees and stockholders of BioMarin Pharmaceutical Inc.

Patient Consent Statement

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. Each participant or his/her legally authorized representative provided written informed consent before entering the study in compliance with the applicable local regulations.

Contributions of Individual Authors

Adrian Quartel contributed to the conception and research design, performed data analysis, and contributed to the writing of the manuscript.

Christian J. Hendriksz contributed to the conception and research design, acquisition of data, and revising manuscript critically for important intellectual content.

Rossella Parini contributed to the conception and research design, acquisition of data, and revising manuscript critically for important intellectual content.

Sue Graham contributed to the conception and research design and revised manuscript critically for important intellectual content.

Ping Lin contributed to the research design, developed statistical methodologies, performed statistical analyses and interpretations, and revised manuscript critically for important intellectual content.

Paul Harmatz contributed to the conception and research design and acquisition of data and contributed to the writing of the manuscript.

Guarantor: Adrian Quartel

All authors approved the final version of the manuscript and the decision to publish.

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RESEARCH REPORT

Treatment Adherence in Type 1 Hereditary Tyrosinaemia (HT1): A Mixed-Method Investigation into the Beliefs, Attitudes and Behaviour of Adolescent Patients, Their Families and Their Health-Care Team

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Abstract *Background*: Type 1 hereditary tyrosinaemia (HT1) is a rare metabolic disorder caused by an enzymatic defect in the metabolism of the amino acid tyrosine. Primary treatment for HT1 is nitisinone (Orfadin) in conjunction with a low-tyrosine/phenylalanine diet. The appropriate use of nitisinone medication and adhering to specialist diet is thus central to the successful management of HT1.

Objective: To date, no published research has examined adherence (to medication and diet) and factors that influence it in the context of HT1. This study aimed to ascertain the extent to which non-adherence is a problem in this patient population, identify perceived barriers and facilitators to treatment adherence and explore the role of illness beliefs and treatment perceptions in treatment management.

Methods: The present study used a combination of qualitative interviews and quantitative survey methods with patients, carers and health-care professionals (HCPs).

Results: This study found adherence to medication to be high amongst patients with HT1 and their carers who administer it. However, adherence to diet was reported to be much lower. A key factor influencing adherence to diet was age, with adolescents reported to have most difficulty adhering.

Conclusions: The results indicate that adherence to dietary instructions becomes more problematic as children with HT1 grow older. Greater involvement in managing their condition and in their consultation at an early stage may have a positive impact on future adherence by increasing their investment and understanding of the treatment regime, potentially making adherence rates more stable and less influenced by moving through different life stages.

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Introduction

Type 1 hereditary tyrosinaemia (HT1) is an ultra-orphan disease caused by an enzymatic defect in the metabolism of the amino acid tyrosine. It typically presents in infancy as failure to thrive and, if left untreated, can lead to progressive liver and kidney dysfunction as well as neurological crisis and death (Sniderman King et al. 2006).

HT1 has an estimated incidence of 1 case in every 100,000 births (Sniderman King et al. 2006). The latest prevalence estimate available from Orphanet is 1 per 2,000,000 people (Orphanet 2007). Although HT1 is uncommon in all populations, there is considerable variation in its incidence according to region and ethnic background. For example, at a centre for hereditary metabolic disorders in the UK, the frequency of HT1 was

approximately 100 times higher amongst individuals of Pakistani origin compared with those of European descent (3.7 per million vs. 0.04 per million, respectively), and a difference attributed to a high rate of consanguineous marriages amongst Pakistani parents (Hutchesson et al. 1998). Additionally, overall incidence is much higher in Quebec, Canada (1 in 16,000), due to a founder mutation in the French-Canadian population (Sniderman King et al. 2006).

The primary treatment for HT1 is currently nitisinone (Orfadin), which works by inhibiting enzymes that convert excess tyrosine into urea. Nitisinone treatment is orally administered and taken in conjunction with a low-tyrosine/phenylalanine diet. Research has shown that if nitisinone treatment is started before 2 months of age, 4-year survival rates are increased to 94% compared to 29% with a controlled diet alone (Sobi 2010). The appropriate use of nitisinone medication and adhering to a specialist diet is thus central to the successful management of HT1.

There is little research regarding adherence to medication or diet in HT1; however, available research indicates that adherence may be suboptimal, particularly with regard to dietary restrictions (Masurel-Paulet et al. 2008; Wisse et al. 2012). Published research across a range of chronic conditions has shown that treatment regimen adherence is suboptimal across chronic illnesses (Sabaté 2003) and is significantly lower in adolescence (Dean et al. 2010; Salema et al. 2011).

Patients' perceptions and beliefs are consistently found to be important predictors of non-adherence (Nunes et al. 2009). For example, perceptions relating to the *cause* of the illness, its *nature* or identity, its *duration*, the personal *consequences* of suffering from it and the extent to which the illness can be *controlled* or cured, influence disease management (Broadbent et al. 2006). Patients' beliefs around the *necessity* of treatment and *concerns* about taking it also affect their adherence (Horne et al. 1999). Interventions focusing on changing these beliefs have been shown to be effective at improving adherence in the long term (e.g. Petrie et al. 2012).

To date, however, no published research has examined adherence challenges and support needs in the context of HT1 specifically. The present study used a combination of qualitative and quantitative research methods with patients, carers and health-care professionals (HCPs) to (a) ascertain the extent to which non-adherence is a problem in this patient population, (b) identify perceived barriers and facilitators to treatment adherence and (c) explore the role of illness beliefs and treatment perceptions in relation to treatment management. As this is such a small patient group, it was decided to run the study in two countries.

Methods

Sample

HCPs

HCPs from key sites in the UK (Birmingham, Manchester and Bradford) and France (Paris, Lyon and Marseille), who were involved in the care of patients with HT1, were identified and invited to take part in the qualitative arm of the study.

Patients

A total of 40 families with HT1 were identified across the research sites in the UK (Birmingham, Manchester and Bradford) and 37 families in France (Paris, Lyon and Marseille). Convenience sampling was employed, whereby all eligible patients (or their carers if under 12) at research sites in the UK and France were invited to participate in the study. Patients were excluded from the study if they were either under the age of 12, had received a liver transplant or had severe or profound intellectual impairments and learning difficulties. Additionally patients and carers who could not communicate in English or French fluently were excluded from the study.

Procedure

In the UK, ethical approval for the study was obtained from the Fulham Research Ethics Committee and the R&D department in each NHS site. French ethical approval was obtained from the equivalent research committees for the sites in France (CPP[1], CCTIRS[2], CNIL[3] and the CNOM[4]).

Eligible patients were identified by the lead physician at each site, informed about the research project and asked to provide consent for the research team to contact them regarding participation in the study. Those who expressed an interest were sent the study information sheet along with the questionnaire and a stamped addressed envelope for return of the questionnaire and signed consent form.

Qualitative Research

Semi-structured interviews were conducted in English or French, recorded and transcribed verbatim. The French

[0] Comité de Protection des Personnes – ethics committee in France.
[0] Comité consultatif sur le Traitement de l'Information en matière de Santé – a consultative committee specialising in health which gives advice to the data protection committee for medical projects.
[0] Commission nationale de l'informatique et des libertés – data protection committee.
[0] The French physician organisation.

interviews were translated into English for data analysis. Translations were reviewed by the interviewer in france for validity prior to data analysis.

The qualitative data was analysed using a framework analysis approach (Richie And Spencer 1994), which involves identifying key themes in the data in relation to the specific questions/issues driving the research (Gale et al. 2013).

Quantitative Research

The self-report questionnaire contained questions covering the following:

- Socio-demographic and disease-related information: Patients/carers were asked to indicate their gender, age, main language spoken, employment status, duration of diagnosis, details of treatments currently used for HT1 and any comorbid illnesses.
- Persistence to medication: A single item 'are you still using your prescribed medication?' was used to measure treatment persistence. Participants were asked to indicate yes or no.
- Adherence to medication was measured by a 6-item version of the Medication Adherence Report Scale (MARS) (Horne and Weinman 2002), a reliable and valid self-report measure of non-adherence.
- Adherence to dietary instructions was measured by four items. A single item devised for the study asked patients and carers to rate on a 5-point scale how well they thought they (patients) or their child (carers) usually adhered to dietary instructions. Response options ranged from 1 (not at all) to 5 (completely), with higher scores indicating higher adherence. Two items adapted from the MARS were included (I forget to stick to my diet regime and I stop adhering to my special diet regime for a while). In addition, participants were asked to state the number of days over the past week that they had not deviated from the recommended diet.
- Beliefs about medication: The Beliefs about Medicine Questionnaire (BMQ) (Horne et al. 1999) is a reliable and valid 10-item scale. Five items assess beliefs about the necessity of medication, and five assess concerns about medication.
- Illness perceptions: The Brief Illness Perception Questionnaire (BIPQ) was used to assess patients' and carers' cognitive and emotional representations of illness with a single item representing each illness perception (Broadbent et al. 2006).
- Mood: Anxiety was measured using the General Anxiety Disorder Assessment (GAD-2) (Kroenke et al. 2007). The PHQ 2 (Kroenke et al. 2003) was used to screen for depression.
- Social support: Perceptions of social support were assessed using the Medical Outcomes Study Social Support Survey (MOS-SSS) (Sherbourne and Stewart 1991).
- Relationship with physician: Four items were devised for the study to measure satisfaction and confidence in the physician. Participants were asked to rate their perceptions of care on a scale from 0 to 10, with higher scores indicating more positive perceptions of care (see Table 2).

Unless otherwise stated, the questions were scored in line with published recommendations.

Data were analysed using IBM SPSS Statistics version 21. Descriptive statistics were performed on all data. Pearson's correlations were used to explore relationships between variables, and t-tests were used to assess differences between patient and carer samples.

Results

Sample Characteristics

Qualitative interviews were conducted with four patients, nine carers and eight HCPs (three doctors, two dieticians, two specialist nurses, and one pharmacist).

A total of 27 participants, 22 (81%) who were adult carers and 5 (19%) patients, returned completed questionnaires. The demographic and clinical characteristics of the patient and carer sample who completed the demographic questionnaire are shown in Table 1. The majority of carers were female (15; 68%) with a mean age of 38 (SD = 8.9).

Due to the low response rate of this study, the quantitative data lacked the power to detect predictors of adherence. Data from the quantitative survey and qualitative interviews were thus combined to allow us to further explore and interpret trends identified through the statistical analyses.

Psychological Well-Being and Social Support

The majority of participants reported levels of anxiety and depression that were under the threshold for clinical caseness; however, 4 (15%) participants had scores on the GAD2 of >3, and 6 (22%) participants had scores of ≥2 on the PHQ2 that warrant clinical caseness. Patients reported more depressive symptoms than carers; conversely carers reported more symptoms of anxiety than patients. However these differences did not reach statistical significance.

Table 1 Demographic and clinical characteristics of the sample

			Overall ($n = 27$)	Patients ($n = 5$)	Carers ($n = 22$)
Country	UK	*n* (%)	10 (37.0)	2 (40.0)	8 (36.4)
	France	*n* (%)	17 (63.0)	3 (60.0)	14 (63.6)
Patient/Carer Gender	Male	*n* (%)	11 (40.7)	2 (40.0)	9 (40.9)
	Female	*n* (%)	16 (59.3)	3 (60.0)	13 (59.1)
Patient/Carer age		Mean (SD)	13.1 (4.3)	16.8 (4.2)	9.3 (4.4)
First language	English	*n* (%)	7 (25.9)	1 (20.0)	6 (27.3)
	French	*n* (%)	12 (44.4)	2 (40.0)	10 (45.5)
	Moroccan	*n* (%)	1 (3.7)	0	1 (4.5)
	Arabic	*n* (%)	3 (11.1)	1 (20.0)	2 (9.1)
	Russian	*n* (%)	1 (3.7)	0	1 (4.5)
	Gujarati	*n* (%)	1 (3.7)	0	1 (4.5)
	Punjabi	*n* (%)	2 (7.4)	1 (20.0)	1 (4.5)
Employment	Full time	*n* (%)	10 (37.0)	1 (20.0)	9 (40.9)
	Part time	*n* (%)	3 (11.1)	0	3 (13.6)
	Self-employed	*n* (%)	3 (11.1)	0	3 (13.6)
	Student	*n* (%)	4 (14.8)	3 (60.0)	1 (4.5)
	Unemployed	*n* (%)	3 (11.1)	0	3 (13.6)
	Other	*n* (%)	4 (14.8)	1 (20.0)	3 (13.6)
Duration of diagnosis		Mean (SD)	12.7 (3.8)	16.2 (3.2)	9.2 (4.3)
Prescribed nutritional supplements		*n* (%)	26 (96.3)	5 (100)	21 (95.5)
Prescribed co-medicines		*n* (%)	6 (22.2)	2 (40.0)	4 (18.2)

Table 2 Psychological well-being, social support and relationship with physicians

Psychological well-being and social support	Overall ($n = 27$) Mean (SD)	Patients ($n = 5$) Mean (SD)	Carers ($n = 22$) Mean (SD)
GAD2 anxiety	1.6 (1.7)	0.4 (0.9)	1.9 (1.7)
PHQ2 depression	0.9 (1.3)	1.8 (1.7)	0.8 (1.1)
MOS-SSS emotional/informational support	3.3 (0.9)	3.9 (0.9)	3.1 (0.9)
MOS-SSS tangible support	3.3 (0.9)	3.8 (0.8)	3.1 (0.9)
MOS-SSS affectionate support	4.2 (0.9)	5.0 (0.0)	4.1 (0.9)*
MOS-SSS positive social support	4.1 (0.8)	4.5 (0.5)	3.9 (0.9)
Relationship with physicians			
How satisfied are you with the care you/your child receives from your/their doctor	8.0 (1.7)	7.8 (1.9)	8.1 (1.7)
How likely is it that you would talk to your/your child's doctor if you had queries about your/your child's condition and the treatments you/they have been asked to use?	7.5 (2.4)	6.6 (1.5)	7.7 (2.5)
How much confidence do you have in your/your child's doctor with regard to tyrosinaemia?	8.0 (2.0)	7.2 (2.3)	8.2 (2.0)
How much do you think your physician understands you and your/your child's condition?	8.0 (2.0)	7.2 (2.6)	7.7 (2.0)

*$p < 0.005$

Descriptive statistics for depression, anxiety and social support are shown in Table 2.

The qualitative research amongst patients and carers found that the social and emotional impact of the condition was more evident in carers than patients. The diagnosis of HT1 was particularly devastating. Carers' descriptions of their concerns may explain the increased anxiety they reported. Primarily, there was concern for the present and future health of the child. In addition, carers were concerned for the child's emotional well-being, such as

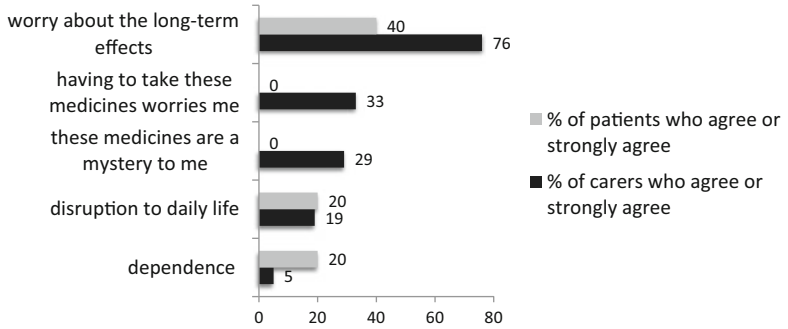

Fig. 1 Types of concerns held by patients and carers about nitisinone

their feelings of social exclusion and of being different. Feelings of isolation were common amongst carers, particularly at the time of diagnosis. This was attributed to the rarity of the condition and therefore the lack of available information and support.

> "I feel like we've been left a lot of the time to fend for ourselves" Carer 001, parent of a 10-year-old

Some carers often reported feelings of stress, anxiety and of being emotionally overwhelmed by the change in lifestyle and the difficulty of caring for a child with this condition. Carers often felt guilty because the condition is genetic, and one carer reported experiences of stigma and discrimination within her community. HCPs also reported that several parents seemed to experience a sense of stigma surrounding the condition and appeared reluctant to openly acknowledge that their child had a serious medical condition, to avoid setting them apart from other children.

Relationship with HCPs

Overall, participants reported a high level of satisfaction with their doctor and confidence in their doctor with regard to HT1 (see Table 2). Ratings were slightly lower amongst patients than carers; however, no significant differences were found.

The qualitative research confirms this level of satisfaction with HCPs in general, with patients perceiving HCPs as knowledgeable and reporting satisfaction with the support they had received. However, some carers reported unmet needs, such as beliefs that HCPs lacked understanding of the psychosocial impact of the condition and the difficulties involved in maintaining adherence to diet and managing the condition on a day-to-day basis. Carers also felt that HCPs should provide greater opportunities for the child to ask questions.

> "I don't think his consultants [and] dieticians explain it to him because he's a child and they see him as a child so they don't want to like bombard him with all the scientific information" Carer 004, parent of a 14-year-old

Perceptions of Illness and Treatment

All participants reported high beliefs in necessity for treatment (>scale midpoint), and these were significantly higher amongst carers than patients (carers: mean 4.6 (SD = 0.5); patients: mean 3.9 (SD = 0.3), $t = 3.455$; $p = 0.002$). Over half of the participants ($n = 16$; 59%) reported high concerns about their medication, in particular long-term effects (>scale midpoint); see Fig. 1. Descriptive statistics are shown in Table 3. Overall, participants perceived treatment to be helpful in controlling HT1; however, carers were significantly more convinced than patients that treatment was helpful (IPQ treatment control, carers: mean = 9.3 (SD = 0.9); patients: mean = 7.8 (SD = 1.4); $t = 2.920$; $p = 0.007$).

Adherence

Overall a high level of adherence to medication was reported, with a mean score of 29.1 (SD = 2.4) on the MARS. The mean number of days per week patients reported non-adherence was 0.1 (SD = 0.6). Patients reported lower adherence on all scales compared to carers. Only four carers (18%) scored <30 on the MARS (see Table 4), compared to 4 (100%) of patients (one patient had missing data). Adherence to medication was uniformly high

Table 3 Beliefs about illness and treatment

	Overall ($n = 27$) Mean (SD)	Patients ($n = 5$) Mean (SD)	Carers ($n = 22$) Mean (SD)
BMQ necessity (range = 1–5)	4.5 (0.5)	3.9 (0.3)	4.6 (0.5)*
BMQ concerns (range = 1–5)	2.7 (0.7)	2.4 (0.4)	2.8 (0.7)
IPQ consequences (range = 0–10)	6.8 (2.1)	6.6 (2.1)	6.8 (2.1)
IPQ timeline (range = 0–10)	8.9 (1.9)	9.4 (1.3)	8.8 (2.0)
IPQ personal control (range = 0–10)	6.6 (2.6)	6.0 (2.3)	6.7 (2.7)
IPQ treatment control (range = 0–10)	9.0 (1.2)	7.8 (1.4)	9.3 (0.9)**
IPQ identity (range = 0–10)	4.1 (2.9)	4.4 (3.8)	4.0 (2.8)
IPQ concern (range = 0–10)	8.1 (1.8)	7.4 (1.8)	8.3 (1.7)
IPQ coherence (range = 0–10)	7.5 (1.9)	6.4 (1.5)	7.8 (1.9)
IPQ emotional representations (range = 0–10)	7.1 (1.8)	6.4 (1.1)	7.2 (1.9)

*$p < 0.005$; **$p < 0.01$

Table 4 Adherence to medication and dietary recommendations

	Overall ($n = 27$) Mean (SD)	Patients ($n = 5$) Mean (SD)	Carers ($n = 22$) Mean (SD)
Medication MARS total	29.1 (2.4)	25.5 (5.1)	29.8 (0.4)
Medication MARS forget to take medication	4.4 (1.0)	2.6 (0.9)	4.9 (0.4)*
Medication MARS alter the dose of medication	4.9 (0.4)	4.5 (1.0)	5.0 (0.2)
Medication MARS stop taking medication	4.9 (0.4)	4.5 (1.0)	5.0 (0.0)
Medication MARS decide to miss out a dose	4.9 (0.6)	4.3 (1.0)	5.0 (0.0)
Medication MARS take less medication than instructed	4.9 (0.4)	4.5 (1.0)	5.0 (0.0)
Medication MARS take more medication than instructed	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)
Medication Number of days non-adherent	0.1 (0.6)	1.0 (1.7)	0.0 (0.0)
Diet how well do you comply with instructions	4.1 (0.8)	3.0 (1.0)	4.3 (0.7)*
Diet adherence total (2 items)	7.8 (2.4)	6.0 (3.2)	8.1 (2.2)
Diet-forget to stick to diet regimen	3.7 (1.3)	2.8 (1.7)	3.9 (1.1)
Diet-stop adhering for a while	4.0 (1.3)	3.3 (1.5)	4.2 (1.2)
Diet number of days non-adherent	0.7 (1.2)	2.3 (2.1)	0.4 (0.8)

*$p = < 0.005$

on all MARS scales measuring intentional non-adherence (e.g. altering the dose intentionally). The findings show that patients were significantly more likely to report unintentional non-adherence (e.g. forgetting) than carers (patients mean (SD) = 2.6 (0.9); carers mean (SD) = 4.9 (0.4); $t = 5.562$; $p = 0.004$).

There was greater variation in rates of adherence to dietary recommendations. Overall non-adherence was reported on a mean of 0.7 day/week, with more days of non-adherence reported by patients (mean = 2.3 day/week; SD = 2.1) than carers (mean = 0.4 day/week; SD = 0.8). Carers reported a higher level of adherence to dietary instructions than patients (mean (SD) = 4.3 (0.7) amongst carers; 3.0 (1.0) amongst patients ($t = 2.821$; $p = 0.010$).

The qualitative research amongst patients, carers and HCPs supported this finding that adherence to dietary restrictions and protein supplements was often less successful than adherence to nitisinone.

> "The biggest problem is compliance with the diet rather than compliance with the medicines and in general they're relatively compliant with the medicine but diet less so" Physician, Birmingham

Factors Associated with Adherence to Diet and Medication

Illness Experience and Perceptions

Carers of older children and those who had been diagnosed for a longer period of time reported higher adherence to medication ($r = 0.436$, $p = 0.048$; $r = 0.456$, $p = 0.038$, respectively). Carers who believed that their child's illness would last a long time (IPQ Timeline: $r = 0.472$, $p = 0.036$) and those who attributed fewer symptoms to HT1 (IPQ Identity: $r = -0.496$, $p = 0.026$) were more adherent to medication. There was a significant positive correlation between concerns about HT1 and adherence to dietary recommendations (IPQ concern: $r = 0.399$; $p = 0.048$). Those who were more concerned about HT1 reported higher adherence. Amongst carers, anxiety was inversely correlated with adherence to medication (GAD2: $r = -0.547$, $p = 0.013$), with a similar trend for depression (PHQ2: $r = -0.419$, $p = 0.066$).

Interviews with patients and carers suggest that the perception of consequences of non-adherence, such as experiencing symptoms after eating protein, can also be a driver for better adherence. Indeed, HCPs noted that if the patient eats restricted foods, they don't immediately become ill, which can lead both patients and their carers to believe that full adherence to dietary recommendations is not necessary.

HCPs felt that a lack of understanding about the condition, its seriousness and the role of diet and medication was linked to non-adherent behaviour.

Treatment Perceptions and Experiences

In the sample as a whole, there was a significant positive correlation between BMQ necessity beliefs and adherence to treatment, indicating that those who were more convinced of the necessity of nitisinone were more adherent ($r = 0.494$; $p = 0.014$). The qualitative interviews amongst patients and carers found that participants were convinced that treatment was effective at controlling the condition and maintaining good health. Adherence was driven by a strong perceived need for treatment (both medication and dietary restrictions).

> "She has got to have her medicine, if she doesn't have it, she will become poorly". Carer 002, parent of a 10-year-old

Adherence to treatment was also significantly associated with a stronger belief that treatment can successfully control HT1 (IPQ treatment control: $r = 0.533$; $p = 0.007$).

In addition, the unpleasant taste of protein supplement drinks was reported by many participants as a particular difficulty, especially for parents of small children, who were often adamant in their refusal to take them.

Patient and Family Factors

A significant inverse correlation was found between child age and adherence within the overall sample ($r = -0.634$; $p = 0.0001$) and amongst the carers ($r = -0.526$; $p = 0.012$), indicating that both carers and patients experience increasing difficulty with adherence to dietary recommendations as the patient grows older. As children grow older, social situations, including school dinner times, posed particular difficulties. Problems arise as children grow older because they become more independent and become aware of the differences between their own diet and those of their family or peers and start to take control of their own medication/eating habits. HCPs also suggested that since patients have no recollection of being unwell, they don't always perceive that they have a serious condition.

> "I've found him in the middle of the night, some nights, coming down to fridge and you'll go what are you doing? And he goes I want some ham". Carer 003, parent of 3- and 6-year-olds

The qualitative interviews amongst patients and carers and HCPs suggest that establishing a daily routine and advanced planning are important for adherence. For example, ensuring adequate supplies of medicines or dietary supplements and preparation for being away from home seem to be important for successful adherence in this population.

Although many had good support networks, both carers and patients talked of a lack of understanding from others about the severity and potential consequences of the condition and the importance of adhering to a strict diet.

> "And then telling people, they kind of just went, Oh she's vegan … there was no real understanding". Carer 001, parent of a 10-year-old

Discussion

This mixed-method study amongst patients with HT1, their carers and HCPs provided valuable insight into the extent of adherence to treatment recommendations, namely, diet and medication (nitisinone). Findings from the qualitative and quantitative phases revealed that reported adherence to nitisinone was high. However, adherence to dietary recommendations was more problematic. These findings support previous research which indicates that adherence to dietary restrictions may be suboptimal (Masurel-Paulet et al. 2008; Wisse et al. 2012).

Patients reported lower levels of adherence to medication and dietary restrictions than carers. This finding may be influenced by carers' strong perceived necessity for treatment, whereas patients were more likely to have doubts about their personal necessity for treatment and were less convinced than carers that their treatment was effective in controlling their condition. In addition those carers who perceived their children's illness to have a chronic timeline and those who attributed fewer symptoms to HT1 were more adherent. Although it is not possible to determine causal relationships due to the cross-sectional design of the study, it is important to consider that the latter finding may be a consequence of, rather than a cause of, high adherence. In addition, carers who reported higher levels of anxiety and depression were less adherent to their children's medication regime. These differences between carers and patients provide insight into key factors to target amongst patients to support their continued adherence into adolescence and adulthood.

The results indicate that adherence to dietary instructions becomes more problematic as children with HT1 grow older. Published research across a range of chronic conditions has shown that treatment regimen adherence is significantly lower in adolescence than in adult populations (Dean et al. 2010; Salema et al. 2011). One explanation for this could be the unique physical, social and emotional challenges experienced by all young people during adolescence, thus making the management of a strict treatment regime seem like an additional and often unnecessary burden that sets them apart from their peers (Salema et al. 2011). In addition, it was reported that young patients are often not actively involved in their consultations with HCPs. Greater involvement in managing their condition and in their consultation at these early stages (as appropriate) may have a positive impact on future adherence by increasing their investment and understanding of the treatment regime, potentially making it more stable and less influenced by moving through different life stages.

The study has several limitations including a very small sample size, particularly for the quantitative phase of the research. The small sample size and lack of variation in adherence mean that the findings of this study should be interpreted with caution, as the study lacked power to detect predictors of adherence. Also, due to the small sample size and anonymity of the survey responses, it was not possible to explore potential similarities and/or differences in the response pattern of patient/carer dyads. Since patients in this study typically reported lower levels of adherence than carers, this could be an interesting avenue to explore in future research. Additionally, since a cross-sectional design was employed, it was not possible to infer the direction of relationships. It should also be considered that self-report measures of adherence may be prone to bias. However, despite the small sample, the present study successfully gained insight from a range of perspectives into the experiences of living with or caring for someone with HT1 through a mixed-method approach. Mixed-method research designs are often used to expand the scope of enquiry by accessing a wider range of data (O'Cathain and Thomas 2006) and are ideal in areas where there is currently little information such as the study of rare diseases.

Recommendations and Conclusions

Additional research, using prospective designs and larger samples, is required to track and examine levels of adherence and barriers to/strategies for maintaining adherence as children and adolescence grow older and reach adulthood. In addition, it is important for HCPs to provide practical and emotional support for both parents and children to facilitate adherence to dietary restrictions, with a focus on social situations and when away from home (e.g. psychological support to address stigma and address feelings of being different to others; practical support to plan for risk situations and facilitate appropriate food choices). Children should also be encouraged to ask questions about their condition and their treatment regimen, with a view to making them more actively involved in their care, while they still have the full support of their carer. Greater support may be needed when transitioning from child to adult services, with recognition of the potential psychosocial factors that may influence an unhelpful change in behaviours relating to medication and dietary management.

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Take-Home Message

Patients with HT1 and their carers need greater support adhering to the low tyrosine/phenylalanine diet necessary for successful management of this condition, through education and increased involvement in consultations.

Compliance with Ethics Guidelines

The submitting authors have circulated the article and secured final approval of the version to be peer-reviewed from all co-authors prior to article submission. We can also conclude:

- Absence of previous similar or simultaneous publications.
- That all authors have inspected and approved the manuscript.
- That all authors have made a substantial contribution to the work (all authors should have been involved in (a) conception and design, or analysis and interpretation of data, and (b) drafting the article or revising it critically for important intellectual content).
- All authors have agreement to submission.

Conflict of Interest

Dr Sumaira Malik, Dr Sinead NiMhurchadha, Dr Christina Jackson and Dr Lina Eliasson PhD are all employed by Atlantis Healthcare, which received funds from Swedish Orphan Biovitrum AB to carry out this project.

Professor John Weinman has received consulting fees from Swedish Orphan Biovitrum AB, to carry out this project and attend scientific meetings.

Dr Sandrine Roche has received Professor Weinman is also a part time employee of Atlantis Healthcare. Support from Swedish Orphan Biovitrum AB, to attend scientific meetings and to carry out data collection for this study in France.

Professor John H. Walter has received support from Swedish Orphan Biovitrum AB, to attend scientific meetings.

Informed Consent

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

Details of the contributions of individual authors:

SM: study conception and design, data collection, analysis and interpretation and drafting of the manuscript. SNM: data collection, analysis and interpretation and drafting of the manuscript. CJ and LE: data interpretation and critical revision of the manuscript. Prof JW: study conception and design and critical revision of the manuscript. SR: data collection and critical revision of the manuscript. JW: data collection and critical revision of the manuscript. All authors gave final approval of the version to be published.

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RESEARCH REPORT

Regression of Hepatocellular Adenomas with Strict Dietary Therapy in Patients with Glycogen Storage Disease Type I

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Abstract Hepatocellular adenomas (HCAs) are a common complication in patients with glycogen storage disease type I (GSD I). In this series, we report regression of HCAs in a cohort of patients who achieved metabolic control with strict dietary therapy. A retrospective review of the clinical records for all patients with GSD I was performed at our institution. All available imaging studies were reviewed in patients with reported regression of HCAs in the medical record. The charts of 163 patients with GSD Ia and 42 patients with GSD Ib were reviewed, and HCAs were documented in 47 subjects (43 Ia/4 Ib). After review of all available imaging studies, eight patients met criteria of being followed with both magnetic resonance imaging and ultrasound and were found to show evidence of regression of HCAs. In these individuals, regression of the HCAs occurred once metabolic control was obtained, as determined by decreasing levels of serum triglyceride levels. The average triglyceride level in all patients prior to regression of HCAs was 753 mg/dL (SD ± 293). The average serum triglyceride level in all patients at the time of regression of HCAs was 340 mg/dL (SD ± 164). These findings suggest that strict dietary therapy may cause regression of HCAs. If HCAs are documented in a patient with suboptimal metabolic control, intensive medical therapy may be an alternative to surgical intervention in some individuals.

Abbreviations

CT	Computed tomography
GSD	Glycogen storage disease
HCA	Hepatocellular adenoma
MRI	Magnetic resonance imaging

Introduction

Glycogen storage disease type I (GSD I) is an autosomal recessive inborn error of metabolism caused by defects in the glucose-6-phosphatase complex. All endogenous glucose synthesis is impaired in affected individuals, which leads to metabolic derangements including hypertriglyceridemia, hyperuricemia, and hyperlactatemia. Disordered glucose metabolism results in marked hepatomegaly. Hepatocellular adenoma (HCA) formation occurs frequently in patients with GSD I and can be seen in up to 70% of patients greater than the age of 25 (Wang et al. 2011; Lee 2002; Rake et al. 2002a; Weinstein and Wolfsdorf 2002). The management of HCA has been a source of debate due to the risk of hepatocellular carcinoma, and invasive procedures including embolization, ablation, surgical resection, and transplantation have been proposed as methods for treating growing or suspicious lesions (Rake et al. 2002b; Davis and Weinstein 2008; Reddy et al. 2009). We report regression of HCAs in several patients who experienced metabolic improvement after presenting with suboptimal metabolic control.

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Methods

Institutional review board approval and informed written consent was obtained from patients participating in an observational study at the University of Florida Glycogen Storage Disease Program. Subjects have been followed in a longitudinal and prospective manner. A retrospective review of the clinical records for all patients with GSD I was performed. Longitudinal records were available from 205 patients of whom 163 patients had GSD Ia and 42 patients had GSD Ib. The diagnosis for GSD I was established biochemically or by assessment of enzymatic testing from a liver biopsy specimen, and the diagnosis was confirmed with DNA analysis. As part of routine clinical care, patients discovered to have HCAs were subsequently monitored with serial imaging through either magnetic resonance imaging (MRI) or ultrasonography. In some patients, contrast enhanced computed tomography (CT) was also performed. The MR protocol used at our institution for evaluation of liver lesions involves imaging the patient on either a 1.5 or 3.0 Tesla MR unit and obtaining pre-contrast axial in and out of phase T1-weighted images, pre-contrast axial T1-weighted image, and pre-contrast coronal T1- and T2-weighted images. A hepatocellular-specific MR contrast agent, gadoxetate disodium, is then administered intravenously. Axial post-contrast T1-weighted images are then obtained after 25 s, 60 s, 90 s, 3 min, and 20 min. A coronal T1-weighted image is also obtained 20 min after contrast administration. A post-contrast fat-saturated T2-weighted axial image and diffusion weighted imaging are also included in the protocol.

The total number of patients with documented HCAs was 47 (43 GSD Ia/4 GSD Ib) (Tables 1 and 2). The diagnosis of HCA was made based on imaging appearance. Biopsy of the liver lesions was not performed in this group of patients since it is no longer standard of care for this population. The lesions identified on imaging are presumed to represent HCAs given their characteristic imaging appearance and location within the liver. Ultrasound and CT appearance can vary depending on intratumoral hemorrhage and lipid content; however, HCAs tend to be homogenous and hyperechoic with ultrasound. HCAs with CT tend to be isodense to background liver on non-contrast studies and are hyperdense after administration of contrast during the arterial phase and hypodense on delayed phase. Lesions are typically heterogeneous on MRI and demonstrate no significant uptake of hepatocellular specific contrast agents. None of the lesions identified were thought to represent focal fatty deposition or focal nodular hyperplasia. Optimal metabolic control is defined as triglyceride level less than 200 mg/dL, lactate less than 2.2 mmol/L, and serum glucose greater than 75 mg/dL. The clinical records, laboratory data, and all radiographic imaging for these patients were reviewed.

Results

There were ten patients (all with GSD Ia) who experienced regression of their HCAs. Eight of these patients were included in this report as they met criteria of having clear regression of their HCAs and have been followed with both MRI and ultrasonography (Table 2). Regression was defined as a minimum of 10% decrease in size or complete resolution of the lesion. Representative images demonstrating regression of HCAs are displayed in Figs. 1, 2, 3, and 4. In the individuals with documented regression, shrinkage of the HCAs occurred once improved metabolic control was obtained with strict dietary therapy as determined by decreasing levels of serum triglycerides. Dietary therapy included uncooked cornstarch given in a slurry of water or artificially sweetened fluid and is given at 3–5 h intervals during the day and 4–6 h intervals overnight. Each patient's optimal schedule was based on metabolic and clinical monitoring. Intake of fructose, galactose, and sucrose was restricted to 2.5 g of non-utilizable sugar per meal. Of note, other laboratory values were followed in this group of patients including total serum cholesterol, serum glucose, uric acid, AST/ALT, and lactate. However, these biochemical parameters did not correlate as well with long-term metabolic control compared with serum triglycerides because uric acid can be affected by use of medication; serum glucose and lactate concentrations are short-term markers that can fluctuate rapidly. There were no confounding variables in these patients that could influence triglyceride levels, such as use of oral contraception, pregnancy, medications, or supplements.

The average serum triglyceride concentration in this group prior to regression of HCAs was 753 mg/dL (SD $\pm$ 293). In contrast, the average serum triglyceride level in all patients at time of regression of HCAs was 340 mg/dL (SD $\pm$ 164) (Table 2). Each patient's average serum triglyceride concentration prior to regression presented in the table was calculated by averaging all their serum triglyceride concentration values prior to documented HCA regression on imaging. Each patient's average serum triglyceride concentration since beginning of regression presented in the table was calculated by averaging all their serum triglyceride concentration values starting at the time imaging first demonstrated regression and during the follow-up period since regression was first reported. In six of the eight patients, complete regression of the HCAs was noted. The average total duration of patient follow-up was 93.8 months, while duration of follow-up since the beginning of HCA regression was 63.6 months. For the

Table 1 Patients with HCAs that did not regress

	Age	Sex	GSD type	DNA analysis	Age at HCA discovery (years)	Average triglyceride level (mg/dL)	Number of HCAs	Largest HCA size (cm)	HCA change in last 5 years	Avg. follow-up (years)
1	20	M	GSD Ia	35X/Q347X	16	584 ± 297	1	0.8	Stable	5
2	21	F	GSD Ia	R83C	16	325 ± 110	2	1.0	Stable	6
3	10	M	GSD Ia	R83C/E110K	3	289 ± 65	1	1.3	Stable	6
4	47	M	GSD Ia	R83C/Q347X	19	546 ± 118	3	1.3	Stable	6
5	15	M	GSD Ia	R83C/G270V	9	295 ± 69	1	1.4	Stable	5
6	14	M	GSD Ib	464del10ins3C/1211delCT	6	146 ± 41	2	1.8	Stable	8
7	59	F	GSD Ia	R170Q/Q347X	56	250 ± 61	2	3.1	Stable	1
8	32	M	GSD Ia	R83C	??	1,348 ± 953	3	3.2	Stable	7
9	25	M	GSD Ib	G339C/1042delCT	23	143 ± 25	1	3.3	Stable	2
10	30	F	GSD Ia	R83C	16	905 ± 299	15	3.6	Stable	15
11	40	F	GSD Ia	R83C	Teenager	314 ± 48	3	3.8	Stable	7
12	43	F	GSD Ia	R83C/212X	13	1,004 ± 113	4	8.0	Stable	12
13	18	M	GSD Ia	R83C/Q242X	8	890 ± 500	4	2.5	Possible regression of adenomas; however, no MR for confirmation	9
14	20	M	GSD Ib	1211delCT/ C381 + 1G > T	15	79 ± 25	2	1.3	Decrease in size with possible resolution, but no MR for confirmation	4
15	36	F	GSD Ia	R83C/Q347X	31	474 ± 129	2	3.5	Largest lesion stable, questionable disappearance of smaller lesion	8
16	38	M	GSD Ia	R83C	19	342 ± 86	2	2.2	Largest lesion stable, smaller 9 cm lesion has developed	7
17	16	F	GSD Ia	T108I	12	259 ± 147	4	1.3	Largest increased by 0.5 cm	3
18	38	F	GSD Ia	??	19	501 ± 145	5	3.9	Largest increased by 0.7 cm	12
19	43	M	GSD Ia	R83C/G188S	??	340 ± 107	>20	5.5	Largest increased by 1 cm	5
20	18	F	GSD Ia	Q347X	13	551 ± 359	>20	4.9	Largest increased by 1 cm	6
21	12	F	GSD Ib	G68R/Q248X	6	189 ± 39	4	4.0	Largest grew by 1.3 cm	6
22	41	M	GSD Ia	A124T/Q347X	37	341 ± 137	8	4.0	Largest increased by 1.7 cm (previously had a 13.5 cm lesion which was resected)	4
23	28	M	GSD Ia	Q347X	15	349 ± 91	>20	4.8	Largest increased by 2 cm with new lesions developing	4
24	27	M	GSD Ia	Q347X	16	237 ± 116	6	4.0	Largest increased by 2 cm	12
25	35	M	GSD Ia	Q347X/Q242X	25	498 ± 102	4	5.6	Largest increased by 2 cm	3
26	24	F	GSD Ia	35X/D38V	14	479 ± 107	7	5.6	Increase in size by 2.5 cm	5
27	39	F	GSD Ia	R83C	11	888 ± 302	8	4.2	Largest increased by 3 cm with new lesions developing	13

(continued)

Table 1 (continued)

	Age	Sex	GSD type	DNA analysis	Age at HCA discovery (years)	Average triglyceride level (mg/dL)	Number of HCAs	Largest HCA size (cm)	HCA change in last 5 years	Avg. follow-up (years)
28	29	F	GSD Ia	R83C	16	729 ± 77	>20	6.1	Largest increased by 4 cm with new lesions developing	6
29	46	M	GSD Ia	R83C	15	404 ± 140	>20	17.4	Increase by 6 cm	2
30	33	F	GSD Ia	T108I	19	232 ± 55	1	Prior 14 cm lesion	Lesion resected, no new lesions	11
31	28	F	GSD Ia	R83C	12	947 ± 365	1	Prior 4.3 cm lesion	Lesion resected, no new lesions	7
32	30	M	GSD Ia	Q347X/Q242X	21	315 ± 113	8	6.5	Right hepatectomy, otherwise stable	2
33	19	M	GSD Ia	158delC	10	632 ± 135	12	6.3	Largest resected. Next dominant lesion growth by 4 cm	8
34	23	M	GSD Ia	R83C/Q347X	13	1,082 ± 301	6	3.6	Largest resected, other adenomas small with slow growth	9
35	23	M	GSD Ia	R83C/R170Q	17	596 ± 353	6	6.0	Largest resected after it grew by 5 cm, other adenomas small with slow growth	9
36	22	M	GSD Ia	R83C	10	555 ± 198	>20	5.3	Largest resected, other adenomas continue to grow with new lesions appearing	9
37	56	F	GSD Ia	??/Q347X	45	725 ± 387	3	1.7	Prior liver transplant, no new lesions	5
38	41	F	GSD Ia	R83C	19	202 ± 3	4	7.1	Unknown	1
39	29	M	GSD Ia	R83C	18	1,044 ± 514	2	2.1	Unknown	0

?? unknown

Table 2 Patients with regression of HCA

	Age	Sex	GSD Type	DNA analysis	Age at HCA discovery (years)	Age at Time of HCA regression (years)	# of HCAs	Size of Largest HCA (cm)	Size of HCA after regression	Duration of follow-up since beginning of regression (months)	Duration it took for HCAs to completely resolve (months)	Duration of follow-up since complete resolution (months)	Total duration of follow-up (months)	Average triglyceride level prior to HCA regression in mg/dL	Average triglyceride level since beginning of HCA regression in mg/dL
1	18	M	GSDIa	R83C/ 130X	5	9	14	1.5	Complete resolution	86	65	23	131	345 ± 68	211 ± 59
2	31	F	GSDIa	R83C/ R295C	27	28	4	1.9	Complete resolution	29	7	24	152	957 ± 276	444 ± 140
3	34	F	GSDIa	Q347X	21	30	2	1.9	Complete resolution	60	19	41	81	591 ± 131	207 ± 22
4	32	F	GSDIa	R83C	12	23	5	2.0	Complete resolution	123	23	100	147	961 ± 403	294 ± 84
5	23	M	GSDIa	W77R/ G118S	15	17	4	3.1	Complete resolution	57	36	21	57	814 ± 243	718 ± 169
6	35	M	GSDIa	R83C/ R295C	22	30	2	3.3	Complete resolution	64	34	40	73	835 ± 184	312 ± 66
7	31	F	GSDIa	Q347X	19	25	>10	2.6	1.5 cm (most small lesions resolved)	41	NA	NA	60	314 ± 84	187 ± 14
8	34	F	GSDIa	R83C	29	30	5	13.2	9.1 cm	49	NA	NA	49	1,205 ± 852	343 ± 110
Average values										63.6	30.7	41.5	93.8	753 ± 293	340 ± 164

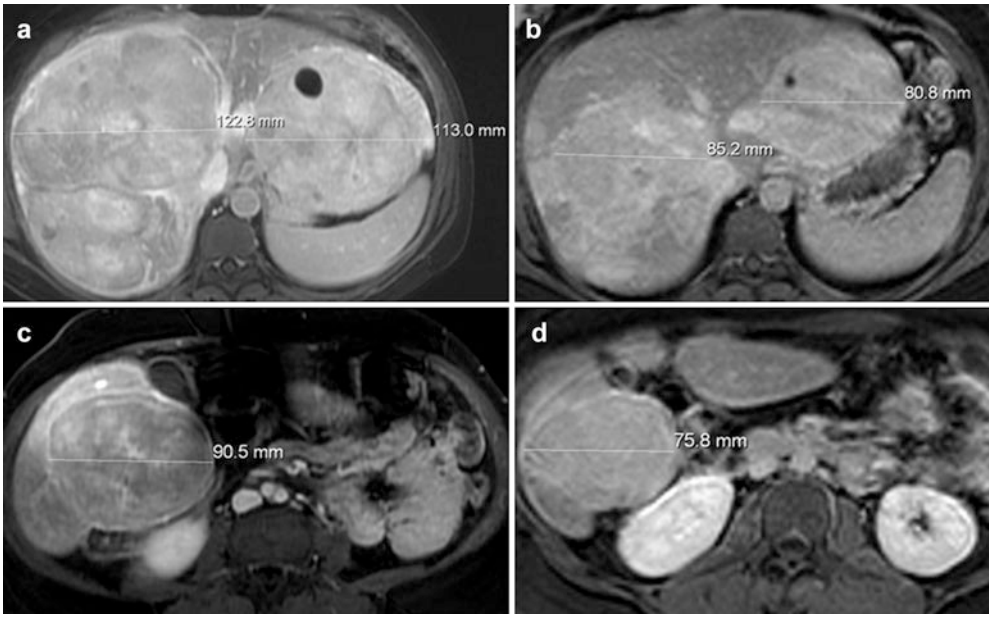

Fig. 1 Post-contrast (gadoxetate disodium) MRI of a patient with GSD I with HCAs. (**a**) Fat-saturated T1-weighted post-contrast axial MR image of the superior liver obtained after intravenous bolus injection of gadoxetate disodium demonstrates multiple large lesions in the liver consistent with HCAs, two of the lesions are measured. (**b**) Fat-saturated T1-weighted post-contrast axial MR image of the superior liver obtained after intravenous bolus injection of gadoxetate disodium in the same patient 4 years later demonstrates decrease in size of the multiple HCAs, two of which are measured for comparison. (**c**) Fat-saturated T1-weighted post-contrast axial MR image of the inferior liver obtained after intravenous bolus injection of gadoxetate disodium in the same patient at the same time as the image in (**a**) demonstrates a large HCA involving the inferior right hepatic lobe (lesion is measured). (**d**) Fat-saturated T1-weighted post-contrast axial MR image of the inferior liver obtained after intravenous bolus injection of gadoxetate disodium in the same patient four years later, at the same time as the image in (**b**), demonstrates significant decrease in size of the HCA, measured for comparison

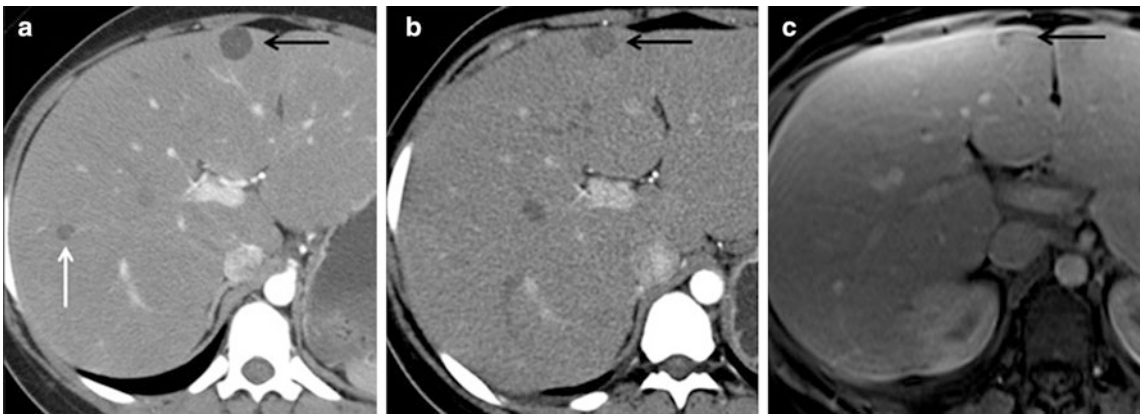

Fig. 2 Computed tomography (CT) and MRI of a patient with GSD I with HCAs. (**a**) Axial CT image of the liver after administration of iodinated contrast demonstrates two hypodense lesions within the liver representing HCAs (*black and white arrows*). (**b**) Axial CT image of the liver after administration of iodinated contrast in the same patient 18 months later demonstrates decrease in size of the HCA in segment 4 of the liver (*black arrow*). The lesion in the right hepatic lobe, white arrow in (**a**), has completely resolved. (**c**) Axial fat-saturated T1-weighted post-contrast MR image of the liver obtained after intravenous bolus injection of gadoxetate disodium in the same patient 8 years later demonstrates continued decrease in size of the segment 4 HCA (*black arrow*)

six patients with complete HCA resolution, the mean duration to disappearance of the lesions was 30.7 months from the beginning of adenoma shrinkage.

In contrast, there were eight patients who showed a significant increase in the size of their HCAs (>2 cm). None of these of patients ever achieved optimal metabolic control, and all of these patients struggled with dietary compliance with the prescribed treatment regimen. Of note, pharmacologic therapy was used in all of these patients to lower their triglycerides, but chronic hyperlactatemia remained due to the suboptimal metabolic control.

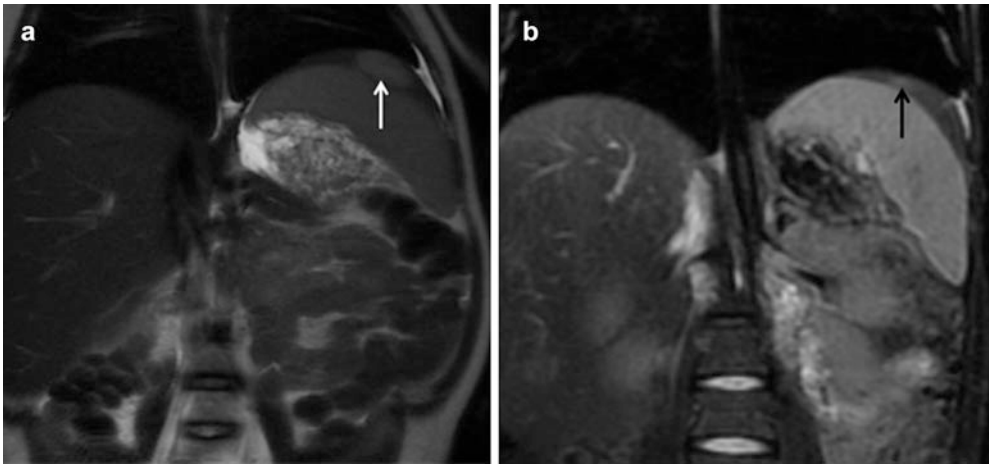

Fig. 3 MRI of a patient with GSD I with HCAs. (**a**) Coronal non-contrast T2-weighted MR image of the abdomen in a patient with GSD I demonstrates a mildly hyperintense lesion (*white arrow*) in the left lobe of the liver inferior to the diaphragm consistent with a HCA. (**b**) Coronal non-contrast fat-saturated T2-weighted MR image of the abdomen in the same patient 4 years later demonstrates significant decrease in size of the HCA in the left hepatic lobe (*black arrow*)

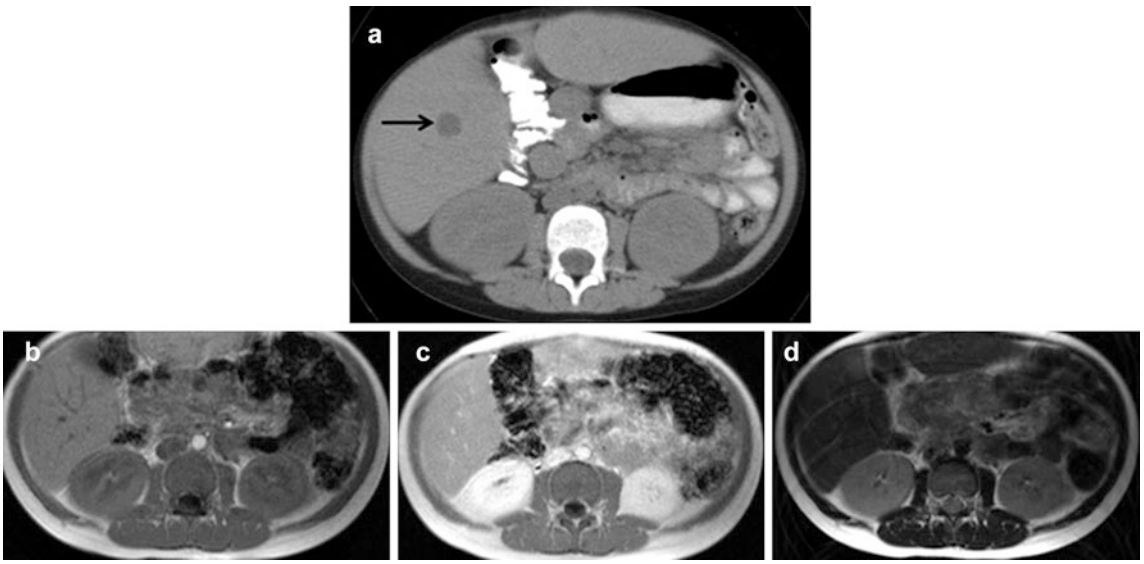

Fig. 4 CT and MRI of a patient with GSD I with HCAs. (**a**) Axial CT image of the abdomen with oral contrast and without intravenous contrast in a patient with GSD I demonstrates a hypodense lesion in the inferior right hepatic lobe consistent with a HCA (*black arrow*). (**b**–**d**) Axial non-contrast T1-weighted MR image (**b**), axial post-contrast T1-weighted MR image (**c**), and axial non-contrast T2-weighted MR image (**d**) at the same level as (**a**) in the same patient 14 months later demonstrate complete resolution of the inferior right hepatic lobe HCA

Discussion

HCA formation occurs frequently in patients with GSD I. Proposed mechanisms of HCA formation include hormonal stimulation, oxidative stress from disordered fatty acid metabolism, and proto-oncogenic activation (Wang et al 2011; Bianchi 1993). Complications of HCAs include local compression and hemorrhage (Rake et al. 2002a, b; Weinstein and Wolfsdorf 2002; Davis and Weinstein 2008). Another complication is the transformation of a HCA into hepatocellular carcinoma (Bianchi 1993; Kudo 2001; Limmer et al. 1988), which may occur in as much as 11% of patients (Coire et al. 1987).

While the risk of malignant transformation may be less than previously reported due to improvements in medical care, an aggressive approach toward managing the lesions has been favored. Once HCAs are detected, ultrasound or MRI is recommended every 3–6 months (Rake et al. 2002a, b), and intervention has been recommended when lesions are rapidly growing or reach 5 cm in diameter (Ault et al.

1996). HCA requires special attention during pregnancy due to risk of increased growth and rupture, especially when lesions are greater than 5 cm (Cobey and Salem 2004). It is appropriate to closely observe patients with HCA during pregnancy if lesions are less than 5 cm with frequent ultrasound surveillance every 6–12 weeks (Bröker et al. 2012). Due to the risk of adenoma growth during pregnancy, some investigators have proposed performing surgery, radiofrequency ablation, or embolization prior to pregnancy (Cobey and Salem 2004; Bröker et al. 2012), but intensive medical treatment offers another option for managing these patients.

Surgical resection of growing lesions has been successfully performed, but the procedure is not without risk due to the associated metabolic instability during surgery (Reddy et al. 2007). Radiofrequency ablation and embolization have also been utilized in treating HCAs. These methods are less invasive than surgery with decreased morbidity and mortality and have the ability to treat multifocal disease. However, these methods can be limited based on the location of the lesions (ablation), may need multiple treatments, may show inadequate response due to heat sink effect (ablation), and decreased efficacy with large (>5 cm) tumors (McDaniel et al. 2013; Rhim et al. 2008; Deodhar et al. 2011). Liver transplant cures the primary hepatic enzyme defect, but does not cure the disease (Davis and Weinstein 2008; Reddy et al. 2009; Maheshwari et al. 2012; Labrune 2002). The kidneys in people with GSD I are not normal, and most recipients of a liver transplant have progressed to renal failure (Matern et al. 1999). Medical management of growing HCAs has not been emphasized. We report successful stabilization and regression of HCAs with improved metabolic control, which may eliminate the need for surgical intervention in certain individuals.

The pathogenesis of HCA development is likely multifactorial. Prior work by Wang et al. (2011) demonstrates that poor metabolic control may play a role in HCA formation. In a case control cohort study, they found a statistically significant difference in the serum triglyceride concentration at the time of HCA discovery compared with age-matched control subjects with a mean triglyceride concentration of 737 mg/dL (SD ± 422) in the case group and 335 mg/dL (SD ± 195) in the control group. At the time of HCA presentation, our patient population had an average triglyceride level of 753 mg/dL (SD ± 293) and an average triglyceride level of 340 mg/dL (SD ± 164) at the time of HCA regression. Because some of the subjects in this study with HCAs are members of the study population that comprised the case–control study by Wang et al. (2011), it is not a surprise that the triglycerides in the HCA group prior to regression were similar. However, it is interesting that the triglyceride levels after regression of HCAs were similar to the control group without HCAs. Another observation that was made is that some patients had regression of HCAs even though triglyceride levels were not in the normal range. The goal with dietary therapy is to get triglyceride levels within normal range; however, reduction of triglyceride levels, even if suboptimal, may be sufficient for regression of HCAs. Within our group of patients, complete resolution occurred in patients with smaller lesions (<3.3 cm) whereas the larger lesions showed decrease in size. This suggests that intensive dietary therapy and optimization of biochemical control is more likely to result in complete resolution of HCAs before the HCAs become large. Genetic and chromosomal alterations may also play a role in HCA formation and progression, such as chromosomal aberration on chromosome 6 with simultaneous gain of 6p and loss of 6q. This may also explain why some HCAs increase in size in some patients with GSD I despite improving metabolic control (Kishnani et al. 2009).

There are a limited amount of cases in the literature documenting disappearance or reduction in the size of HCAs. Parker et al. 1981 demonstrated two cases of disappearance of HCAs and a third case of reduction in the size of a HCA in GSD type Ia patients with strict dietary therapy. In contrast to this prior report which depended upon Tc-99 m sulfur colloid liver-spleen scans, all of our patients were followed with serial MRI and ultrasound imaging over several years. In the cohort followed by the European Study on Glycogen Storage Disease Type I, three cases demonstrated remission of the HCAs, but no further details about these cases were reported (Rake et al. 2002a, b).

These findings suggest that if HCAs are documented in a patient with suboptimal metabolic control, intensive medical therapy should be considered before recommending surgical excision or liver transplantation. These observations add to the body of evidence showing that optimal metabolic control may delay, prevent, or reverse complications in patients with GSD I.

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Take Home Message

Hepatocellular adenomas can regress after metabolic control is achieved suggesting intensive medical therapy may be an alternative to surgical intervention in some individuals with GSD I.

Compliance with Ethics Guidelines

Conflict of Interest

Richard D. Beegle, Laurie M. Brown, and David A. Weinstein declare that they have no conflicts of interest.

Financial Disclosure

Richard D. Beegle, Laurie M. Brown, and David A. Weinstein have no financial relationships relevant to this article to disclose. The authors confirm independence from the sponsors. The authors confirm the content has not been influenced by the sponsors. The content does not financially affect the sponsors.

Other Relationships

There are no other relationships that present a potential conflict of interest.

Ethics Approval

Institutional review board approval and informed written consent was obtained from patients participating in an observational study at the University of Florida Glycogen Storage Disease Program.

IRB Approval Number: 533–2005

IRB Approval Name: Correlation of Markers of Metabolic Control with Long-Term Complications in Glycogen Storage Disease

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. There is no identifying information about patients in this article.

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

Richard Beegle: Involved in design of the project and analysis and interpretation of the data. Involved in interpretation of all radiological images. Drafted the revised manuscript and approved the final manuscript as submitted.

Laurie Brown: Involved in conception and design as well as data interpretation. Involved in data collection and review of medical charts. Coordinated all regulatory aspects of studies. Assisted in drafting the revised manuscript and approved the final manuscript as submitted.

David Weinstein: Involved in conception and design of studies, supervised the clinical and laboratory investigations, and involved in data collection, data interpretation, and analysis. Editor of manuscript and approved the final manuscript as submitted. Guarantor of the study and controlled decision to publish.

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RESEARCH REPORT

Proteasome Inhibitor Bortezomib Enhances the Activity of Multiple Mutant Forms of Lysosomal α-Glucosidase in Pompe Disease

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Abstract Pompe disease is an autosomal recessive myopathic disorder caused by the deficiency of lysosomal acid α-glucosidase (GAA). Recently, we showed that function of mutant GAA in fibroblasts derived from Pompe disease patient carrying c.546G>T mutation is improved by treatment with proteasome inhibitor bortezomib as well as pharmacological chaperone (PC). However, bortezomib-responsive GAA mutations are not fully characterized. In this study, we showed the effect of bortezomib on different mutants of GAA in patient fibroblasts and transiently expressed HEK293T cells. Bortezomib increased the maturation and residual activity of GAA in patient fibroblasts carrying PC-responsive M519V and PC-unresponsive C647W mutations. Enhanced colocalization of GAA with lysosomal marker LAMP2 was also observed in patient fibroblasts after treatment with bortezomib. When four distinct mutant GAAs, which show different response to PC, were overexpressed in HEK293T cells, bortezomib improved the activity of M519V, S529V, and C647W in them (1.3–5.9-fold). These results indicate that bortezomib enhances the activity of some PC-unresponsive GAA mutants as well as PC-responsive mutants.

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Introduction

Pompe disease (OMIM 232300) is an autosomal recessive lysosomal storage disorder arising from a deficiency of acid α-glucosidase (GAA), leading to progressive accumulation of glycogen in multiple tissues including skeletal and cardiac muscles (Hers 1963). The clinical presentation of Pompe disease spans a broad spectrum of severity, ranging from a rapid infantile-onset form to a slow late-onset form. The infantile-onset form displays a near complete loss of GAA activity and is characterized by generalized muscle weakness and severe cardiomyopathy, leading to premature death in the first year of life (van den Hout et al. 2003). The late-onset form typically displays some residual GAA activity and manifests as slowly progressive respiratory failure and muscle weakness without cardiac involvement (van der Ploeg and Reuser 2008). In 2006, enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) was approved for the treatment of Pompe disease. ERT extends life expectancy, improves cardiac muscle pathology and walking distance, and stabilizes pulmonary function in patients with Pompe disease (Kishnani et al. 2007; van der Ploeg et al. 2010), but the clearance of accumulated glycogen in skeletal muscle remains an ongoing challenge. ERT also facilitates the formation of anti-rhGAA antibodies that can reduce the efficacy of treatment (de Vries et al. 2010; Kishnani et al. 2010; Banugaria et al. 2011).

Recently, pharmacological chaperone (PC) therapy has emerged as a promising treatment for several lysosomal storage disorders including Pompe disease. PCs are small molecules that stabilize misfolded enzymes and prevent their degradation, thereby rescuing their intracellular trafficking and activity (Fan 2008). In Pompe disease, imino sugars such as deoxynojirimycin (DNJ) and *N*-butyl-DNJ (NB-DNJ) have

shown to improve the function of several mutant GAAs (Okumiya et al. 2007; Parenti et al. 2007; Flanagan et al. 2009). We previously reported that the proteasome inhibitor bortezomib as well as imino sugars can exert a positive effect on mutant GAA in fibroblasts from a patient with Pompe disease carrying the c.546G>T mutation (Shimada et al. 2011). In addition, bortezomib, which is a therapeutic drug for multiple myeloma, has recently shown to be a safe treatment to induce the effective reduction of anti-rhGAA antibody titers in patients with infantile Pompe disease who received ERT (Banugaria et al. 2013). Thus, bortezomib is attracting interest as a novel treatment for Pompe disease. However, it has not been fully characterized as an enzyme enhancement molecule that is effective for multiple GAA mutations.

In this study, we investigated the effect of bortezomib treatment on mutant GAAs in patient fibroblasts and transiently expressed cells.

Materials and Methods

Chemicals and Antibodies

Bortezomib was purchased from Toronto Research Chemicals Inc. (North York, Canada). Protease inhibitor cocktail (PIC) was obtained from Roche Diagnostics (Indianapolis, IN) Anti-GAA antibody was a gift from Genzyme Corporation (Cambridge, MA). Anti-LAMP2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Cell Signaling Technologies (Beverly, MA), and 4-methylumbelliferyl α-D-glucopyranoside and all other chemicals were obtained from Sigma (St. Louis, MO).

Cell Cultures

HEK293T cells and skin fibroblasts were maintained at subconfluent densities in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. The fibroblasts were obtained from Coriell Institute for Medical Research (Camden, NJ) or established in our laboratory after informed consent was obtained.

Western Blot Analysis

Protein samples for Western blot were prepared as previously described (Shimada et al. 2011). Briefly, fibroblasts were extracted with 50 mM Tris–HCl, pH 7.5 containing 2% SDS, and PIC. Cell lysates were resolved by SDS–PAGE on 4–20% acrylamide gradient gels and transferred onto a nitrocellulose membrane. The membranes were blocked with 50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl, 0.1% gelatin, 0.1% casein, and 0.05% Tween 20, and then incubated with each primary antibody. After a brief washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Nichirei Corp., Tokyo, Japan) and detected using Immunostar LD (Wako Pure Chemicals, Tokyo, Japan).

Immunocytochemistry

Immunofluorescence staining against both GAA and LAMP2 was performed according to a procedure previously described (Shimada et al. 2011). Fibroblasts were incubated with the primary antibodies and visualized by using Alexa 488-conjugated anti-rabbit IgG and Alexa 594-conjugated anti-mouse IgG secondary antibodies (Invitrogen, Carlsbad, CA) followed by counterstaining with DAPI (Vector Laboratories).

Site-Directed Mutagenesis and Expression of Mutated GAA in HEK293T Cells

Human GAA cDNA was purchased from OriGene Technologies (Rockville, MD). Site-directed mutagenesis was performed to introduce mutations into the GAA gene according to the manufacturer's instructions (Toyobo, Tokyo, Japan), and the mutant GAA was subcloned into pCMV-Script (Stratagene, Cedar Creek, TX). HEK293T cells were transiently transfected with 1 μg wild-type and mutant cDNA using X-tremeGENE 9 (Roche Diagnostics) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were incubated with 10 nM bortezomib for 24 h. The cells were harvested and lysed with water by sonicating on ice for the GAA activity assay.

Quantitative Analyses of Proteins and GAA Activity

Protein concentrations were estimated with the DC protein assay (Bio-Rad, Hercules, CA) standardized with bovine serum albumin. GAA activities in fibroblasts and HEK293T cells were assayed using 4-methylumbelliferyl α-D-glucopyranoside as previously described (Shimada et al. 2011).

Results

Bortezomib Improves the Activity of Several Mutant GAAs in Fibroblasts from Patients with Pompe Disease

To investigate the effect of treatment with bortezomib on mutant GAAs, we used 3 cell lines carrying C647W/C647W (P1 cells), M519V/c.1326+1G>A (P2 cells), and S619R/S619R (P3 cells) mutations, respectively. Patient fibroblasts were treated with bortezomib (100 nM) for 24 h followed by analysis of GAA activity in cell lysate.

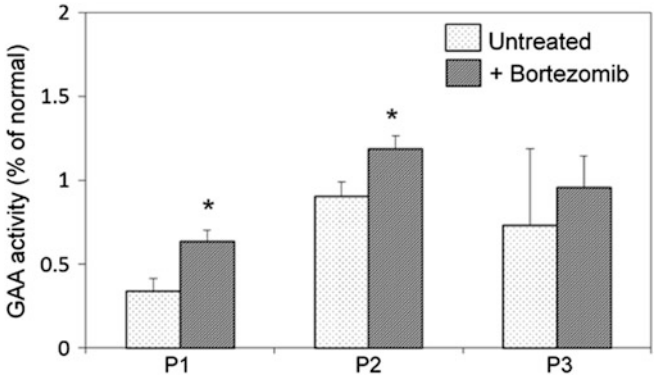

Fig. 1 Enzyme activity of acid α-glucosidase (GAA) in patient fibroblasts treated with bortezomib. Patient fibroblasts (P1, C647W/C647W; P2, M519V/c.1326 + 1G > A; P3, S619R/S619R) were cultured for 24 h with or without 100 nM bortezomib. Fibroblasts were solubilized with ultrapure water, and the activity of GAA was measured in triplicate. GAA activity values in the histograms are expressed as percentage of normal. Statistical analysis of data was performed by Student's *t*-test (*: $p < 0.05$)

P1 and P2 cells showed significant increases in residual GAA activity (1.9-fold and 1.3-fold, respectively), but P3 cells showed no change in activity (Fig. 1). Given that c.1326+1G>A mutation in P2 cells is known to result in lack of GAA mRNA (Raben et al. 1999), this result suggests that M519V and C647W respond to bortezomib but that S619R does not respond to it.

Bortezomib Improves the Maturation and Intracellular Localization of Mutant GAAs in Patient Fibroblasts Carrying M519V and C647W Mutations

To determine whether bortezomib improves the maturation of mutant GAAs in patient fibroblasts, we evaluated the levels of GAA in these cells using Western blotting with anti-GAA antibody. Whereas only the 110-kDa precursor forms were observed in P1 fibroblasts, increased levels of 110-kDa and 95-kDa intermediate and 76-kDa mature forms were observed in them after treatment with bortezomib (Fig. 2). In P2 fibroblasts, mature forms were detected even under basal conditions, but treatment with bortezomib increased the levels of 76-kDa GAA in them (Fig. 2). In contrast, although levels of 95-kDa forms were slightly increased in P3 cells treated with bortezomib, mature GAA forms were not detected in these cells (data not shown).

Next, to determine whether bortezomib improved the trafficking of GAA containing M519V and C647W mutations to the lysosome, we analyzed the subcellular localization of GAA in patient fibroblasts by immunocytochemistry. Most GAA fluorescence did not overlap LAMP2 signal in P1 and P2 fibroblasts, but GAA was colocalized with LAMP2 in these patient fibroblasts after treatment with bortezomib (Fig. 3). These results indicate that bortezomib improves the maturation and lysosomal trafficking of mutant GAAs in patient fibroblasts carrying M519V and C647W mutations.

Bortezomib Increases the Activity of Several Mutant GAAs in HEK293T Cells

The function of the M519V mutant is rescued by treatment with DNJ, whereas the C647W mutant does not respond to this compound (Flanagan et al. 2009). We hypothesized that bortezomib has a spectrum of GAA mutation different from that of DNJ. To test this hypothesis, we generated four different GAA mutants (DNJ-responsive mutants, M519V and S529V; DNJ-nonresponsive mutants, S619R and C647W) and transiently transfected them into HEK293T cells. M519V, S529V, S619R, and C647W mutants showed 4.7%, 9.0%, 1.8%, and 0.23% wild-type activity, respectively (Table 1). Treatment with bortezomib significantly increased the activities of three mutants (M519V by 1.3-fold, S529V by 1.3-fold, and C647W by 5.9-fold increase), but not of S619R (Table 1). Increased levels of GAA activities were also observed in HEK293T cells expressing wild-type GAA (1.1-fold increase) (data not shown). This result indicates that bortezomib can improve the enzyme activity of some GAA mutants regardless of response to DNJ.

Discussion

We previously showed that bortezomib rescues the function of GAA in fibroblasts from a patient with Pompe disease carrying the c.546G>T mutation (Shimada et al. 2011). However, the responsiveness of almost all GAA mutations to bortezomib has not been reported. In this study, we demonstrated that treatment with bortezomib increases the activities of several GAA mutants such as M519V, S529V, and C647W in patient fibroblasts or transiently expressed HEK293T cells (Fig. 1, Table 1). Additionally, we showed the increased lysosomal localization of GAA in fibroblasts

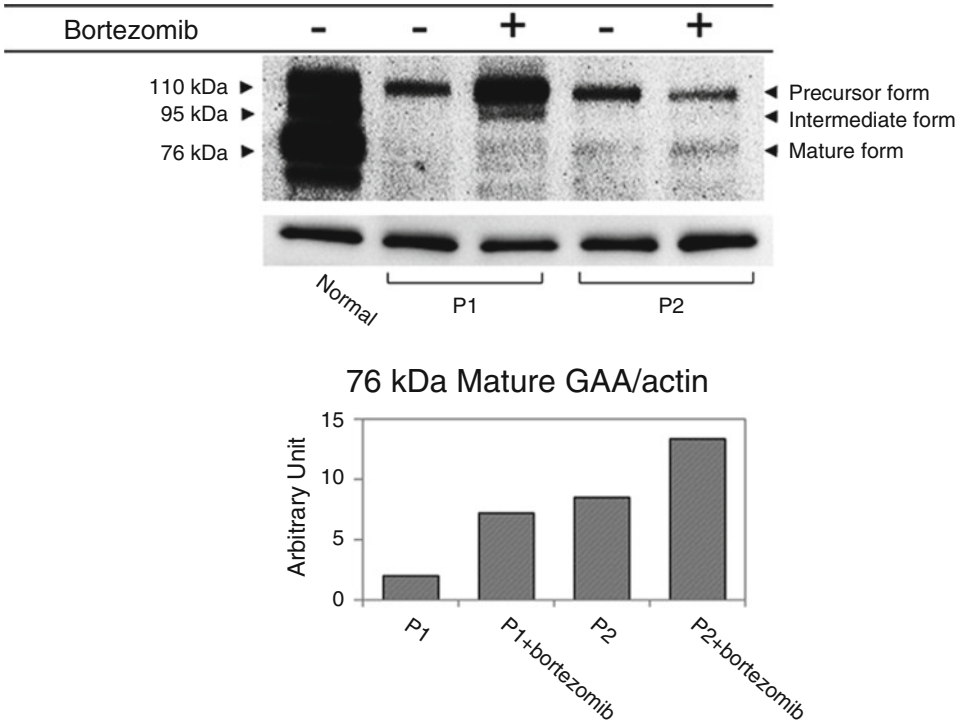

Fig. 2 Acid α-glucosidase (GAA) in Pompe disease patient fibroblasts treated with bortezomib. Patient fibroblasts (P1, C647W/C647W; P2, M519V/c.1326+1G > A) were cultured for 24 h with or without 100 nM bortezomib. Cell lysates from normal and patient fibroblasts were electrophoresed followed by immunoblotting using antibodies against GAA and actin. The band intensities of 76-kDa GAA and actin were quantified by densitometry and normalized to actin

from patients with Pompe disease carrying M519V and C647W mutations (Fig. 3). Our findings provide new evidence that bortezomib improves the function of GAA for at least three different missense mutants.

Bortezomib is a boronic acid dipeptide that binds to the chymotrypsin-like β5 subunit of 20S proteasome and is the first proteasome inhibitor approved by US Food and Drug Administration for treatment of multiple myeloma and mantle cell lymphoma (Richardson et al. 2003). Because proteasomal degradation of ubiquitin-protein conjugates is essential for diverse cellular processes such as cell cycle progression, regulation of gene expression, and quality control of misfolded proteins in ER, bortezomib is proposed to have therapeutic potential for various diseases including genetic diseases (Elliott and Ross 2001; Araujo et al. 2013).

PC therapy in Pompe disease is based on the binding of imino sugars such as DNJ to mutant GAAs, enhancing their stability and trafficking (Okumiya et al. 2007; Parenti et al. 2007; Flanagan et al. 2009). In contrast, treatment with bortezomib is based on the concept that proteasome inhibitors cause inhibition of proteasomal degradation and induction of intracellular chaperones including Hsp40, Hsp70, Hsp90, and Bip, thereby enhancing interaction between GAA mutants and molecular chaperones (Mu et al. 2008). It thus appears that the spectrum of bortezomib-responsive mutated residues is different from that of imino sugar-responsive mutations. Indeed, we showed that bortezomib enhances the activities of the DNJ-unresponsive C647W mutant in patient fibroblasts and HEK293T cells (Fig. 1; Table 1). We also demonstrated that the function of DNJ-responsive M519V and S529V mutants is improved by treatment with bortezomib, indicating that proteasome inhibitor is applicable to some imino sugar-nonresponsive mutations as well as to imino sugar-responsive mutations. This notion is also supported by our previous report that bortezomib improved the function of GAA in patient fibroblasts carrying the NB-DNJ-responsive c.546G>T mutation (Shimada et al. 2011).

Our results showed that although several mutants including S529V are responsive to bortezomib, S619R is unresponsive to it (Table 1). According to the GAA structural model, whereas both S529 and S619 are located in the $(\beta/\alpha)_8$-barrel domain, mutations of these residues have distinct effects on GAA conformation (Flanagan et al. 2009; Tajima et al. 2011). An amino acid substitution of serine with valine at position 529 is suggested to induce a moderate structural change adjacent to the surface of the enzyme far from the active site (Tajima et al. 2011). In contrast, amino acid substitution of serine with arginine at

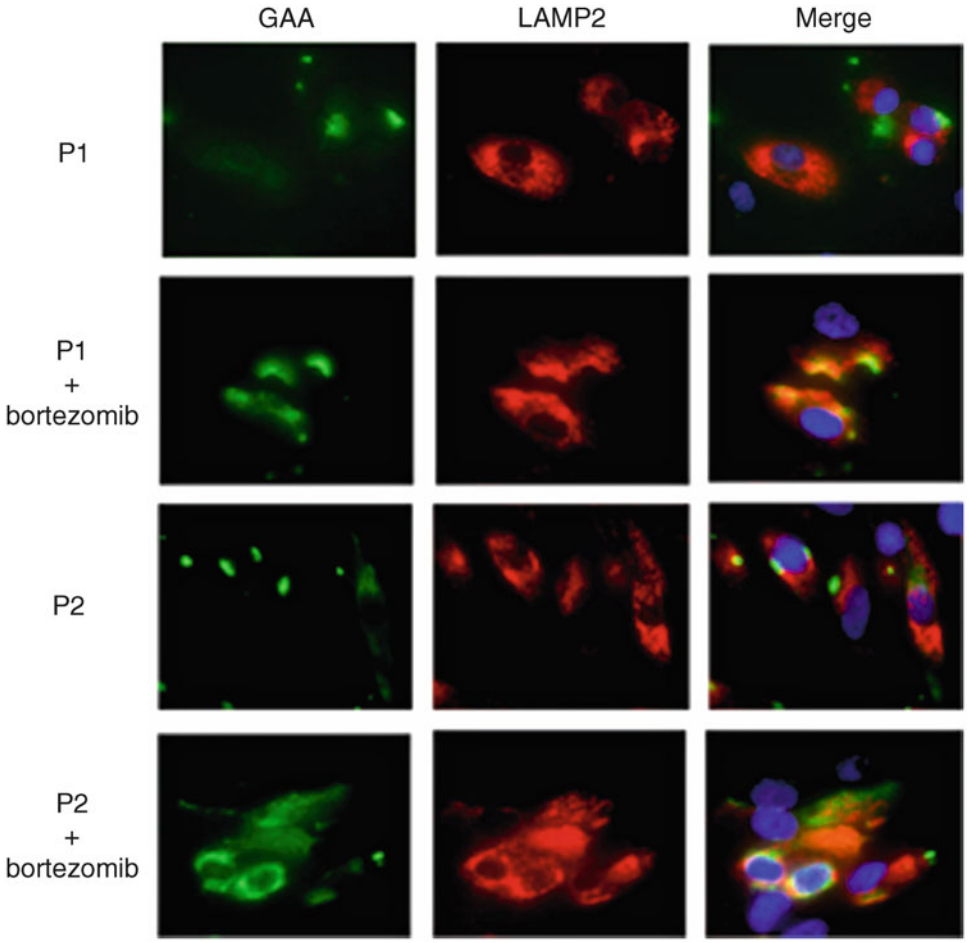

Fig. 3 Subcellular localization of acid α-glucosidase (GAA) in patient fibroblasts treated with bortezomib. Patient fibroblasts (P1, C647W/C647W; P2, M519V/c.1326+1G>A) were grown on cover slips with or without 100 nM bortezomib for 24 h. Cells were fixed and stained with anti-GAA antibody (*green*) and anti-LAMP2 antibody (*red*). Nuclei were counterstained with DAPI (*blue*)

Table 1 Effect of bortezomib on enzyme activities in HEK293T cells expressing acid α-glucosidase (GAA) mutations

	GAA activity (% of wild type)		
GAA mutation	Untreated	+ Bortezomib	Fold increase
M519V	4.7 ± 0.35	6.1 ± 0.67*	1.3
S529V	9.0 ± 0.80	12.0 ± 1.54*	1.3
S619R	1.8 ± 0.71	0.80 ± 0.75	–
C647W	0.23 ± 0.20	1.3 ± 0.48*	5.9

GAA activity values are expressed as percentage of wild type after background subtraction and normalization. Data represent mean ± standard deviation of independent triplicate or quadruplicate samples. Statistical analyses of data were performed by Student's *t*-test (*: $p < 0.05$ compared with untreated)

position 619 is suggested to lead to a large conformational change near the active site pocket of GAA (Tajima et al. 2011). It thus appears that bortezomib is not effective for GAA mutations that induce large conformational perturbations in the enzyme structure.

ERT with rhGAA is the only approved treatment for Pompe disease and shows remarkable success in terms of prolonged survival and improved clinical manifestations such as in cardiac muscle pathology in infantile patients (Kishnani et al. 2007). However, as the formation of antibodies against rhGAA reduces the efficacy of treatment (de Vries et al. 2010; Kishnani et al. 2010; Banugaria et al. 2011), immune response to the infused enzyme remains of high concern. Recently, Banugaria et al. showed that immunomodulatory regimens incorporating bortezomib

are effective for the reduction of high sustained anti-rhGAA IgG antibody titers with no obvious side effects in infantile Pompe disease patients (Banugaria et al. 2013). In this study, we showed that bortezomib represents an effective drug for improving the function of several mutants in patient fibroblasts and HEK293T cells (Figs. 1, 2, and 3; Table 1), suggesting that treatment with bortezomib is a promising approach providing both induction of immune tolerance and enhancement of enzyme effects in patients with Pompe disease carrying certain mutations.

In summary, our results indicate the potency of bortezomib as an enzyme enhancement molecule for Pompe disease patients carrying certain missense mutations.

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Synopsis

Bortezomib improves the function of multiple GAA mutants in Pompe disease.

Compliance with Ethics Guidelines

Conflict of Interest Disclosures

Yoshikatsu Eto has received grant support from Genzyme Corporation.

Hiroyuki Ida has received grant support from Genzyme Corporation.

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Yohta Shimada, Erica Nishimura, Hiroo Hoshina, Hiroshi Kobayashi, and Takashi Higuchi declare that they have no conflict of interest.

These activities have been fully disclosed and are managed under a Memorandum of Understanding with the Conflict of Interest Resolution Board of the Jikei University School of Medicine.

Informed Consent

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

Animal Rights

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

Contribution

Y.S. and T.O. designed and conducted research; Y.S., E.N., and H.H. performed experiments and analyzed data; Y.S. wrote the paper; and H.K., T.H., Y.E., H.I., and T.O. discussed data and edited the paper.

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RESEARCH REPORT

Cognitive Function in Adults Aging with Fabry Disease: A Case–Control Feasibility Study Using Telephone-Based Assessments

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Abstract We examined the feasibility of recruiting US adults ≥45 years old with Fabry disease (FD) for telephone assessments of cognitive functioning. A case–control design matched each FD participant on age, sex, race, and education to four participants from a population-based study. Fifty-four participants with FD age 46–72 years were matched to 216 controls. Standardized cognitive assessments, quality of life (QOL), and medical histories were obtained by phone, supplemented by objective indices of comorbidities. Normalized scores on six cognitive tasks were calculated. On the individual tasks, scores on list recall and semantic fluency were significantly lower among FD participants (p-values < 0.05), while scores on the other four tasks did not differ. After averaging each participant's normalized scores to form a cognitive composite, we examined group differences in composite scores, before and after adjusting for multiple covariates using generalized estimating equations. The composite scores of FD cases were marginally lower than controls before covariate adjustments ($p = 0.08$). QOL and mental health variables substantially attenuated this finding ($p = 0.75$), highlighting the influence of these factors on cognition in FD. Additional adjustment for cardiovascular comorbidities, kidney function, and stroke had negligible impact, despite higher prevalence in the FD sample. Telephone-based cognitive assessment methods are feasible among adults with FD, affording access to a geographically dispersed sample. Although decrements in discrete cognitive domains were observed, the overall cognitive function of older adults with FD was equivalent to that of well-matched controls before and after accounting for multiple confounding variables.

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Competing interests: None declared

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Introduction

Fabry disease (FD) is a rare X-linked lysosomal storage disorder with potential impact on cognitive function. Deficient alpha-galactosidase A results in accumulation of globotriaosylceramide and related glycosphingolipids in cardiomyocytes and in vascular endothelial, neural, and renal cells, leading to tissue remodeling, fibrosis, ischemia, and potential end-organ damage affecting the heart, kidneys, and brain (Desnick et al. 2001), although phenotypic variations are striking (Wilcox et al. 2008).

Reports of cognitive difficulties are not uncommon among persons with FD (Segal et al. 2010; Low et al. 2007), but assessment of cognitive function has been limited. Samples available for clinical cognitive assessments have been small, spanning a broad age spectrum. While some researchers have found a pattern of below average scores in discrete cognitive abilities (Elstein et al. 2012), others have found no pattern of deficits (Segal et al. 2010; Low et al. 2007).

There are multiple pathways whereby cognitive dysfunction might occur. Transient ischemic attacks and strokes are important causes of cognitive impairment and debilitation in FD (Desnick et al. 2001). In the absence of clinical events, cognitive decrements could occur due to nonspecific cerebrovascular changes (Elstein et al. 2012; Moore et al. 2007). Hearing loss also can affect cognitive processing and correlates with neuropathic and vascular damage in FD (Ries et al. 2007). White matter changes also are important findings; however, to date, the relation of brain abnormalities to described minor changes in cognitive function in FD is not compelling (Moore et al. 2007; Muller et al. 2005; Schermuly et al. 2011; Fellgiebel et al. 2012). Chronic distress associated with FD may have more influence on cognitive function than do pathologic alterations in the central nervous system (Segal et al. 2010; Schermuly et al. 2011).

We conducted a pilot study to examine the feasibility of telephone-based cognitive assessments as a means of expanding access to persons with FD. Such methods previously have been found to be reliable and precise (Unverzagt et al. 2007). We focused on persons middle-aged and older to represent patients experiencing a prolonged lifespan attributable to improved renal transplants and the availability of enzyme replacement therapies (ERT). We capitalized on telephone assessment technologies being used in the Reasons for Geographic and Racial Differences in Stroke (REGARDS) population-based cohort study (Howard et al. 2005) and used a subset of that cohort as age-, sex-, race-, and education-matched controls.

We expected that the cooperation rate of FD patients would be higher than that of the REGARDS cohort due to differences in the samples and recruitment methods and that the cognitive assessment would yield usable data even among patients using assistive hearing devices. We hypothesized that FD participants would perform worse than REGARDS control participants on a cognitive composite and component tests of learning, memory, and executive function. We predicted that controlling for effects of quality of life (QOL), perceived stress, depressive symptoms, income, cardiovascular disease, kidney function, and stroke would attenuate group differences in cognitive function. These variables have been associated with cognitive function in REGARDS (Kurella Tamura et al. 2011; Unverzagt et al. 2011; Addison-Brown et al. 2014).

Methods

Participants and Procedures

Participants with Fabry Disease

Participants with FD were enrolled from September 2009 through May 2011. They were recruited primarily through Fabry Registry physicians' offices and secondarily through two patient websites. Community-dwelling, English-speaking men and women with FD $\geq$ 45 years old who were able to communicate by telephone were eligible, whether treated or untreated with ERT. Neither participants nor physicians were provided with incentives for enrollment.

Interested patients called a toll-free number at the Edward R. Roybal Center for Translational Research on Aging and Mobility at the University of Alabama at Birmingham (UAB) to schedule a telephone assessment of demographics, cognitive function, perceived stress, depressive symptoms, and QOL. Verbal informed consent was obtained, and assessments were conducted by the Survey Research Unit (SRU) in the School of Public Health at UAB using standardized scripts used in the REGARDS study. Written informed consent and medication logs were executed by mail.

In October 2012, we recontacted FD participants by phone for self-reported cardiovascular risk factor histories, and we gathered by mail an addendum consent form granting permission to obtain objective medical information from (1) the Fabry Registry, using Registry IDs released by Registry physicians, or (2) from non-Registry physicians primarily responsible for patients' Fabry disease care.

Control Participants

Control participants were drawn from the database of the REGARDS study, a population-based study designed to examine geographic and racial differences in stroke and

cognitive decline (Howard et al. 2005; Wadley et al. 2011). REGARDS participants ($N = 30{,}239$) were recruited from January 2003 to October 2007 through mailings followed by telephone contacts. Eligibility criteria included age ≥45, self-reported race of black or white, English-speaking, residing in the community, no life-limiting illness, and able to communicate by telephone. Following verbal informed consent, an interview assessing demographics, perceived stress, depressive symptoms, QOL, and cardiovascular risk factors was conducted by telephone, followed by a home visit including written informed consent, blood pressure measurement, blood and urine samples, and an electrocardiogram (ECG) that was centrally read. The SRU began conducting telephone-based cognitive assessments of learning, memory, and executive function between 2006 and 2009.

The same personnel, custom computer programs, standardized cognitive assessment and scoring techniques, and abbreviated mental health scales were used in both FD patient and REGARDS control samples. Each telephone assessment lasted less than 30 min on average.

Each enrolled FD participant was matched on age (±2.5 years), education level, sex, and race to 4 REGARDS participants randomly selected from the pool of all potential matches in the REGARDS database. The 4:1 matching scheme was selected to provide stable estimates and adequate power to detect group differences in overall cognitive function after adjustment for covariates. For the present comparison of cognitive function of persons with FD to matched control participants, only initial assessments of REGARDS participants' cognitive function were used.

Measures

A detailed account of study measures, administration, and scoring is available in the Appendix. The measures are listed here only briefly.

Age, sex, race, education, and income were self-reported. Cognitive function was assessed with six tasks drawn from the National Institute of Neurological Disorders and Stroke–Canadian Stroke Network (NINDS-CSN) 5-minute battery (Hachinski et al. 2006) and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) battery (Morris et al. 1989). Learning, memory, and executive function were assessed with CERAD *word list learning (WLL)* and *word list delayed recall (WLDR)*, NINDS-CSN *5-item recall* and *6-item orientation* (Hachinski et al. 2006; Morris et al. 1989; Nasreddine et al. 2005), *letter fluency (letter F)*, and *semantic fluency* (Hachinski et al. 2006; Morris et al. 1989). Health-related QOL was measured using a modified interviewer script version of the Short Form-12 (SF-12; Quality Metrics) survey (Ware et al. 1996). Perceived stress was measured with the 4-item Perceived Stress Scale (PSS-4; Cohen et al. 1983). The Center for Epidemiologic Studies Depression Scale – 4-item version (CES-D-4; Melchior et al. 1993) – was used to evaluate depressive symptoms. A combination of self-report and objective assessments was used to ascertain cardiovascular comorbidities of hypertension, diabetes, dyslipidemia, and heart disease, as well as kidney function and history of stroke.

Statistical Approach

The cooperation rate of the FD sample was calculated (Morton et al. 2006), and the feasibility of the telephone-based assessment approach was assessed via interviewer ratings of participants' hearing, comprehension, and compliance with the assessment. Usable data from at least 85% of FD participants was established a priori as the threshold for acceptable feasibility.

Scores on each cognitive measure were converted to *z*-scores based on the distributions observed in the combined sample of REGARDS and FD participants. The average *z*-score across available measures was calculated to form an overall cognitive composite score for each participant. Composite scores of FD participants were compared to those of REGARDS participants after adjustment for potential confounding variables using linear regression.

Baseline characteristics of the two samples were compared using generalized estimating equation (GEE) models in matched analyses. A compound symmetry covariate structure was fitted to determine whether the cognitive composite *z*-score of FD participants differed from that of REGARDS participants before and after adding covariate groups (QOL and mental health, income, cardiovascular comorbidities, kidney function, stroke history).

Results

Participant Characteristics, Cooperation Rates, and Feasibility

Fifty-six participants were screened for the FD sample; one was ineligible due to severe hearing difficulties and one due to comprehension difficulties unrelated to hearing. The final FD sample included 54 participants (45 who learned about the study from Fabry Registry physicians' offices and nine from patient websites). Four clinicians participating in the Fabry Registry of North America (authors Sims, Warnock, Hopkin, and Laney) contributed the majority of study enrollees. After matching each patient with FD to four REGARDS participants, the full study sample consisted of 270 participants (54 with FD, 216 controls).

Table 1 presents the baseline characteristics of each population. By design, the matching variables of race, sex,

Table 1 Baseline factors and cognitive scores for participants with Fabry disease and controls

	n	Fabry disease ($n = 54$)	*n*	REGARDS controls ($n = 216$)	*p*-value
Age M (SD)	54	55.7 (6.7)	216	56.0 (6.6)	0.074
White race *n* (%)	54	53 (98)	216	212 (98)	0.99
Men *n* (%)	54	17 (31)	216	68 (31)	0.99
Education *n* (%)	54		216		0.99
<HS (%)		2 (4)		8 (4)	
HS grad (%)		8 (15)		32 (15)	
Some college (%)		16 (30)		64 (30)	
College grad (%)		28 (52)		112 (52)	
Quality of life M (SD)	53		214		
SF-12 MCS score[a,b]		47.7 (11.1)		53.1 (9.0)	0.002
SF-12 PCS score[a,b]		38.0 (11.4)		48.0 (9.9)	0.001
Perceived stress, PSS-4 score[a,b] M (SD)	53	6.4 (2.3)	214	3.2 (2.7)	<0.0001
Elevated depressive symptoms CES-D-4[b] *n* (%)	54	15 (28)	214	21 (10)	0.007
Income *n* (%)	54		216		0.33
<$20 K (%)		3 (6)		26 (12)	
$20 K–$34 K (%)		10 (19)		54 (25)	
$35 K–$74 K (%)		20 (37)		67 (31)	
>=$75 K (%)		15 (28)		54 (25)	
Refused (%)		6 (11)		15 (7)	
Systolic blood pressure[c] M (SD) (mmHg)	17	120 (17)	216	120 (15)	0.96
Diastolic blood pressure[c] M (SD) (mmHg)	16	74 (8.9)	216	76 (11.0)	0.96
Hypertension *n* (%)	54	38 (70)	216	81 (38)	<0.0001
Diabetes[d] *n* (%)	54	5 (9)	213	24 (11)	0.56
Heart disease[d] *n* (%)	54	31 (57)	206	36 (17)	<0.0001
Atrial fibrillation[c,d] *n* (%)	38	17 (45)	206	10 (5)	<0.0001
LVH[c,d] *n* (%)	26	19 (73)	81	2 (2)	<0.0001
Dyslipidemia[d] *n* (%)	54	38 (70)	211	102 (47)	0.0044
Total cholesterol[c,d] M (SD) (mg/dL)	26	180 (42)	210	201 (36)	0.021
LDL[c,d] M (SD) (mg/dL)	26	96 (30)	210	119 (31)	0.0006
HDL[c,d] M (SD) (mg/dL)	26	63 (22)	210	53 (17)	0.013
Triglycerides[c,d] M (SD) (mg/dL)	26	102 (56)	211	152 (69)	0.0019
Kidney function, eGFR ml/min/1.73 m^{2}[c,d] M (SD)	27	63 (26)	211	85 (20)	0.0001
History of stroke[e] *n* (%)	54	6 (11)	216	10 (5)	0.071

REGARDS stands for the Reasons for Geographic and Racial Differences in Stroke study

Four controls were randomly selected from all possible REGARDS participants who could be matched to each Fabry disease study participant on age (±2.5 years), race, sex, and education

Variables in bold print were used in subsequent generalized estimating equation models

Missing data:

[a] 1 FD ppt refused to answer these questions

[b] 2 REGARDS ppts refused to answer these questions

[c] Missing data for FD ppts on these variables was due to lack of available information in the Fabry Registry or physicians' medical records

[d] Missing data for REGARDS ppts on these variables was due to missing or inadequate blood samples or ECG data

[e] 14 FD ppts responded "don't know" regarding self-reported stroke and had no stroke history documentation; these ppts were classified as "no stroke"

and education did not differ between groups; mean age differed only marginally. FD participants were 46–72 years old; matched REGARDS participants were 45–74 years old. FD participants had higher prevalence of elevated depressive symptoms, higher perceived stress, and lower QOL than controls. Thirty-two FD participants were using

Table 2 Raw cognitive scores and normalized *z*-scores

	Fabry disease ($n = 54$)		REGARDS controls ($n = 216$)	
	Raw score	*Z*-score (SD)	Raw score (SD)	*Z*-score (SD)
Word list learning	19.4 (4.7)	−0.17 (0.9)	20.2 (5.0)	0.0 (1.0)
Word list delayed recall	6.6 (2.1)	−0.43 (1.0)	7.4 (2.0)	0.0 (1.0)
NINDS-CSN recall	4.4 (1.1)	−0.05 (1.2)	4.4 (0.9)	0.0 (1.0)
NINDS-CSN orientation	5.7 (0.6)	−0.08 (1.3)	5.8 (0.5)	0.0 (1.0)
Semantic fluency	17.6 (5.7)	−0.29 (0.8)	19.7 (7.4)	0.0 (1.0)
Letter fluency	12.8 (5.4)	0.24 (1.3)	11.8 (4.3)	0.0 (1.0)
Average *Z* composite score	–	−0.16 (0.7)	–	0.0 (0.6)

REGARDS stands for the Reasons for Geographic and Racial Differences in Stroke study
Numbers missing for FD participants: word list learning, 3; word list recall, 3; NINDS-CSN recall, 1; NINDS-CSN orientation, 0; semantic fluency, 4; letter fluency, 4; and average *Z* composite, 0. No REGARDS participants were missing cognitive data

ERT (agalsidase beta at 1 mg/kg) at the time of the cognitive assessment.

Cooperation rate (number enrolled divided by number contacted and eligible) for FD participants from the Fabry Registry was 74% (45 enrollees from a pool of 61 Registry patients who were invited and deemed eligible). As expected, this cooperation rate was higher than the 49% cooperation rate of the REGARDS epidemiological population study (Wadley et al. 2011). We were not able to include in the cooperation rate calculation the nine enrollees with FD recruited from patient websites, due to an unknown number of potentially eligible persons who may have viewed the study information but did not contact us.

With respect to feasibility of telephone-based assessments within the FD participants, the mean time for interview completion was 30.0 min (for men, the mean time was 29.2 min [range 12–48], and for women the mean time was 30.9 min [range 18–48]). The REGARDS participant telephone interviews were shorter by 6 min on average because in that study, the verbal consent process and medication inventory were done at baseline visits prior to the cognitive assessments. Interviewer ratings revealed one participant with moderate hearing loss and one displaying modest motivation/effort. Most of the data provided by these two participants was considered usable, but selected cognitive tests affected by the identified issues were set to missing. Interviewer error or equipment malfunction resulted in missing data on a subset of tests for two additional participants. Thus, all FD participants (100%) provided sufficient usable cognitive data for calculating the overall cognitive composite, exceeding the feasibility threshold of 85%. Applying a more rigorous standard of usable and complete data on all cognitive tests, feasibility remained acceptable at 92.5% (50 of 54 participants).

Cognitive Function Analyses

Table 2 presents the two groups' raw scores and *z*-scores for each of the cognitive tests and their average *z*-score composites. Figure 1 displays the pattern of *z*-scores and 95% confidence intervals of FD participants on each test and overall, based on the means and SDs of the full sample. Mean z-scores of participants with FD were significantly lower than controls on WLL delayed recall and semantic fluency tasks and did not differ from the other tasks or the overall composite.

Table 3 presents GEE results examining group differences between average *z*-score composites, with *p*-values for tests of whether each difference score is greater than zero. Model 1 presents the univariate analyses. Models 2 through 6 present the multivariable analyses, with each subsequent model including all variables from the prior models. Model 2 added QOL and mental health factors (PCS, MCS, PSS-4, CES-D-4). Model 3 added income. Model 4 added cardiovascular comorbidities that differed between FD and REGARDS participants in univariate analyses. Model 5 added kidney function (eGFR). Model 6 added stroke history. The two cohorts differed marginally on the overall cognitive composite ($p = 0.08$) prior to consideration of covariates. This effect was attenuated after accounting for QOL and mental health variables ($p = 0.75$) and remained nonsignificant after the entry of each subsequent group of covariates.

Due to possible sex differences in cognitive function, particularly among FD patients, we performed post hoc analyses using unadjusted GEE models to test the interaction between group (FD vs. REGARDS) and sex for each cognitive measure. These interactions were nonsignificant for all measures (*p*-values from 0.19 to 0.79), indicating that score variations due to sex were no different for the FD sample than for the control sample.

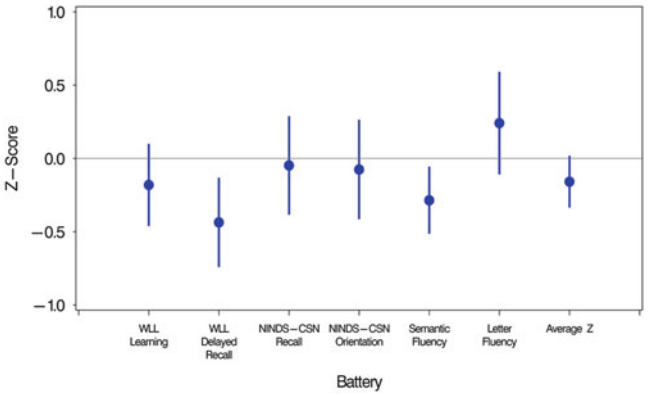

Fig. 1 *Z*-scores of participants with Fabry disease relative to matched controls by cognitive task and overall composite. *Z*-scores (mean = 0, SD = 1.0) on the *y*-axis were computed from raw score means and SDs of the full sample available for each cognitive task (Ns range from 266 to 270) and the average composite *z*-score ($N = 270$). *Error bars* represent 95% confidence intervals

Discussion

We examined cognitive function in middle-aged and older adults with Fabry disease using matched controls and telephone-based assessments. Our results suggest that telephone-based cognitive assessment methods are feasible among many patients with Fabry disease. All participants with FD provided usable data on the primary cognitive composite outcome, and over 90% provided complete data on the cognitive battery. Importantly, we failed to establish a significant difference in overall cognitive function among FD participants relative to controls matched on sex, education, age, and race. A trend for lower cognitive functioning in FD participants was substantially attenuated by adjustment for the expected differences between groups in QOL and depression measures, even prior to accounting for key medical comorbidities. Our matched sample design and adjustment for multiple comorbidities and confounders suggest that this null finding with respect to global cognition is fairly robust.

Among eligible potential participants who learned about the study from their Fabry Registry physicians' offices, 74% enrolled in the study. It is possible that enrollment could be enhanced in future studies with careful consideration of incentives, including provision of individual feedback to participants and providers. Even so, to our knowledge, our FD sample is the largest to participate in a study of cognitive function and is also the oldest, with a mean age of 56 years and a range of 46–72 years.

Our finding of no difference in composite cognitive performance in participants with FD compared to well-matched control participants is similar to that of Fellgiebel et al. (2012). These investigators enrolled 25 FD patients and 20 age-matched controls in a neuroimaging study that included measures of learning and memory. Although patients with FD had significantly smaller hippocampal volumes than controls, memory performance was not associated with hippocampal volume, and patients did not differ from controls on cognitive measures. Similarly, Low et al. (2007) conducted a neuroimaging study in 22 FD patients aged 20–62 years. They demonstrated increased lesion prevalence with age but no differences in cognitive performance compared to normative data on two global screening instruments. While it is possible that cognitive decrements may be too subtle to detect in small patient samples, our results in a somewhat larger sample with a large demographically matched control group are consistent with prior research reporting no global deficits and are strengthened after accounting for key variables related to cognitive function.

Although our global composite did not differ between participants with FD and controls, it is of interest to note performance patterns on the component cognitive tasks. On the individual tests, FD participants' performance was lower than controls on 5 of the 6 tasks and significantly lower on delayed recall and semantic fluency tasks representing memory and executive function. Mild but diffuse cognitive impairments on subdomains of a computerized cognitive battery have previously been reported (Elstein et al. 2012), as have marginal differences between FD patients and controls on subdomain scores of the Neuropsychiatry Unit Cognitive Screen (Low et al. 2007). Semantic fluency and memory deficits are characteristic of Alzheimer's disease and Parkinson's disease (Henry et al. 2004; Henry and Crawford 2004), and white matter lesions contribute independently to memory impairment in preclinical dementia (Grambaite et al. 2011). Thus, linkages between semantic fluency and memory, white matter lesions, and dementia risk in FD warrant future investigation.

Disease-related factors help explain variability in cognitive performance among patients with FD. We found greater prevalence of elevated depressive symptoms, higher ratings of perceived stress, and lower ratings of QOL in patients with FD compared to controls. Twenty-eight percent of our FD sample screened positive for depressive symptoms on the 4-item CES-D, consistent with survey findings using the full CES-D (Cole et al. 2007). Likewise, lower QOL in both mental and physical domains is consistent with previous reports (Low et al. 2007). Prior research found that mild deficits in executive function among 25 patients with FD compared to 20 controls were no longer significant after controlling for depression (Schermuly et al. 2011). In our study, controlling for depressive symptoms, perceived stress, and QOL variables substantially attenuated the trend for lower composite cognitive scores among FD participants. Further control

Table 3 Average composite *z*-score results from GEE models

		Magnitude of difference (SE)	*p*-value
Model 1 ($n = 270$)	Univariate	−0.16 (0.090)	0.08
Model 2 ($n = 266$)	+ MCS, PCS, PSS-4, CES-D-4	0.034 (0.11)	0.75
Model 3 ($n = 266$)	+ Income	−0.043 (0.11)	0.69
Model 4 ($n = 254$)	+ Hypertension, dyslipidemia, heart disease	−0.051 (0.12)	0.67
Model 5 ($n = 229$)	+ eGFR	−0.14 (0.19)	0.46
Model 6 ($n = 229$)	+ Stroke	−0.14 (0.18)	0.45

GEE stands for generalized estimating equation. SE stands for standard error. A negative magnitude of difference indicates that participants with Fabry disease have a lower *z*-score than REGARDS control participants

MCS stands for mental component score. PCS stands for physical component score. PSS-4 stands for the 4-item Perceived Stress Scale. CES-D-4 stands for the 4-item the Center for Epidemiologic Studies Depression Scale. eGFR stands for estimated glomerular filtration rate

for comorbid cardiovascular, renal, and cerebrovascular disease did not change these results, despite significantly higher prevalence in the FD sample of all comorbidities except diabetes. It is notable, however, that our sample attained high levels of education and income (82% with at least some college, 28% with household incomes over $75K) in spite of disease-related restrictions. Further, it may be considered a testament to their coping capacities that they generally scored in ranges that were only mildly abnormal on scales of depressive symptoms and quality of life.

Our findings, though largely consistent with those of smaller clinical studies with control groups, should be interpreted in light of certain limitations. Our sample of persons with FD was able to participate meaningfully in telephone-based cognitive assessments but may not represent the full spectrum of persons with FD, particularly those with prominent hearing loss and survivors of severe stroke. In addition, men with FD were underrepresented in our study, with women comprising 70% of our sample. Disease severity in women with FD can range from asymptomatic carriers to classic symptomatic cases with involvement of multiple organ systems (Wang et al. 2007). Our study design allowed us to test whether cognitive score differences attributable to sex were different in our FD sample than in the control sample. The relationship of sex to cognitive scores did not differ across the two groups on any of the cognitive tests, thus minimizing the possibility of confounding due to sex differences in FD severity.

Strengths of this research are the relatively large sample, collection of important covariates, and a control group matched on four key demographic variables. The older mean age of our study sample extends previous research by capturing a population that is aging with Fabry disease. We demonstrate the feasibility of a low-cost and accessible telephone cognitive assessment methodology that is amenable to future studies of FD and other rare disorders. Such studies might apply this data collection method to children, young adults, and non-English-speaking persons both within and outside of the United States. Future investigations also are needed in which ERT effects on cognitive function in FD are explicitly examined.

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Synopsis

In a relatively large sample of older adults with Fabry disease matched to controls on age, sex, race, and education, we demonstrated the feasibility of telephone assessments of cognitive function, accounted for important psychosocial and disease comorbidities, and found no substantial differences between groups in overall cognitive function based on six tasks.

Compliance with Ethics Guidelines

Conflict of Interest

Virginia Wadley has received an investigator-initiated award from Genzyme: a Sanofi Company. David Warnock

has received investigator-initiated awards from Genzyme: a Sanofi Company and Amgen, Inc.; he is a consultant for Genzyme: a Sanofi Company and Amgen, Inc.

Robert Hopkin has received research funding from Genzyme: a Sanofi Company.

Dawn Laney has received investigator-initiated awards from Genzyme: a Sanofi Company, Amicus Therapeutics, and Shire Corporation.

Virginia Clarke has received a coinvestigator award from Genzyme: a Sanofi Company. Katherine Sims has received research funding from Genzyme: a Sanofi Company, Synageva, and Biogen Idec.

Leslie McClure, Caroline Lassen-Greene, Manjula Kurella Tamura, and George Howard declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committees 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.

Author Contributions

Virginia Wadley designed the study, obtained funding, collected data, contributed to statistical analyses and interpretation, and drafted the manuscript.

Leslie McClure contributed to study design, randomly selected matched controls from the REGARDS study, created and merged the datasets, analyzed and interpreted the data, and critically revised the manuscript.

David Warnock contributed to the study conception and design, recruited participants, contributed to data interpretation, and critically revised the manuscript.

Caroline Lassen-Greene collected data, created databases, contributed to data interpretation, and critically revised the manuscript.

Robert Hopkin recruited participants, contributed to data interpretation, and critically revised the manuscript.

Dawn Laney recruited participants, contributed to data interpretation, and critically revised the manuscript.

Virginia Clarke recruited participants, contributed to data interpretation, and critically revised the manuscript.

Manjula Kurella Tamura contributed to data interpretation and critically revised the manuscript.

George Howard designed the REGARDS study, contributed to the design of the present study, contributed to statistical analyses and interpretation, and critically revised the manuscript.

Katherine Sims contributed to the study design, recruited participants, contributed to data interpretation, and critically revised the manuscript.

Appendix: Detailed Methods

Demographics

Matching variables of age, sex, race (black or white), and education level (less than high school, high school, some college, college graduate) were self-reported. Income also was self-reported (<$20 K, $20 K–$34 K, $35 K–$75 K, ≥$75 K, Refused).

Cognitive Function

Cognitive function was assessed with six tasks drawn from the National Institute of Neurological Disorders and Stroke–Canadian Stroke Network (NINDS-CSN) 5-minute battery (Hachinski et al. 2006) and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) battery (Morris et al. 1989). The NINDS-CSN 5-minute battery was recommended for use in studies calling for very brief assessments, epidemiological studies, and/or telephone administration (Hachinski et al. 2006).

Learning and Memory

Learning and memory were assessed with *word list learning (WLL)* and *word list delayed recall (WLDR)* from the CERAD battery and NINDS-CSN *5-item recall* and *6-item orientation* (Hachinski et al. 2006; Morris et al. 1989; Nasreddine et al. 2005). WLL consists of three learning trials of a list of ten words which are presented in fixed orders that vary across the three trials, followed by a free recall trial (WLDR) after a 5-minute delay (Welsh et al. 1995). WLL and WLDR were administered according to standard protocols with minor modifications for telephone administration (Addison-Brown et al. 2014). Instructions and word lists were administered via a recording so that all participants were exposed to the same narrator. For WLL, correct responses on the three trials are summed. Scores range from 0 to 30. For WLDR, participants recall as many of the ten words as possible. Scores range from 0 to 10. Scores for NINDS-CSN 5-item recall and 6-item orientation from the Montreal Cognitive Assessment (Nasreddine et al. 2005) range from 0–5 and 0–6, respectively.

Executive Function

Executive function was assessed with *letter fluency (letter F)* from the NINDS-CSN battery and *semantic fluency (animals)* from the CERAD battery (Hachinski et al. 2006; Morris et al. 1989). Letter fluency and semantic fluency tests prompt participants to name as many words as they can beginning with the letter "F" in one minute and, subsequently, to name as many animals as they can in one minute. Scores on each consist of the total number of valid responses produced by each participant in 60 s. With explicit verbal permission, the assessments were recorded in digital waveform audio files and then played back later for scoring following standard scoring protocols.

Quality of Life

Health-related QOL was measured using a modified interviewer script version of the Short Form-12 (SF-12) survey (Ware et al. 1996; Quality Metrics). The survey yields Physical and Mental Component Summary scores (PCS-12 and MCS-12), each with a mean of 50 and SD of 10, facilitating comparisons to general population norms.

Perceived Stress

Perceived stress was measured with the 4-item Perceived Stress Scale (PSS-4; Cohen et al. 1983). This questionnaire elicits perceptions of stress during the past month. Each response is assigned a value of 0–4. Scores range from 0 (no stress) to 16 (high stress).

Depressive Symptoms

The Center for Epidemiologic Studies Depression Scale – 4-item version (CES-D-4; Melchior et al. 1993) – was used to evaluate depressive symptoms. Each of the 4 items assesses emotional symptoms of depression; no somatic symptoms are included in the scale. Each response is assigned a value of 0–3. Total scores range from 0 to 12; a score $\geq$4 suggests a clinically significant level of psychological distress (Melchior et al. 1993).

Cardiovascular Comorbidities

Cardiovascular comorbidities of hypertension, diabetes, dyslipidemia, and heart disease were assessed, as well as kidney function and history of stroke. A combination of self-report and objective assessments was used. In the FD participants, objective measures were drawn from Fabry Disease Registry information or medical records; for REGARDS participants, they were measured during the baseline home visit. Hypertension was defined as systolic blood pressure $\geq$140 mmHg, diastolic pressure $\geq$90 mmHg, documented or self-reported physician diagnosis, or use of antihypertensive medications. Diabetes was defined as fasting glucose >126 mg/dL, non-fasting glucose >200 mg/dL, documented or self-reported physician diagnosis, or use of diabetes medications. Dyslipidemia was defined as total cholesterol $\geq$240 mg/dL, low-density lipoprotein cholesterol $\geq$160 mg/dL, or high-density lipoprotein cholesterol <40 mg/dL; documented or self-reported physician diagnosis; or use of lipid-lowering medications. Heart disease was defined as presence of atrial fibrillation, left ventricular hypertrophy (LVH), coronary artery disease (CAD), or heart failure. Atrial fibrillation and LVH were defined by ECG or documented diagnosis. CAD was defined by ECG or documented or self-reported physician diagnosis of myocardial infarction or coronary revascularization. Heart failure was defined by self-reported or documented physician diagnosis of orthopnea or paroxysmal nocturnal dyspnea.

Kidney function was a continuous variable derived from blood assays. In REGARDS, serum creatinine was measured with isotope dilution mass spectrometry, and estimated glomerular filtration rate (eGFR) was calibrated using the Modification of Diet in Renal Disease equation. In FD participants, local laboratory values for eGFR were used, representing variable creatinine calibration methods.

For FD participants, stroke was defined as documented or self-reported physician diagnosis. In REGARDS, baseline history of stroke was defined by self-reported physician diagnosis, and reported incident strokes occurring during follow-up but prior to the initial cognitive battery assessment were confirmed by retrieval of medical records, which were adjudicated by REGARDS study physicians using the World Health Organization and clinical stroke criteria. For cases in which death occurred with no medical records surrounding the event, death certificates were examined, and proxy interviews were conducted for detection and adjudication of stroke events.

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RESEARCH REPORT

Clinical, Biochemical, and Molecular Characterization of Novel Mutations in *ABCA1* in Families with Tangier Disease

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Abstract Tangier disease is a rare, autosomal recessive disorder caused by mutations in the *ABCA1* gene and is characterized by near absence of plasma high-density lipoprotein cholesterol, accumulation of cholesterol in multiple tissues, peripheral neuropathy, and accelerated atherosclerosis. Here we report three new kindreds with Tangier disease harboring both known and novel mutations in *ABCA1*. One patient was identified to be homozygous for a nonsense mutation, p.Gln1038*. In a remarkably large Tangier disease pedigree with four affected siblings, we identified compound heterozygosity for previously reported missense variants, p.Arg937Val and p.Thr940Met, and show that both of these mutations result in significantly impaired cholesterol efflux in transfected cells. In a third pedigree, the proband was identified to be compound heterozygous for two novel mutations, a frameshift (p.Ile1200Hisfs*4) and an intronic variant (c.4176-11T>G), that lead to the creation of a cryptic splice site acceptor and premature truncation, p.Ser1392Argfs*6. We demonstrate that this mutation arose de novo, the first demonstration of a pathogenic de novo mutation in *ABCA1* associated with Tangier disease. We also report results of glucose tolerance testing in a Tangier disease kindred for the first time, showing a gene–dose relationship between ABCA1 activity and glucose tolerance and suggesting that Tangier disease patients may have substantially impaired islet function. Our findings provide insight into the diverse phenotypic manifestations of this rare disorder, expand the list of pathogenic mutations in *ABCA1*, and increase our understanding of how specific mutations in this gene lead to abnormal cellular and physiological phenotypes.

Introduction

Plasma levels of high-density lipoprotein cholesterol (HDL-C) are inversely related to risk of atherosclerotic cardiovascular disease. Much of our knowledge of HDL particle function and metabolism has been derived from the study of rare, inherited disorders of HDL. Tangier disease is one such extremely rare, autosomal recessive disorder and is caused by mutations in the ATP-binding cassette transporter, subfamily A, member 1 (*ABCA1*) gene (OMIM entry #205400). Tangier disease is characterized by a near absence of HDL-C in plasma and cholesterol accumulation in the cells of the reticuloendothelial system leading to the classic clinical manifestations of enlarged orange tonsils, peripheral neuropathy, and hypersplenism. Patients with Tangier disease are also reported to be at increased risk for atherosclerosis (Clee et al. 2000; van Dam et al. 2002), although the relationship between *ABCA1* gene mutations, HDL, and atherosclerosis is controversial (Brunham et al. 2008a). Heterozygous mutations in *ABCA1* also cause familial HDL deficiency and are a common cause of low HDL-C in the general population (Cohen et al. 2004; Alrasadi et al. 2006; Tietjen et al. 2012).

The *ABCA1* gene is expressed widely throughout the body, and studies in mice with targeted inactivation of *Abca1* in specific tissues have elucidated discrete phenotypes associated with *Abca1* function in the liver, intestine, macrophages, adipocytes, pancreatic islets, and brain (Brunham et al. 2006a, 2007, 2009; Karasinska et al. 2009; Timmins et al. 2005; Chung et al. 2011). However, the study of human phenotypes associated with complete *ABCA1* deficiency has been more challenging, owing to the rarity of this condition. To date, approximately 180 mutations have been reported in the *ABCA1* gene, and these are associated with a range of clinical, biochemical, and cellular phenotypes (Singaraja et al. 2003; Brunham et al. 2006b; Stenson et al. 2009). Understanding the clinical, biochemical, and cellular impact of pathogenic mutations in *ABCA1* has greatly increased our understanding of the crucial physiological role of this transporter in regulating cellular cholesterol homeostasis throughout the body. Here we investigated three kindreds in which one or more individual presented with extremely low levels of HDL-C. We identified homozygosity or compound heterozygosity for pathogenic mutations in *ABCA1* in 6 individuals, thus confirming the diagnosis of Tangier disease, and assessed the impact of these mutations on clinical and biochemical phenotypes.

Materials and Methods

Genomic DNA Sequencing and Mutation Detection

Genomic DNA was extracted from the blood or saliva using Qiagen DNeasy kits (Qiagen GmbH). The coding exons of the *ABCA1* gene were amplified using PCR primers spanning intron/exon boundaries (primer sequences available on request). Gel- or column-purified PCR products were subject to sequencing using an ABI 3700 instrument in the forward and reverse directions. Sequence trace files were assembled into contigs and aligned to the *ABCA1* consensus sequence using CodonCode aligner, and mutations were detected by manual inspection. All mutations were named according to Human Genome Variation Society guidelines (Uehara et al. 2008) with respect to the *ABCA1* reference sequences NM_005502.3 (in which nucleotide position 1 corresponds to nucleotide A of the ATG start methionine) and protein sequence NP_005493.2. This study was approved by the institutional review board of the University of British Columbia (UBC-C&W CREB: H96-70297) and the University Hospital Gasthuisberg.

cDNA Synthesis and Sequencing

For RNA isolation, whole blood was collected in PAXgene Blood RNA tube (Qiagen GmbH). The tubes were incubated at room temperature for a minimum of 4 h to ensure complete lysis of blood cells. RNA was isolated and purified with the PAXgene Blood RNA Kit according to the manufacturer's instructions (Qiagen GmbH). A total of 0.5–1 μg of isolated RNA was primed with 50 μM oligo $(dT)_{20}$. First-strand cDNA synthesis was performed from RNA using Superscript III according to the manufacturer's instructions (Invitrogen). The *ABCA1* cDNA was PCR amplified using eight sets of primer pairs designed to amplify the *ABCA1* cDNA sequence including the entire 5′UTR and parts of the 3′UTR (sequences available on request). All PCR products were purified using either QIAquick PCR purification or gel extraction according to the manufacturer's instructions (Qiagen GmbH) and sequenced in the forward and reverse directions. Mutations were detected as described above. Cryptic splice sites were predicted using the Automated Splice Site and Exon Definition Analyses (ASSEDA) tool (http://splice.uwo.ca).

Immunoblotting and Immunofluorescence

ABCA1 cDNA constructs containing wild-type ABCA1, ABCA1-Ala937Val, and ABCA1-Thr940Met were obtained from Blue Heron Biotech. HEK293 cells were transiently transfected with pCI-neo (empty vector), pCMVV6AC-hABCA1 (wild type), pCMVV6AC-hABCA1-Ala937Val, or pCMVV6AC-hABCA1-Thr940Met using Fugene6 (Promega) for 48 h. Transiently transfected HEK293 cells were lysed in lysis buffer (10 mM Tris pH 8.0, 1% Triton X-100, complete protease inhibitors [Roche]) for 30 min on ice with vortexing every 10 min. Thirty micrograms of total protein was loaded and separated on 7.5% acrylamide gels. Protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). ABCA1 was immunoblotted using an anti-ABCA1 monoclonal antibody generated to its C-terminus (Wellington et al. 2002a). Anti-GAPDH primary antibody (Millipore), and anti-mouse-HRP-conjugated secondary antibody (Jackson IR Laboratories) were also used in immunoblotting analyses.

For immunofluorescence, human embryonic kidney (HEK293) cells were grown on 20 mm coverslips. After 36 h of transfection with ABCA1-GFP constructs, cells were fixed with ice cold 100% methanol for 6 min followed by three washes with 1X PBS and permeabilized by three washes with 1X PBS containing 0.3% Triton X-100. After blocking with 4% BSA for 45 min, cells were incubated with rabbit anti-GFP antibody (Abcam) at a 1:200 dilution at 4°C overnight, followed by Alexa 555-labeled goat anti-rabbit secondary IgG (Molecular Probes) at a 1:200 dilution for 1 h. The cells were then mounted on slides with mounting medium containing DAPI (H-1200, Vector Labs) and imaged for ABCA1 and DNA using an Olympus FV1000 confocal microscope.

Cholesterol Efflux

HEK293 cells were plated to near confluency in 24-well tissue culture dishes in DMEM containing 4,500 mg/L glucose and supplemented with 10% FBS and 4 mM L-glutamine. Twenty-four hours after cells were transiently transfected with ABCA1 vectors as described above, 1 μCi/mL of [^{3}H] cholesterol (Perkin–Elmer) was added to the cells. Sixteen hours following loading with [^{3}H] cholesterol, cells were equilibrated in DMEM for 1 h, followed by efflux in the presence or absence of 20 μg/mL of apoA-I (Lee Biosolutions) for 4 h. Radioactivity was measured from the collected supernatant and from cells lysed with 0.1 N NaOH. Both the supernatant and cell lysates were diluted and measured in high flash-point scintillation cocktail fluid (Perkin–Elmer). Efflux data represent the mean plus or minus standard error of six independent experiments, each performed in triplicate. Efflux was calculated as the number of counts in supernatant divided by the number of counts in cell lysates plus supernatant and represents the difference of the efflux in the presence and absence of apoA-I, normalized to wild-type ABCA1. Differences between groups were compared using a one-way ANOVA test with a Neuman–Keulls posttest (GraphPad Prism).

Results

Clinical Description

The proband of Family 1 was a 22-year-old female who presented with gastrointestinal complaints. Clinical examination did not disclose evidence of corneal opacities, yellowish tonsils, or peripheral neuropathy. Abdominal imaging revealed evidence of splenomegaly. Upper and lower gastrointestinal endoscopy was performed with biopsies of the stomach, duodenum, and colon. Staining of colon biopsy specimens with Oil Red O revealed dramatic lipid accumulation in the colonic mucosa (Fig. 1a). Electron micrographs of the biopsied tissue revealed lipid-laden macrophages in the lamina propria with cholesterol crystals, as previously described (Meersseman et al. 2010). Biochemical analysis revealed extremely low HDL-C (0.08 mmol/L [3.1 mg/dL]), low total cholesterol (1.3 mmol/L [50 mg/dL]) and LDL-C (0.7 mmol/L [27 mg/dL]), and undetectable apoA-I levels. Triglycerides were 1.22 mmol/L (108 mg/dL). Both parents displayed low HDL-C (7th and 11th percentile for age and gender in the father and mother, respectively), while the proband's sister had normal plasma lipoprotein levels (Fig. 1b).

The proband of Family 2 was a 26-year-old male referred because of low levels of HDL-C and thrombocytopenia. He was clinically well and free of cardiovascular symptomatology. Previously, he had complained of numbness in his fingers. Nerve conduction studies had shown reduction in amplitude in the right median nerve on motor conduction and in the right median, right ulnar, and left median nerves on sensory conduction, interpreted by the patient's neurologist to be consistent with neuropathy of mixed origin. Clinical examination did not reveal evidence of corneal opacities or yellowish tonsils. He took isoretinoic acid for the treatment of acne. Three siblings of the proband were clinically well. There was no family history of premature coronary artery disease in any members of the family. The proband's father had died of cancer.

Laboratory investigations in the proband revealed low levels of total plasma cholesterol (1.68 mmol/L [65 mg/dL]) and LDL-C (0.89 mmol/L [34 mg/dL]). HDL-C was extremely low (0.2 mmol/L [7.7 mg/dL]) and apoA-I was undetectable. Triglycerides were 1.28 mmol/L (114 mg/dL).

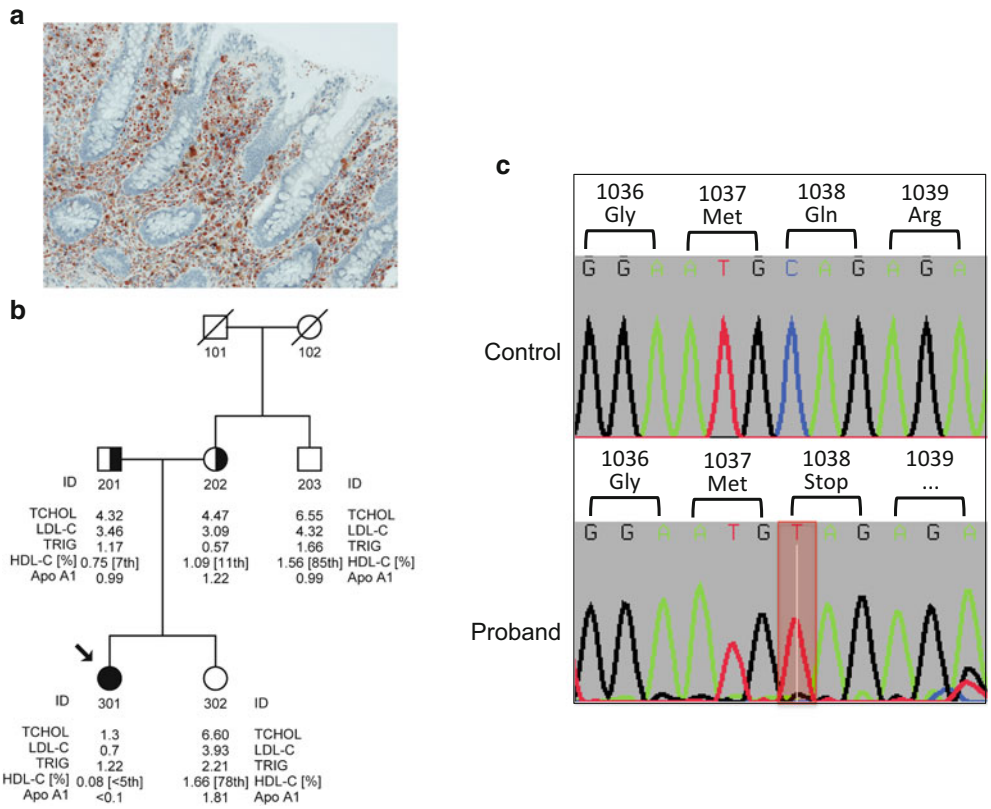

Fig. 1 (**a**) Photomicrograph of a colonic biopsy from the proband of Family 1 with staining of neutral lipids with Oil Red O. Marked accumulation of neutral lipids is observed within the colonic mucosa. (**b**) Pedigree of Family 1. The proband is indicated by an *arrow*. Homozygous affected individuals are shown as *filled symbols*; heterozygous individuals are shown as *half-filled symbols*. Plasma lipid levels are shown below each individual. *Circles*, females; *squares*, males; *diagonal lines*, deceased. Tchol, total cholesterol; Trig, triglycerides. Units are in mmol/L. (**c**) Sequence chromatogram showing *ABCA1* consensus sequence and the sequence of the proband with a homozygous C>T mutation leading to p.Gln1038*. The amino acid translation of the sequence is shown

The proband's father and mother both had moderately low HDL-C levels, 0.86 mmol/L (33 mg/dL) and 0.74 mmol/L (29 mg/dL), representing the 14th and less than the 5th percentile, respectively. All three of the proband's siblings had extremely low levels of HDL-C and apoA-I in keeping with a clinical diagnosis of Tangier disease. Screening of the extended family revealed three additional relatives with low levels of HDL-C (individuals 204, 207, and 318; Fig. 2a).

The proband of Family 3 presented at 4 years of age with abdominal pain without vomiting, steatorrhea, or constipation. She was subsequently found to have splenomegaly, thrombocytopenia (platelet count 143 $\times$ 10^9/L, normal range 200–490 $\times$ 10^9/L), and anemia (hematocrit 0.298, normal range 0.310–0.390). Extended investigations including bone marrow aspirate and biopsy did not reveal an explanation for these findings. On clinical examination she had no corneal opacities and the neurological examination was normal with no evidence of peripheral neuropathy. She was noted to have abnormal yellowish coloration of her tonsils. Other investigations revealed levels of total cholesterol, HDL-C, LDL-C, and apoA-I that were below than the lower limits of detection of the reference laboratory. Plasma triglycerides were elevated at 5.08 mmol/L (452 mg/dL), arguing against the possibility of abetalipoproteinemia. The proband's father had moderately reduced HDL-C (0.64 mmol/L [25 mg/dL], less than the 5th percentile). However, the proband's mother had normal levels of HDL-C (1.36 mmol/L [52 mg/dL], 49th percentile) (Fig. 3a).

Genetic Analysis

In each of these three families, a diagnosis of Tangier disease was suspected on the basis of the characteristic extremely low levels of HDL-C. We applied dideoxy terminator sequencing of the 49 coding exons of the *ABCA1* gene in each of these cases to establish a molecular diagnosis of Tangier disease. In the proband of Family 1, we identified a homozygous c.3112C>T mutation in exon 22 that leads to a stop mutation in the encoded protein, p.Gln1038* (Fig. 1c). Sequencing of the remaining family members indicated that both parents were heterozygous for

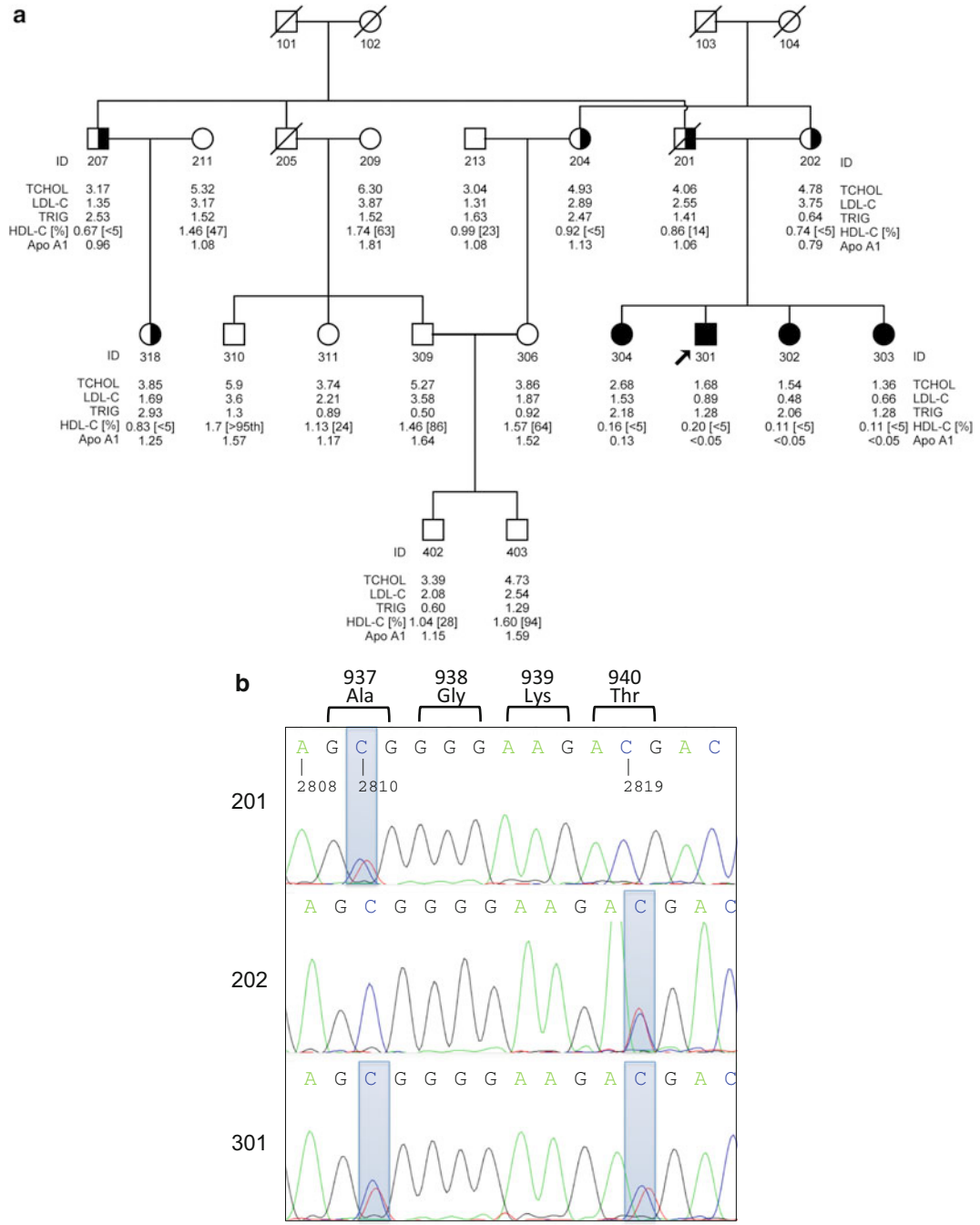

Fig. 2 (**a**) Pedigree of Family 2. The proband is indicated by an *arrow*. Homozygous-affected individuals are shown as *filled symbols*; heterozygous individuals are shown as *half-filled symbols*. Plasma lipid levels are shown below each individual. *Circles*, females; *squares*, males; *diagonal lines*, deceased. Tchol, total cholesterol; Trig, triglycerides. Units are in mmol/L. (**b**) Sequence chromatogram from individuals 201, 202, and 301. The proband (301) is compound heterozygous for c.2810C>T, resulting in a p.Arg937Val substitution, and c.2819C > T, resulting in p.Thr940Met. The proband's father (201) is heterozygous for c.2810C>T while the mother (202) is heterozygous for c.2819C>T, indicating that these two mutations reside on different alleles

this mutation while the proband's sister was homozygous wild type. This mutation was previously reported in a patient with Tangier disease who was compound heterozygous for this mutation and a second missense variant (Candini et al. 2010).

Sequencing of the *ABCA1* gene in the proband of Family 2 disclosed two heterozygous missense mutations, both in exon 19, c.2810C>T, resulting in a p.Arg937Val substitution, and c.2819C>T, resulting in p.Thr940Met (Fig. 2b). We sequenced exon 19 in the remaining members of the

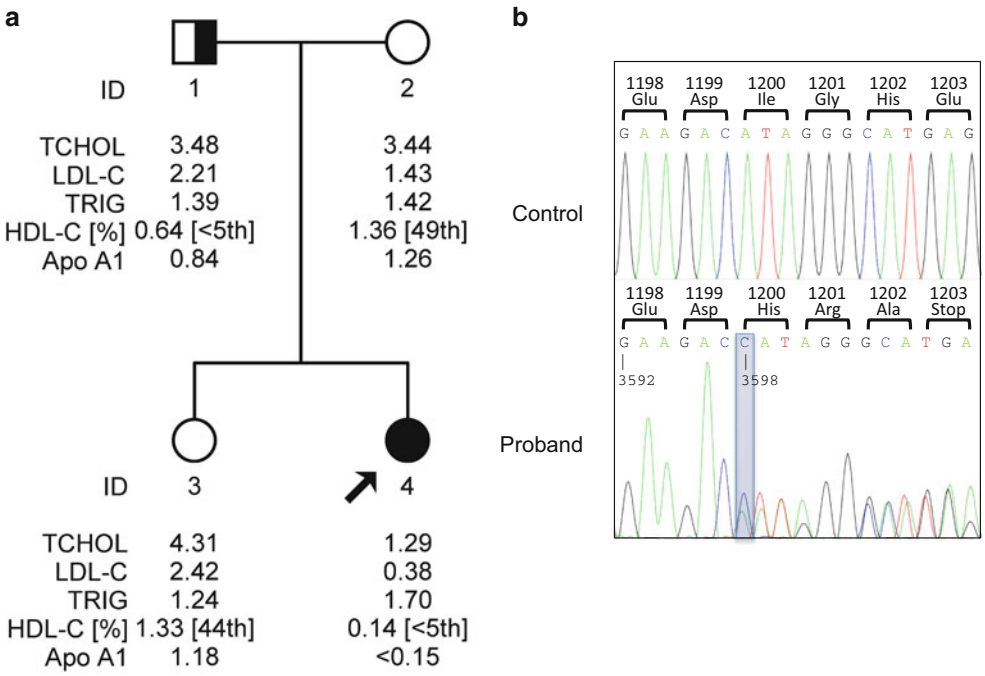

Fig. 3 (**a**) Pedigree of Family 3. The proband is indicated by an *arrow*. Homozygous-affected individuals are shown as *filled symbols*; heterozygous individuals are shown as *half-filled symbols*. Plasma lipid levels are shown below each individual. *Circles*, females; *squares*, males; *diagonal lines*, deceased. Tchol, total cholesterol; Trig, triglycerides. Units are in mmol/L. (**b**) Sequence chromatogram showing *ABCA1* exon 25 consensus sequence (control) and that of the proband. The chromatogram depicts a single nucleotide insertion, c.3597_3598insC, predicted to result in a frameshift and premature truncation at amino acid 1203 (p.Ile1200Hisfs*4)

immediate family and found that the father was heterozygous for p.Arg937Val while the mother was heterozygous for p.Thr940Met, indicating that these two mutations reside on different alleles (Fig. 2b). All three siblings of the proband were compound heterozygotes for these two mutations. Sequencing of the extended family members identified two additional heterozygotes for p.Arg937Val (individuals 207 and 318) and one additional heterozygote for p.Thr940Met (individual 204), with complete co-segregation of these mutations with the low HDL-C phenotype in this pedigree (Fig. 2a). Both p.Arg937Val and p.Thr940Met have been previously reported in patients with Tangier disease (Bodzioch et al. 1999; Uehara et al. 2008).

Sequencing of the *ABCA1* gene in the proband of Family 3 revealed a novel heterozygous insertion mutation in exon 25, c.3597_3598insC, leading to a frameshift and premature protein truncation at amino acid 1203 (p.Ile1200Hisfs*4) (Fig. 3b). We confirmed that the proband's father also carried this mutation, while the mother did not. Subsequent analysis of the remaining coding sequence of the *ABCA1* gene in the proband did not reveal a second mutation. However, based on the proband's characteristic phenotype, we hypothesized that she must harbor a second *ABCA1* mutation. We therefore examined the flanking intronic sequences of the *ABCA1* gene and identified a T>G mutation in intron 29, 11 nucleotides upstream of the start of exon 30 (c.4176-11T>G) (Fig. 4a). This variant is absent in the 1,000 genomes database, indicating that it is extremely rare or private. Intriguingly, this mutation is strongly predicted to create a cryptic splice acceptor site 10 nt upstream of the natural acceptor (T at position −11: −2.9 bits versus G at position −11: 5.8 bits) (Fig. 4b).

To determine the impact of this intronic variant on mRNA splicing, we extracted RNA from the whole blood from the proband and performed RT-PCR, followed by PCR amplification and sequencing of the *ABCA1* cDNA. This identified an insertion mutation at the boundary of exons 29 and 30, corresponding to the insertion of the final 10 nucleotides of intron 29 and thus confirming that c.4176-11T>G creates a cryptic splice acceptor (Fig. 4b). This mutation leads to a frameshift and premature stop mutation in the protein at amino acid position 1397, p. Ser1392Argfs*6. Sequencing of both genomic DNA and reverse-transcribed cDNA and from the proband's mother revealed that she neither carried the intronic mutation nor had abnormal mRNA splicing (data not shown). Sequence analysis of the father's genomic DNA showed that he also did not carry c.4176-11T>G, indicating that this mutation occurred de novo in the proband.

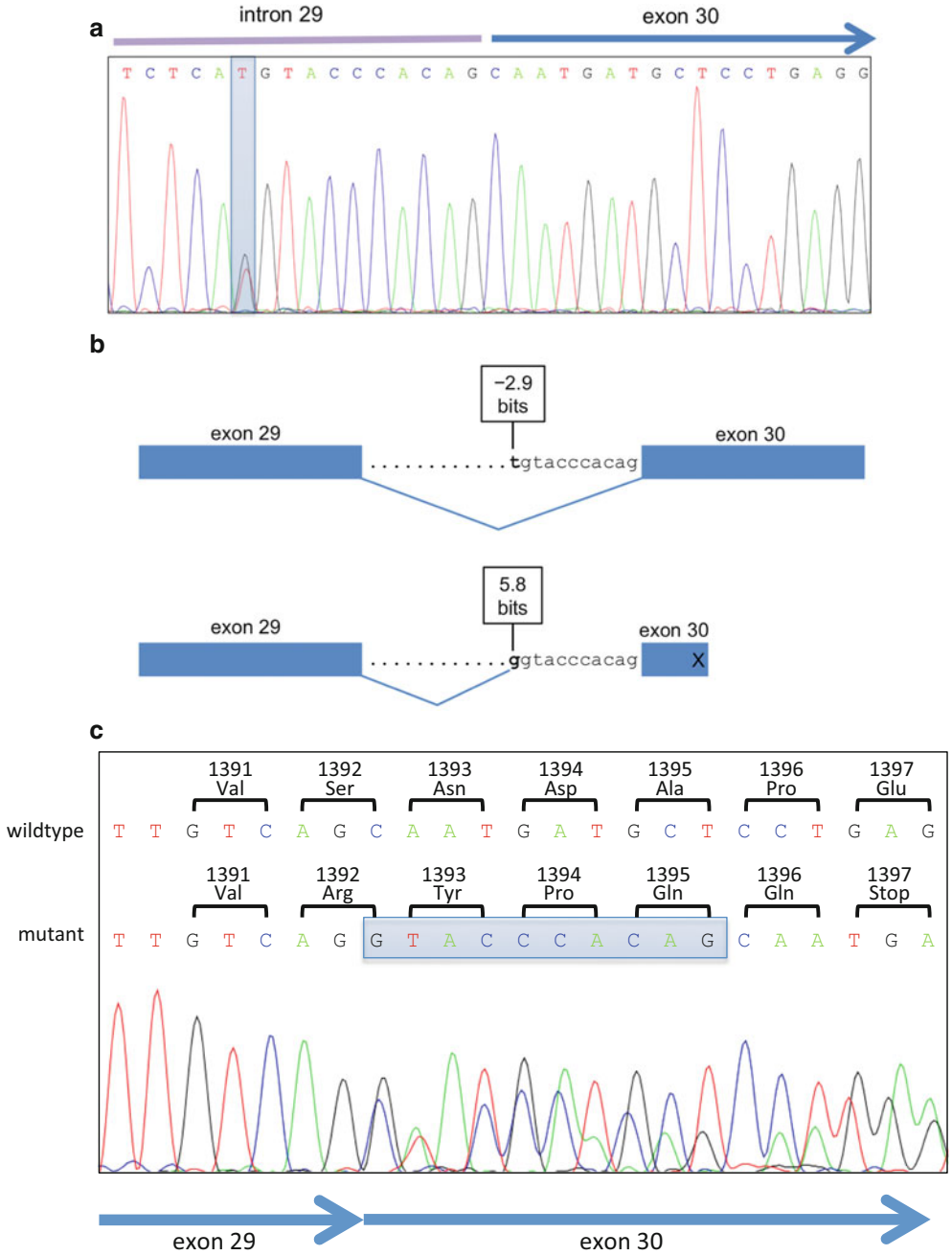

Fig. 4 An intronic mutation in *ABCA1* leads to the creation of a cryptic splice site acceptor and a frameshift in the mRNA. (**a**) Sequence chromatogram from genomic DNA from the proband depicting a heterozygous T>G mutation 11 nucleotides upstream of the start of exon 30. (**b**) This mutation is strongly predicted to increase the strength of a cryptic splice site acceptor that would lead to aberrant mRNA splicing. (**c**) Sequence chromatogram of reverse-transcribed cDNA from the proband depicting a 10 bp insertion mutation (highlighted in the *blue box*) at the beginning of exon 30 corresponding to the last 10 bp of the intron 29 sequence (gtacccacag). The sequence of the wild-type and mutant alleles is shown above the chromatogram with the amino acid translation. The insertion mutation leads to premature truncation of the protein at amino acid 1397 (p.Ser1392Argfs*6). This indicates that the intron 29 mutation in panel A results in creation of a cryptic splice acceptor with abnormal mRNA splicing

Cholesterol Efflux

While premature truncation mutations in *ABCA1* have been shown to be uniformly deleterious (Wellington et al. 2002b), missense mutations in *ABCA1* are associated with a wide range of phenotypes, ranging from no functional impact to complete loss of function (Singaraja et al. 2006). While p.Arg937Val has been shown to be a functionally null mutation (Landry et al. 2006), the functional impact of p.Thr940Met has not been previously assessed. To

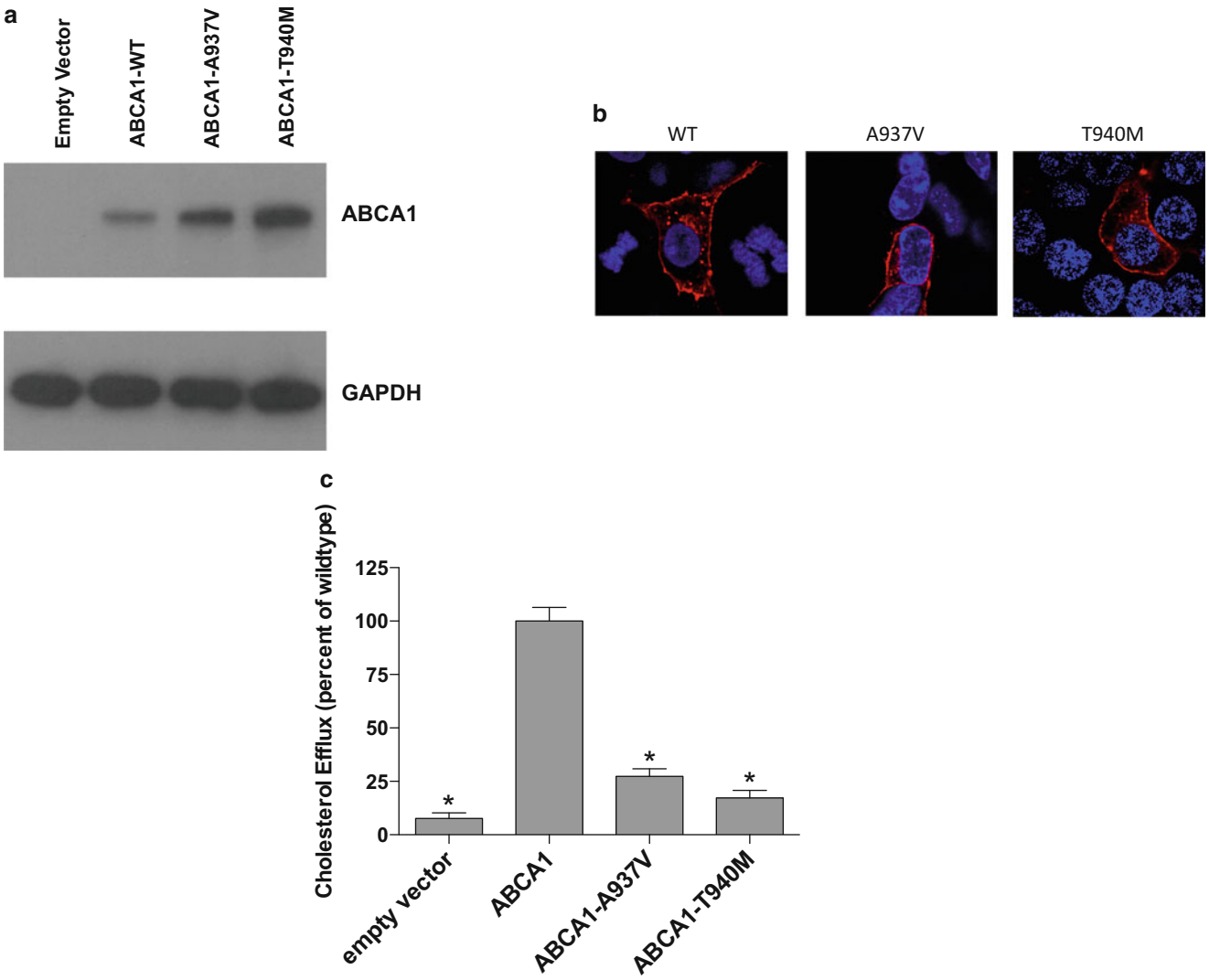

Fig. 5 Functional analysis of *ABCA1* missense mutations. (**a**) cDNA constructs containing wild-type ABCA1, or the p.Arg937Val or p. Thr940Met mutations, were obtained. HEK293 cells were transiently transfected with either empty vector or these ABCA1 alleles and protein expression determined by Western blot. Both mutations were robustly expressed relative to wild-type ABCA1. (**b**) Immunofluorescence revealed a normal pattern of subcellular localization of both mutants. hABCA1 was probed with an anti-GFP primary antibody. *Blue*: DAPI, Red: hABCA1. (**c**) Cholesterol efflux assays demonstrated that both mutations had significantly impaired apoA-I-dependent cholesterol efflux activity relative to wild-type ABCA1. *, $p < 0.01$ compared to wild-type ABCA1

determine the functional impact of this mutation on the ABCA1 protein, we obtained cDNA clones expressing wild-type ABCA1 as well as ABCA1-Arg937Val and ABCA1-Thr940Met. HEK293 cells were transiently transfected with these plasmids and ABCA1 protein expression was determined by Western blot. Both mutations were robustly expressed (Fig. 5a). Subcellular localization was determined by immunofluorescence and revealed a similar pattern of expression of these mutations compared to wild-type ABCA1, with prominent localization to the plasma membrane as well as intracellular compartments (Fig. 5b). We next quantified apoA-I -dependent cholesterol efflux in HEK293 cells transiently transfected with these alleles. Consistent with previous reports, we observed significantly less cholesterol efflux in cells which express p.Arg937Val compared to wild-type ABCA1 (Landry et al. 2006). Cells expressing p.Thr940Met also elicited significantly less efflux compared to wild-type ACBA1 (Fig. 5c). These data indicate that both p.Thr940Met and p.Arg937Val result in near complete loss of ABCA1-mediated cholesterol efflux and are functionally null alleles.

Glucose Metabolism

ABCA1 plays a crucial role in glucose homeostasis, and previous studies have shown that heterozygous *ABCA1*

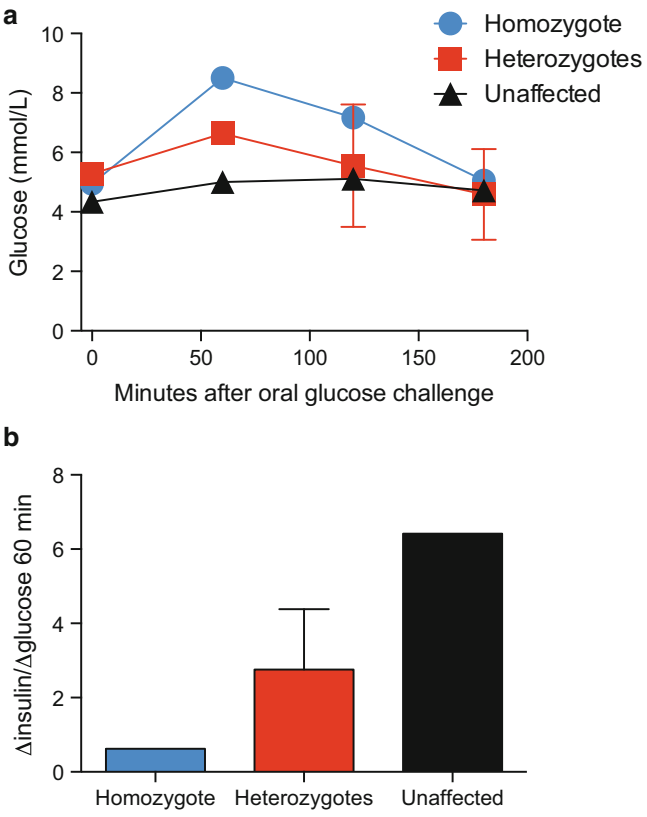

Fig. 6 Glucose metabolism in Family 1. (**a**) Oral glucose tolerance testing results. A standard 75 g oral glucose load was administered to fasting individuals and plasma glucose measured at the indicated time points. (**b**) Acute insulin response to glucose. The change in insulin divided by the change in glucose over the first 60 min of the oral glucose tolerance test is shown. The homozygous-affected individual has markedly blunted acute insulin response to glucose, suggesting impaired islet function

mutation carriers have impaired islet function (Brunham et al. 2007; Vergeer et al. 2010). However, there is a paucity of data regarding glucose homeostasis in Tangier disease subjects. We performed glucose tolerance testing in the proband and the immediate family members of Family 1. The proband did not have a previous history of dysglycemia and was lean (body mass index 22.4) with no evidence of insulin resistance. Despite her normal fasting blood glucose (4.9 mmol/L, normal range 3.6–5.5 mmol/L) and glycosylated hemoglobin level (4.6%, normal range 4.0–6.0%), the proband displayed substantial glucose intolerance relative to her unaffected sister, with the two heterozygous parents displaying an intermediate phenotype (Fig. 6a). The acute insulin response to glucose, a measure of islet function, was markedly reduced in the proband, with a lesser reduction in the heterozygous parents (Fig. 6b), suggesting a gene–dose relationship between ABCA1 activity and glucose intolerance and islet dysfunction in this family.

Discussion

Here we report three Tangier disease kindreds harboring both known and novel mutations in the *ABCA1* gene. To date ~100 patients have been described with molecularly confirmed Tangier disease. Approximately 50 mutations in the *ABCA1* gene have been reported in association with Tangier disease and a further ~130 mutations associated with familial low HDL-C or other phenotypes (Brunham et al. 2006b; Stenson et al. 2009). We report an additional six individuals with Tangier disease, as well as the description of two novel *ABCA1* mutations.

We show that the missense mutations p.Arg937Val and p.Thr940Met both result in near complete loss of apoA-I-dependent cholesterol efflux activity, despite normal subcellular localization to the plasma membrane. Both of these mutations occur near the first nucleotide-binding domain of the ABCA1 protein, a region known to be functionally critical for the transporter (Singaraja et al. 2003), and our results showing normal subcellular localization suggest that these mutations may have impaired ATP binding, thus resulting in defective cholesterol efflux. Very recently, p.Thr940Met was also reported in a targeted re-sequencing study of more than 6,000 individuals in which it was observed in a single individual with low cholesterol (Service et al. 2014). Our in vitro functional characterization of this mutation showing defective cholesterol efflux provides a biological basis and functional validation of that association.

In Family 1, we demonstrated that despite a low BMI, the affected proband had substantial islet dysfunction and that the heterozygous parents had a more mild impairment in glucose tolerance. ABCA1 is highly expressed in the pancreatic beta cell where it is essential for preventing excess cholesterol accumulation. Absence of beta cell *Abca1* in mice leads to islet cholesterol overload and dysfunctional exocytosis of insulin-containing granules (Brunham et al. 2007; Kruit et al. 2012). Heterozygous carriers of *ABCA1* mutations have impaired glucose tolerance and islet function (Vergeer et al. 2010), but the phenotype of homozygous Tangier disease patients as regards glucose homeostasis is less clear. One hypothesis is that the marked reduction in total plasma cholesterol and LDL cholesterol that typically accompanies Tangier disease may limit the degree of islet cholesterol accumulation and glucose intolerance, similar to what has been observed in mice, thereby limiting any impact on islet dysfunction in Tangier disease patients (Brunham et al. 2008b). However, whether the level of residual ABCA1 activity or the plasma cholesterol concentration is more important for determining islet function in humans is unknown. One previous study reported impaired glucose tolerance in four unrelated Tangier disease probands, but that study lacked appropriate

controls and, in addition, all four Tangier disease patients in that study met diagnostic criteria for diabetes based on fasting blood sugar, suggesting ascertainment bias in the results (Koseki et al. 2009). Our results in Family 1 using family member controls with normal fasting blood sugars show a gene–dose relationship with glucose tolerance and suggest that Tangier disease patients may have even greater islet dysfunction than heterozygous *ABCA1* mutation carriers. Our data suggest that Tangier disease patients should be screened for glucose tolerance even in the presence of normal fasting blood glucose or glycosylated hemoglobin. However, it is important to note that our results are from a single, small family and further investigation of glucose homeostasis in larger Tangier disease families using well-matched, related controls will be required to clarify the phenotype of glucose metabolism in Tangier disease.

A remarkable feature of Family 2 is the observation that all four siblings inherited two mutant alleles of *ABCA1* and have clinical and genetically confirmed Tangier disease. The probability of observing this pattern of inheritance based on Mendelian segregation is 1/256. Preferential transmission of the Tangier disease chromosome has not been previously reported, suggesting that this represents a chance occurrence. Nevertheless, the large size of this pedigree with 4 Tangier disease patients, 3 heterozygotes, and multiple unaffected individuals provides an opportunity for future investigation into disease phenotypes associated with Tangier disease in a controlled setting.

The proband of Family 3 initially posed a diagnostic dilemma for two reasons: firstly, this individual's biological mother had normal levels of HDL cholesterol (49th percentile for age and gender), arguing against a carrier status for a functional *ABCA1* mutation, and, secondly, examination of the coding sequence of the *ABCA1* gene in the proband disclosed only one mutation (c.3597_3598insC). This puzzle was solved by the identification of a novel, de novo intronic mutation in the proband (c.4176-11T>G) that resulted in aberrant mRNA splicing and protein truncation, thus both explaining the severe phenotype of the proband (being compound heterozygous for two truncation mutations) and providing a mechanism for why her mother is phenotypically normal. To our knowledge, this represents the first documentation of a de novo mutation in the *ABCA1* gene causing Tangier disease and suggests that even in the absence of phenotypically affected parents, investigation of patients with extremely low HDL cholesterol for Tangier disease may be warranted in the appropriate clinical setting.

The patient we report in Family 3 is among the youngest patients described with Tangier disease. One previous study described Tangier disease in a four-month-old infant with gastrointestinal complaints (Lachaux et al. 1998), although molecular confirmation of the diagnosis was not available at that time. In general, reports of Tangier disease in extremely young children are unusual. However, the determinants of age of onset of the clinical manifestations of Tangier disease are not known. The most common presenting complaints in patients ultimately identified as having Tangier disease are peripheral neuropathy and yellowish tonsils (Assmann et al. 2013). Both the four-year-old proband we describe in Family 3 of this report and the four-month-old patient previously described (Lachaux et al. 1998) presented with abdominal symptoms, suggesting that this may be a more common presentation in very young children with Tangier disease. While the confirmation of Tangier disease in this patient provides a molecular explanation for her plasma lipoprotein abnormalities, given her atypical presentation, it remains possible that not all features of her presentation are attributable to *ABCA1* deficiency.

In summary, we provide molecular evidence of Tangier disease in 6 patients and report two novel mutations in the *ABCA1* gene, as well as document the functional impact of two previously reported missense mutations in *ABCA1*. We also report the first documented de novo mutation in *ABCA1*, a novel intronic mutation that creates a cryptic splice site acceptor leading to a frameshift in the mRNA and premature truncation. Our results expand our understanding of the clinical and biochemical phenotypes associated with specific mutations in the *ABCA1* gene.

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Compliance with Ethics Guidelines

Conflict of Interest

The authors of this manuscript 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. No identifying information about patients is included in this manuscript.

Animal Rights

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

Author Contributions

Liam R. Brunham: Designed and conducted experiments, characterized patients, analyzed data, wrote manuscript, guarantor

Martin H. Kang: Conducted experiments

Clara Van Karnebeek: Characterized patients, wrote manuscript

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Jennifer A. Collins: Wrote manuscript

Lin-Hua Zhang: Conducted experiments

Bryan Sayson: Wrote manuscript

Fudan Miao: Conducted experiments

Sylvia Stockler: Characterized patients, wrote manuscript

Jiri Frohlich: Characterized patients

David Cassiman: Characterized patients

Simon W. Rabkin: Characterized patients

Michael R. Hayden: Designed experiments, characterized patients, wrote manuscript

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CASE REPORT

Early Umbilical Cord Blood-Derived Stem Cell Transplantation Does Not Prevent Neurological Deterioration in Mucopolysaccharidosis Type III

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Abstract Mucopolysaccharidosis type III (MPS III), or Sanfilippo disease, is a neurodegenerative lysosomal storage disease (LSD) caused by defective lysosomal degradation of heparan sulfate (HS). No effective disease-modifying therapy is yet available. In contrast to some other neuronopathic LSDs, bone marrow-derived hematopoietic stem cell transplantation (HSCT) fails to prevent neurological deterioration in MPS III patients. We report on the 5-year outcome of early transplantation, i.e., before onset of clinical neurological disease, in combination with the use of umbilical cord blood-derived hematopoietic stem cells (UCBT), in two MPS III patients. Both patients had a normal developmental quotient at the time of UCBT. One patient had a combination of mutations predicting a classical severe phenotype (MPS IIIA), and one patient (MPS IIIB) had mutations predicting a very attenuated phenotype. Transplantation was uncomplicated with full engraftment of donor cells in both.

Both patients showed progressive neurological deterioration with regression of cognitive skills and behavioral disturbances during 5 years after successful UCBT, comparable to the natural history of patients with the same combination of mutations. The concentration of HS in CSF in the patient with the attenuated phenotype of MPS IIIB 2 years after UCBT was very high and in the range of untreated MPS III patients.

We conclude that the course of cognitive development, behavioral problems, and absence of biochemical correction in CSF demonstrate the absence of relevant effect of UCBT in MPS III patients, even when performed before clinical onset of CNS disease.

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Introduction

Mucopolysaccharidosis type III (MPS III), or Sanfilippo disease, is a rare autosomal recessive lysosomal storage disease, comprising of four subtypes, types A–D, each caused by a specific enzyme deficiency and all involved in the degradation of the glycosaminoglycan (GAG) heparan sulfate (HS). MPS IIIA (OMIM #252900), caused by a deficiency of heparan-*N*-sulfamidase (NS, EC 3.10.1.1),

and MPS IIIB (OMIM #252920), caused by a deficiency of a-*N*-acetylglucosaminidase (NAGLU, EC: 3.2.1.50), are the most common subtypes (Valstar et al. 2008). Clinically, all subtypes are characterized by the same signs and symptoms. The most prominent symptom in MPS III is progressive neurocognitive impairment after an initial symptom-free interval of 1–4 years, ultimately leading to severe dementia and early death (Valstar et al. 2008). Behavioral difficulties are an early symptom and consist of hyperactivity, impulsivity, obstinacy, anxious behaviors, and autistic-like behaviors. Other symptoms include sleeping problems; frequent diarrhea; ear, nose, and throat infections; and conductive hearing loss. Death usually follows in the second or third decade of life. However, in recent years, a remarkably broad phenotypic spectrum, with attenuated patients living well into adulthood and expressing a developmental delay that may remain stable for many years, has been recognized (Valstar et al. 2010a). To date, there is no disease-modifying treatment available for any of the subtypes of Sanfilippo disease.

Hematopoietic stem cell transplantation (HSCT) is the treatment of choice for patients with the severe, neuronopathic, Hurler phenotype of MPS I (MPS I-H) when performed early, i.e., before the age of 2.5 years (de Ru et al. 2011), as HSCT may prevent or halt cognitive decline (Peters et al. 1996, 1998). HSCT for MPS I-H and other neuronopathic lysosomal storage disorders, including metachromatic leukodystrophy and Krabbe disease, is based on the engraftment of non-affected hematopoietic stem cells in various tissues including the CNS (Boelens et al. 2010). Presumably, donor-derived monocytes can cross the blood–brain barrier and differentiate into microglia cells. These microglia cells may secrete lysosomal enzymes into the extracellular space leading to cross-correction of the enzyme deficiency in neuronal cells (Krivit et al. 1995). Early transplantation, before the clinical onset of irreversible central nervous system (CNS) disease, is key for successful UCBT in neuronopathic lysosomal storage disorders. However, in MPS III patients, HSCT with bone marrow as the stem cell source (bone marrow transplantation, BMT) failed to prevent neurological deterioration (Klein et al. 1995). This might be caused by the relatively late timing of transplantation as diagnosis before clinical symptoms of CNS disease is exceptional due to the fact that first symptoms are almost invariably due to CNS disease. Early HSCT, using bone marrow as donor source, in MPS III has been reported in two neurologically unaffected siblings with MPS IIIB, both <2 years of age (Vellodi et al. 1992) and in one asymptomatic MPS IIIA patient, aged <1 year (Sivakumur and Wraith 1999), all diagnosed because of an affected older sibling. Unfortunately, neurological deterioration was not prevented by the early transplantation. This lack of efficacy might be due to the stem cell source, as in all three transplants a (carrier) parent was used as donor. The lower enzyme activity in the heterozygous donor cells may have impacted on the efficacy of enzymatic cross-correction, as has been demonstrated for MPS I (Wynn et al. 2009). Indeed, a more recent study suggested a moderate positive effect of UCBT on CNS disease in MPS III patients, with a more prominent effect in patients that were transplanted at a younger age (Prasad et al. 2008).

We report on the outcome in two MPS III patients (one with MPS IIIA and one with MPS IIIB) who had a successful UCBT at an age of 2.5 and 3 years, respectively, before the onset of neurological symptoms.

Case Histories

The experimental nature of the UCBT in MPS III was discussed with the parents of both children, and informed consent was obtained. The experimental interventions of UCBT in MPS III were approved by the local IRB.

Patient A

This male patient was born after an uncomplicated pregnancy and delivery as the first child of non-consanguineous parents. He had frequent ENT infections and, at the age of 2 years and 3 months, he was evaluated because of a cardiac murmur detected during a routine physical examination. He was found to have an enlarged liver, and cardiac ultrasound revealed significant mitral valve insufficiency, a ventricular septal defect and as a result dilatation of the left ventricle. A month later he was admitted to the Pediatric Intensive Care Unit (PICU) because of a cardiovascular collapse due to cardiac failure aggravated by supraventricular tachycardia. Because of the presence of mild coarse facial features in combination with hepatomegaly and cardiac defects, a metabolic screening for inborn metabolic diseases was performed, with a focus on lysosomal storage diseases. This led to the enzymatic diagnosis of MPS IIIA. Mutation analysis showed compound heterozygosity for the p.R245H (c.734G>A) and p.Q380R (c.1139A>G) mutations. Both mutations are associated with the classical severe phenotype (Weber et al. 1997; Valstar et al. 2010b). Testing at the age of 25 months revealed a normal neurocognitive development (GQ 106 on the Griffith scale). At the age of 30 months, a UCBT was carried out.

Patient B

This male patient was born as the second child of non-consanguineous parents after an uncomplicated pregnancy

and delivery. He had frequent upper airway infections and an adenotonsillectomy with placement of grommets done at the age of 13 months. At the age of 19 months, hepatomegaly was found on routine physical examination. Metabolic screening led to the enzymatic diagnosis MPS IIIB. Mutation analysis showed compound heterozygosity for the p.R297X (c.889C>T) and the p.S612G (c.1834A>G) mutations. This combination of mutations has been reported previously in two brothers with an attenuated phenotype of MPS IIIB (Valstar et al. 2010a). Neurocognitive testing at the age of 21 months demonstrated a normal cognitive development (DQ 94, BSID-II-NL). At the age of 25 months, a UCBT was carried out.

Materials and Methods

Umbilical Cord Blood Transplantation (UCBT)

The conditioning regime in both patients consisted of busulfan (day −9 until day −7; cumulative dose 360 mg/m^2), cyclophosphamide (day −5 until day −2; cumulative dose 200 mg/kg), and thymoglobulin (day −4 until day −2; cumulative dose 10 mg/kg). Ciclosporin (blood level 0.20–0.25 mg/L, started day −1, phased out from day 28) and prednisone (1 mg/kg/day; started day 0, phased out from day 28) were used as graft-versus-host disease prophylaxis. Donor specifications are as follows: patient A cord blood 6/6, DR mismatch, ABO incompatibility; patient B cord blood 5/6, DR mismatch, ABO incompatibility.

Donor Cell Engraftment

Full donor chimerism (on whole blood) was defined as the presence of >95% donor-derived hematopoietic cells. Neutrophil engraftment was defined as the first day of achieving an absolute neutrophil count of $\geq 0.5 \times 10^9$/L for 3 consecutive days.

Glycosaminoglycans in Urine and Cerebrospinal Fluid (CSF)

Urine

Total GAGs in urine were measured by the DMB test which involves binding of GAGs to the dye dimethylene blue and subsequent spectrophotometric analysis of the GAG-DMB complex (de Jong et al. 1992).

CSF

Heparan sulfate levels in liquor were analyzed essentially as described by de Ru and coworkers (de Ru et al. 2013). In short, 25 μL of CSF was incubated with 5 mIU each of heparinase I, II, and III in 100 mM NH_4Ac (pH 7.0), 10 mM $Ca(Ac)_2$, and 2 mM DTT at 30°C for 2 h for digestion of heparan sulfate into disaccharides (final volume of 150 mL). Next, 125 ng of internal standard (4UA-2S-GlcNCOEt-6S, HD009, Iduron) in 15 μL of 150 mM EDTA (pH 7.0) was added. Samples were boiled for 5 min and centrifuged at 20,000 × *g* for 5 min and the supernatant was centrifuged at 14,000 × *g* for 15 min at 25°C over an Amicon Ultra 30 K centrifugal filter (Millipore). The disaccharides in the filtrate were quantified on a Waters Quattro Premier XE (tandem) mass spectrometer (Waters Corporation, Milford, MA, USA) coupled to an ACQUITY UPLC system. All samples were digested and analyzed in triplicate. The concentrations of D0A0, D0S0, D0A6+D2A0, and D0S6+D2S0 (following the nomenclature of (Lawrence et al. 2008)) were calculated using a calibration curve for each of the disaccharides. The HS level in liquor was calculated as the sum of these disaccharides.

Neurocognitive Testing

Developmental testing was repeatedly performed in both patients within the scope of care. Different tests were used to assess neurocognitive development, appropriate for the developmental age of the child: the second edition of the Bayley Scales of Infant Development (BSID-II), which is the most recent version of the Bayley Scales that is available in Dutch (Van der Meulen et al. 2002), the Griffiths Mental Development Scales (Griffiths 1970), the Snijders-Oomen Nonverbal Intelligence Test 2.5–7 (SON-R) (Moore et al. 1998), the Vineland Adaptive Behavior Scales survey version (VABS) (De Bildt and Kraijer 2003), and the shorter Vineland Screener (VABS-S) (Scholte et al. 2008).

The results of the different tests were expressed as a Developmental Quotient (DQ). DQ was calculated as *(age equivalent (AE) in months/age in months)*100*. This DQ is comparable to IQ, with a mean score of 100 in the case of normal development.

Cardiac Surgery in Patient A

Successful mitral valve repair was performed in patient A at the age of 3 years.

Results

UCBT

The transplantation procedure was well tolerated in both patients, and no serious complications or side effects occurred.

Donor Cell Engraftment

The chimerism posttransplantation of patient A was 100% on day 15 and at 11 months and 5 years after transplantation. The chimerism in patient B was 96% on day 12 and 100% 4 months and 2.5 years after transplantation.

Neutrophil counts of $> 0.5 \times 10^9$/L for 3 consecutive days were achieved on day +13 in patient A and on day +25 in patient B.

Enzyme activities in lymphocytes were measured in patient A and patient B at 5 years and 5 months and 2.5 years after transplantation, respectively, and were all normal.

GAG Levels

The urinary GAG excretion was increased in both patients prior to transplantation and was found to be normal at 5 months and 5 years after HSCT in patient A and at 5 years after HSCT in patient B.

Heparan sulfate in CSF was measured only in patient B, 2 years after HSCT, and was found to be increased (2,035 ng/mL; range in MPS III patients: 809–2,261 ($n = 6$); range in healthy controls: 36–181 ng/mL ($n = 16$)).

Cognitive Development

The developmental quotient shortly before HSCT was normal in both patients (Table 1). The course of DQ after transplantation is presented in Table 1 and Fig. 1.

Behavior

No behavioral problems were noted in both patients before UCBT. Both patients developed hyperactivity, temper tantrums, and a short attention span in the years following transplantation. Both patients have used risperidone for control of the behavioral problems.

Clinical Course

Patient A developed severe mitral valve insufficiency with left ventricular failure, for which a mitral valve repair was performed.

Patient B developed no somatic symptoms of interest.

Table 1 Cognitive development in patients A and B. AE: age equivalent; DQ: developmental quotient

Age (months)	Test (scale)	AE (months)	DQ
Patient A			
29	Griffith scales	–	106
45	BSID-II	17	38
56	BSID-II	15	27
68	VABS	23	34
92	VABS	19	21
Patient B			
21	BSID-II	20	95
33	BSID-II	23	70
39	SON-R	29	74
45	VABS	31	69
58	SON-R	39	67
83	SON-R	49	59
	VABS	48	58

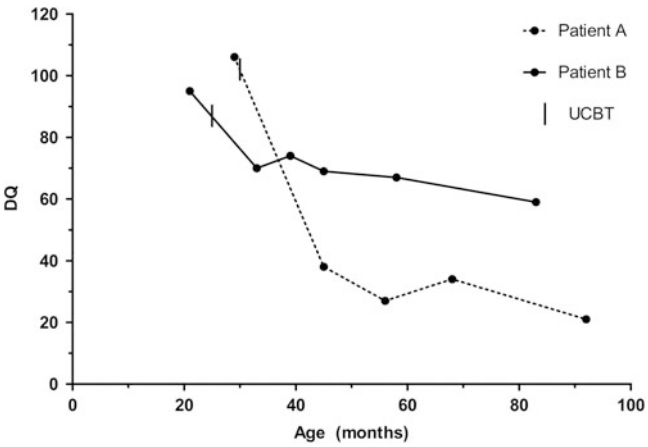

Fig. 1 Developmental quotient in patients A and B relative to the UCBT. DQ: developmental quotient

Discussion

We show that early, i.e., before the clinical onset of CNS disease, and successful umbilical cord stem cell transplantation (UCBT) in two patients with MPS III did not significantly influence the course of the neurological disease. It is not clear why HSCT failed to correct the neurological disease in MPS IIIA and IIIB patients. Indeed, this lack of efficacy is in strong contrast with the results observed in patients with the severe phenotype of MPS I (MPS I-H). A potential explanation is that the donor-derived microglia cells secrete insufficient amounts of enzymes for cross-correction of the neuronal tissue in MPS III (Shapiro et al. 1995; Sivakumur and Wraith 1999). A recent study in MPS IIIA mice indeed showed that ex vivo lentiviral-mediated HSCT, probably resulting in

high expression of SGSH, normalized heparan sulfate storage in brain and significantly improved neuroinflammation and behavior, while wild-type HSCT did not (Langford-Smith et al. 2012).

Our observations demonstrate that it is essential to take into account the predicted severity of the phenotype when assessing efficacy of a therapeutic intervention in MPS III patients. While the clinical course of the disease in patient A (MPS IIIA) essentially followed the course observed in patients with the classical phenotype of Sanfilippo disease (Buhrman et al. 2014), the disease in patient B (MPS IIIB) showed a much more attenuated course and his cognitive development still progressed at the age of 5 years. This may erroneously lead to a conclusion of clinical efficacy of the UCBT in this patient. However, the mutation combination in this patient has been reported previously in two brothers (Valstar et al. 2010a). One of them died at the age of 41 due to pneumonia. The other patient is still alive at the age of 52 years. He could still walk and read small sentences at the age of 48 years. The disease course in patient B closely resembles the clinical history of these brothers, and we can therefore conclude that the UCBT in this patient was ineffective. Mutations and mutation combinations conveying an attenuated phenotype have been reported frequently in MPS IIIB (Weber et al. 1999; Valstar et al. 2010a) and, though more rare, also occur in MPS IIIA (Meyer et al. 2008; Valstar et al. 2010b).

The complete normalization of the urinary GAG excretion in both patients suggests a full biochemical correction of the somatic enzymatic deficiency by the UCBT. Our observation that the HS concentration in CSF was still very high 2 years after transplantation in patient B shows that even full engraftment after UCBT does not lead to biochemical correction of the disease in the CSF which may explain the lack of clinical efficacy. The lack of clinical efficacy in both patients is further supported by the fact that both developed the typical behavioral problems associated with MPS III in the years following the UCBT.

Our study has some limitations. The patients were not studied in a single prospective study, and we did not use a standardized complete protocol for neurocognitive testing, and testing was done at different sites. In addition, no complete standardized assessment of behavior and sleep difficulties was done. This might have resulted in a less precise assessment of the potential benefits of the transplantation. However, the course of cognitive development in combination with the severe behavioral difficulties in both patients in combination with the high concentration of HS in CSF in patient B demonstrates that efficacy of UCBT in Sanfilippo disease is at its best very limited. Finally, we cannot exclude that a transplantation earlier in life might lead to clinical benefit in patients with MPS III. However, a very early diagnosis in MPS III is very rare and in general only possible in case of an older sibling with MPS III.

Several studies on alternative treatment strategies for MPS III are ongoing, including intrathecal enzyme replacement therapy in MPS IIIA (clinicaltrials.gov, identifier: NCT01299727), gene therapy in MPS IIIA (clinicaltrials.gov, identifier: NCT01474343), and high dose genistein in MPS IIIA, B, and C (public.ukcrn.org.uk; UKCRN ID 16209). Given the failure of early UCBT in MPS III and the unmet clinical need in this severe neurodegenerative disease, these studies are of the highest importance.

Compliance with Ethics Guidelines

Conflicts of Interest

Lindsey Welling, Jaap Jan Boelens, Jan Pieter Marchal, Peter van Hasselt Ans T. van der Ploeg, and Frits A. Wijburg declare that they have no conflict of interest.

Informed Consent

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

Contributions of the Individual Authors

Lindsey Welling: reporting

Jaap Jan Boelens: treating physician, coordinating the performed examinations

Jan Pieter Marchal: interpreting neuropsychological test results, reporting

Peter van Hasselt: treating physician, reporting

Ans T. van der Ploeg: reporting

Frits A. Wijburg: reporting

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RESEARCH REPORT

Biochemical and Hematologic Manifestations of Gastric Intrinsic Factor (GIF) Deficiency: A Treatable Cause of B_{12} Deficiency in the Old Order Mennonite Population of Southwestern Ontario

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Abstract Intrinsic factor deficiency (OMIM #261000, IFD) is a rare inherited disorder of vitamin B_{12} metabolism due to mutations in the gastric intrinsic factor (GIF) gene.

We report three individuals from an Old Order Mennonite community who presented with B_{12} deficiency. Two cases are siblings born to consanguineous parents and the third case is not known to be closely related. The older male sib presented at 4 years with gastrointestinal symptoms, listlessness, and pallor. He had pancytopenia with megaloblastic anemia. Serum B_{12} was 61 (198–615 pmol/L). Methylmalonic aciduria was present. C3 was elevated on acylcarnitine profile. Homocysteine was high at 16.7 (5.0–12.0 umol/L). His asymptomatic female sibling was also found to have B_{12} deficiency. Genetic testing for methylmalonic aciduria (*MMAA*), transcobalamin deficiency (*TCN2*), and Imerslund-Gräsbeck syndrome (*AMN*) showed no mutation in both siblings. The third patient, a 34-year-old woman, had presented in infancy with a diagnosis of pernicious anemia. Mutation analysis of *GIF* revealed compound heterozygosity for a c.79+1G>A substitution and a c.973delG deletion in all three individuals. Oral or parenteral vitamin B_{12} has led to complete recovery of clinical parameters and vitamin B_{12} levels. Newborn screening samples on the siblings revealed normal methylcitrate, C3, and C3/C2 ratios thus indicating no disruption of propionic or methylmalonic acid metabolism.

A high index of suspicion should be maintained if children present with megaloblastic anemia since GIF deficiency is a treatable disorder and newborn screening may not be able to detect this condition.

Abbreviations

AMN	Amnionless gene
B_{12}	Vitamin B_{12} (cobalamin)
CUBN	Cubilin gene
GIF	Gastric intrinsic factor
GIF	Gastric intrinsic factor gene
IFD	Intrinsic factor deficiency
IGS	Imerslund-Gräsbeck syndrome
MCEE	Methylmalonic CoA epimerase gene
MMA	Methylmalonic aciduria
MMAA	Methylmalonic acidemia CblA type gene
MMAB	Methylmalonic acidemia CblB type gene
MMADHC	Methylmalonic acidemia CblD type gene
MUT	Methylmalonic CoA mutase gene
TC	Transcobalamin deficiency
TCN2	Transcobalamin II gene

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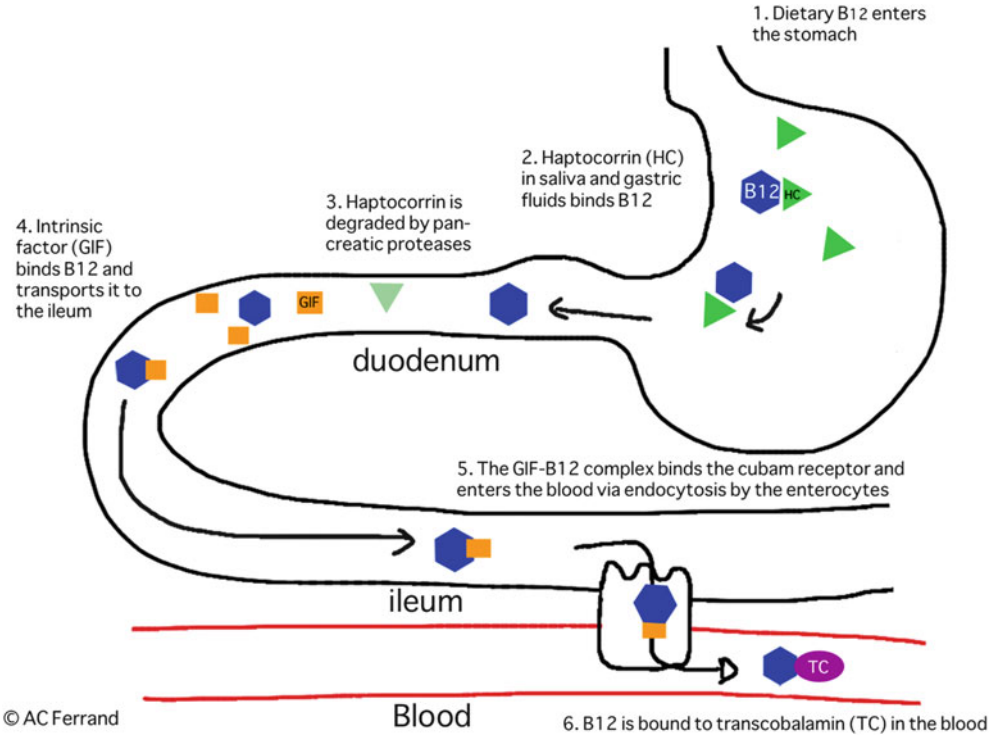

Fig. 1 Enteral metabolism of B_{12} and GIF

Introduction

Inherited disorders of vitamin B_{12} metabolism can be due to many etiologies such as Imerslund-Gräsbeck syndrome (IGS; OMIM #261100, *CUBN* or *AMN* mutations) (Grasbeck 2006; Ament et al. 2009), transcobalamin deficiency (TC; OMIM #275350, *TCN2* mutation) (Prasad et al. 2008; Trakadis et al. 2013), methylmalonic aciduria (MMA; OMIM #251100, *MMAA* mutation) (Trakadis et al. 2013), and intrinsic factor deficiency (IFD; OMIM #261000, *GIF* mutation) (Tanner et al. 2005).

Gastric intrinsic factor (GIF) is a cofactor produced by the parietal cells of the stomach. GIF binds vitamin B_{12} in the duodenum and transports it to the terminal ileum (Kozyraki and Cases 2013). The GIF-B_{12} complex allows for endocytosis of B_{12} by the mucosal cells of the distal ileum via a cubam receptor composed of two proteins, cubilin and amnionless (Watkins and Rosenblatt 2011). Serum vitamin B_{12} is primarily transported by haptocorrin and transported into cells by transcobalamin (Trakadis et al. 2013) (see Fig. 1).

Congenital IFD is a rare disorder of vitamin B_{12} metabolism presenting in infancy or early childhood. In congenital IFD, gastric acid secretion is normal and B_{12} deficiency results from a mutation in *GIF* leading to a low level or lack of GIF in gastric juices, abnormal susceptibility of GIF to pepsin degradation, or reduced affinity for ileal GIF-B_{12} receptor (Gordon et al. 2004; Chery et al. 2013). IFD differs from adulthood-acquired cobalamin deficiencies associated with atrophic gastritis in which normal GIF can be produced but is reduced in quantity due to decreased parietal cells or an autoimmune disorder with production of antibodies against GIF. The Schilling test can be used to differentiate between acquired and inherited causes of B_{12} deficiency; however this test is expensive, invasive, and rarely available in practice (Tanner et al. 2005).

Patients with GIF deficiency present with low serum cobalamin levels and megaloblastic anemia in comparison to TC and MMA deficiencies where B_{12} levels are usually normal (Trakadis et al. 2013). Other presentations include pancytopenia, splenomegaly, hepatomegaly, peripheral neuropathy, joint pain and swelling, anorexia, diarrhea, or infantile death (Gordon et al. 2004; Overgaard et al. 2010).

Material and Methods

Case Report

The proband (II-1 from Family 1) (see Fig. 2) presented at 4 years of age to the gastroenterology clinic with constipation and bloody stools. He is of Old Order Mennonite descent and his parents are consanguineous. Prenatal and birth history were unremarkable.

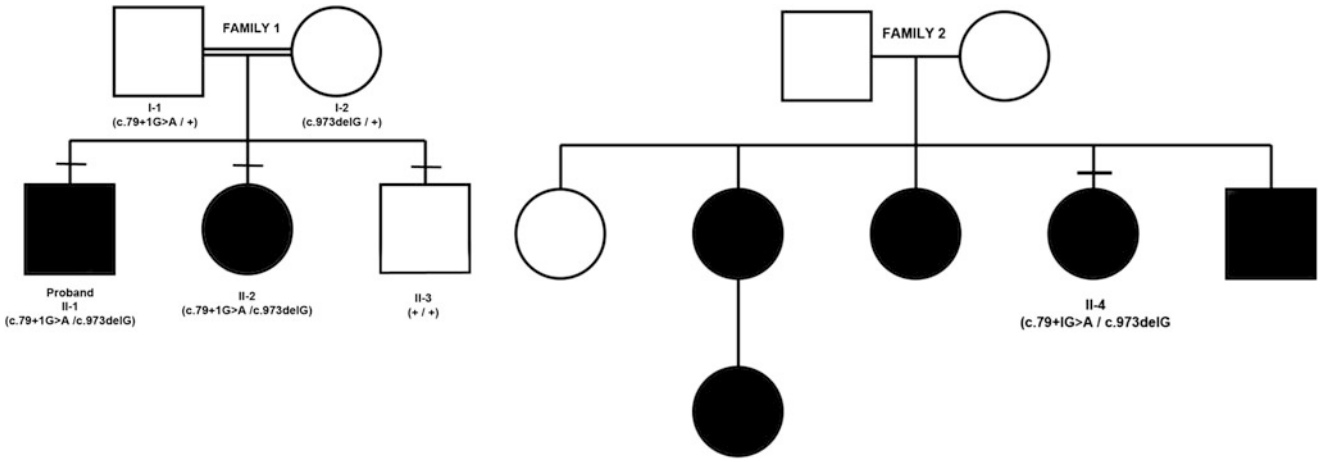

Fig. 2 DNA molecular analysis of *GIF* identified heterozygous mutations for a c.79+1G>A substitution and a c.973delG deletion in Family 1 and Family 2. Closed symbols signify individuals with low serum vitamin B_{12}

Prior to presentation he experienced gastrointestinal symptoms including loose stools, reduced appetite, vomiting, and mild jaundice as noted by his parents. He underwent dental surgery for caries and was noted to be pale and fatigued 2 weeks following this procedure. Over the next 6 months, he started having difficulty walking and getting up as well as paresthesias in his lower limbs. Initial bloodwork from the GI clinic disclosed the presence of pancytopenia and he was referred to the hematology-oncology clinic. Physical examination revealed a very pale child with a heart rate of 80 beats per minute without lymphadenopathy and organomegaly. He had no dysmorphic features. Cardiac examination was normal. Further hematological investigations revealed the diagnosis of megaloblastic anemia secondary to a very low B_{12} level (see Table 1). Nutritional deficiency and malabsorption causes were excluded. Infectious screen was negative. Barium swallow and 99mTc pertechnetate scintigraphy were negative. Urine organic acids showed elevated urine methylmalonic aciduria (MMA) which reinforced suspicions of a defect in cobalamin metabolism or transport. There was no proteinuria. No Schilling test was performed as it was unavailable.

The proband was started on oral B_{12} vitamin (methylcobalamin) supplementation 1,000 mcg/day. He had a rapid full recovery of his gastrointestinal and neurological symptoms and improvement of all hematological cell lineages as well as serum B_{12} levels. At 7 years of age he is thriving and succeeding at school. His weight is 29.7 kg (90th percentile), and height is 127 cm (97th percentile). He has a normal physical examination and no further urine MMA.

The sister of proband (II-2 from Family 1) was seen at 16 months of age on the basis of family history. She was clinically asymptomatic with a normal physical examination. Her initial hematologic parameters were normal. However, she had low serum B_{12} levels and elevated urine MMA. She was started on oral B_{12} supplementation with restoration of her serum B_{12} stores. A recent evaluation at 4 years of age shows a well child with a normal complete blood count, homocysteine level, and B_{12} serum levels as well as no urinary MMA.

A 24-year-old woman (II-4 from Family 2), also of Old Order Mennonite descent and with the same surname as the proband, had presented with low serum B_{12} and megaloblastic anemia in infancy. She had a bone marrow biopsy and has been on B_{12} injections since then. She had complete recovery of her clinical parameters and vitamin B_{12} levels. Three of her sibs had also been diagnosed with pernicious anemia and were on vitamin B_{12} therapy, but no further clinical details were available.

Results

Genetic Analysis

In Family 1, the proband had full sequencing of the *MMAA*, *AMN*, and *TCN2* with no mutations identified. He then was evaluated for *GIF* mutations and was found to be compound heterozygous for a c.79+1G>A substitution and a c.973delG deletion. Subsequently, his sister (II-2) tested positive for the same *GIF* heterozygous mutations. In Family 2, II-4 had a negative test for *AMN* mutation. Molecular genetic testing for GIF deficiency was positive with the same heterozygous mutations as in Family 1 (See Fig. 2).

Table 1 Hematological and biochemical laboratory values before and after B_{12} supplementation

Patient	Initial bloodwork	After B_{12} supplementation	Normal values
II-1, Family 1			
White blood cells	2	4.7	5–12 × 10e9/L
Neutrophils	0.1	1.6	>1.0 × 10e9/L
Hemoglobin	72	132	110–160 g/L
Mean corpuscular volume	111	78.3	75–87 fL
Mean corpuscular hemoglobin concentration	354	349	305–360 g/L
Mean corpuscular hemoglobin	39.5	28.1	24.0–30.0 pg
Platelets	40	243	150–400 × 10e9/L
Serum B_{12}	61	389	198–615 pmol/L
Urine MMA	Positive	Negative	Qualitative analysis
Serum homocysteine	16.7	2.9	5.0–12.0 umol/L
Creatine kinase	89	–	40–280 U/L
C3	Elevated	–	Qualitative analysis
Serum IgA	2.4	–	0.20–1.00 g/L
Anti-tTG IgA	Negative	–	Qualitative analysis
Anti-tTG IgM	Negative	–	Qualitative analysis
Urine proteinuria	Negative	–	Qualitative analysis
II-2, Family 1			
White blood cells	–	5.6	5–12 × 10e9/L
Neutrophils	–	1.4	>1.0 × 10e9/L
Hemoglobin	–	120	110–160 g/L
Platelets	–	227	150–400 × 10e9/L
Serum B_{12}	69	352	198–615 pmol/L
Urine MMA	Positive	Negative	Qualitative analysis
Urine proteinuria	Negative	–	Qualitative analysis
II-4, Family 2			
Hemoglobin	–	128	110–160 g/L
Mean corpuscular volume	–	29	75–87 fL

Biochemical Analysis

Urine organic acids of the proband showed urine MMA (quantitative values not available) on a qualitative analysis (see Table 1).

C3 was elevated on serum acylcarnitine profile. Serum homocysteine was high at 16.7 (5.0–12.0 umol/L). An initial serum IgA level was elevated at 2.40 g/L (0.20–1.00). Anti-tissue transglutaminase IgG and IgA antibodies were negative. There was no proteinuria on urine analysis. A serum creatine kinase level was normal at 89 U/L (40–280).

The sibling of the proband had urine MMA. Analysis of the newborn screening samples on the proband and his sibling revealed normal methylcitrate, C3, and C3/C2 ratios thus indicating no disruption of propionic/methylmalonic acid metabolism.

Hematological Analysis

See Table 1.

Discussion

The functional deficiency of cobalamin may affect multiple organ systems. Presentation can range from mild gastrointestinal symptoms to severe or fatal anemia (Sturm et al. 2013), pancytopenia, failure to thrive or weakness, leading to a delay in diagnosis or misdiagnosis (Gordon et al. 2004). Neurological symptoms of B_{12} deficiency may be subtle in presentation, making developmental delay a concern with untreated low serum cobalamin (Ament et al. 2009; Sturm et al. 2013).

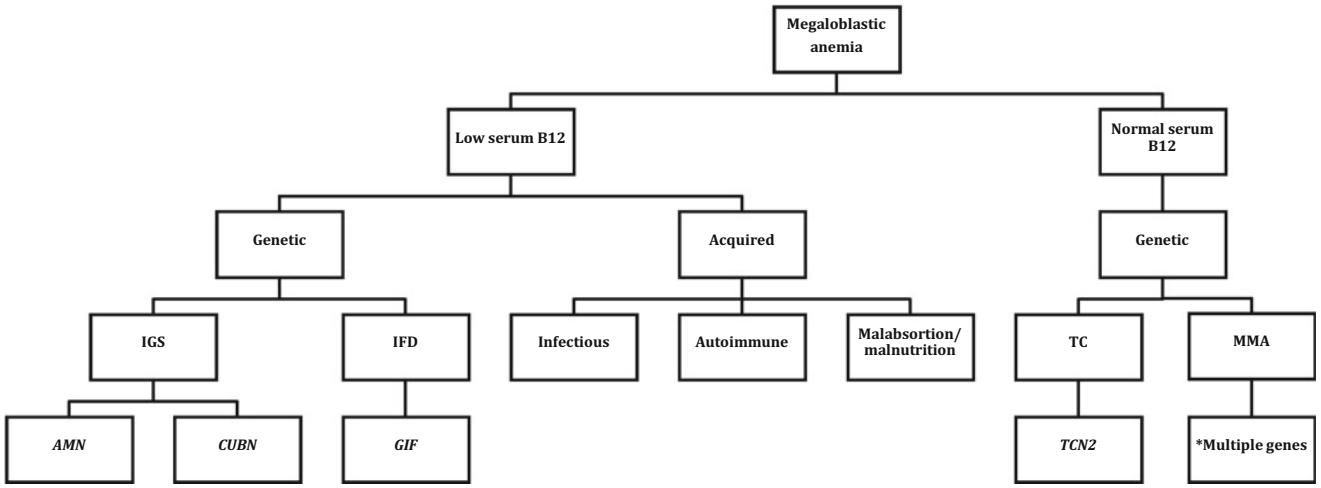

Fig. 3 Diagnostic approach to megaloblastic anemia in childhood. Infectious and dietary causes are becoming less frequent in our society (Sturm et al. 2013). Imerslund-Grasbeck syndrome (IGS) with mutations in *AMN* and *CUBN* and IFD are disease entities that can present with similar phenotypes (Tanner et al. 2005; Ament et al. 2009). Proteinuria is sometimes used to differentiate IGS and IFD and direct the order of genetic testing but is not a specific finding (Grasbeck and Tanner 2011). *Multiple genes include *MUT*, *MMAA*, *MMAB*, *MCEE* and *MMADHC*

When evaluating a patient presenting with low serum B_{12}, many diagnostic investigations and specialists may be involved, making the process lengthy and costly (Carmel et al. 2003). Acquired causes of cobalamin deficiency such as autoimmune gastritis are rare in children. Therefore, in order to accelerate the diagnostic process, genetic causes should be considered in the initial evaluation of megaloblastic anemia in childhood including mutations in AMN, CUBN, GIF, TCN2, and MMAA genes (see Fig. 3) especially in context of consanguinity and Mennonite background. IGS, TC, and IFD are disease entities that can present with similar phenotypes (Overgaard et al. 2010). Lack of proteinuria and response to B_{12} supplementation can sometimes help to differentiate IFD from IGS in order to direct the sequence of genetic testing (Grasbeck and Tanner 2011; Sturm et al. 2013).

Dried blood spot C3 acylcarnitine is the newborn screening metabolite that is elevated in diseases of intracellular cobalamin metabolism, methylmalonic and propionic acidemias. TC deficiency can be detected on newborn screening (Prasad et al. 2012). C3 acylcarnitine was not elevated in newborn screening tests of GIF deficient patients II-1 and II-2 of Family 1. Presumably the infants are protected by hepatic stores of maternally originating cobalamin and not initially dependent on GIF-mediated cobalamin intestinal absorption.

Hewitt and Gordon et al. first identified *GIF* on chromosome 11 (Hewitt et al. 1991). IFD resulting from a mutation in *GIF* was first described in 2004 by Yassin et al. in a patient presenting with severe megaloblastic anemia (Yassin et al. 2004). Since then, multiple case reports have identified other *GIF* mutations resulting in B_{12} deficiency and megaloblastic anemia. The previous case reports have been summarized in Table 2.

The mode of inheritance of IFD was previously unclear (Gordon et al. 2004). With studies demonstrating homozygous and compound heterozygote mutations, the inheritance has been established as autosomal recessive for IFD (Yassin et al. 2004; Chery et al. 2013).

The Human Genome Mutational Database identifies 18 mutations in *GIF* (http://www.hgmd.org, retrieved from 2014/04/21). The three patients described here were compound heterozygous for c.79+1G>A and c.973 delG. The c.79+1G>A mutation (HGMD #CS051254) affects the intron 1 invariant donor splice site and has been described by Tanner et al. (2005). The c.973 delG is a novel mutation and results in a frame shift starting at codon 325 and produces a premature stop at codon 337 in the new reading frame.

All three patients are members of an Old Order Mennonite community in Southwestern Ontario, Canada, but interestingly all three are compound heterozygous for two *GIF* mutations. The phenotype associated with homozygosity for either of these two mutations has not yet been described. This report indicates that GIF deficiency should be considered as a cause of vitamin B_{12} deficiency in the Mennonite communities along with AMN deficiency as described by Strauss and Puffenberger in the Pennsylvania Mennonite community (Strauss and Puffenberger 2009).

B_{12} deficiency caused by GIF deficiency is a treatable disorder responding to oral B_{12} supplementation as gastric mucosa is normal. When diagnosed early many or all symptoms can be avoided. A high index of suspicion for

Table 2 Review of the literature of all previously described GIF mutations

Reference	Mutations identified	Clinical presentation	Proteinuria	Ethnicity	Age at onset (year)
Gordon et al. (2004)	hom g.68 A>G (3) g.68 A>G/g.67C>G(1)	Macrocytic anemia Megaloblasts on bone marrow	–	Spanish American	2–11
	g.68 A>G/Normal (1)	Anorexia Joint pain and swelling Diarrhea		Kazachstani	
Yassin et al. (2004)	c,183_186delGAAT (1)	Severe anemia Megaloblastic erythroid hyperplasia Positive Schilling test	No proteinuria	African-American	3
Tanner et al. (2005)	hom c,1175_1176insT(1) hom c.l61delA(1)	Megaloblastic anemia	8 out of 16	Turkish Turkish	1–17
	hom c.137C>T			Turkish	
	(4) homc.80_lG>A(7)			Kuwait	
	hom c.l83-186deIGAAT(1)			Guinea-Bissau/African	
	hom c.79+lG>A (2)			French/Caucasian	
Garcia Jimenez et al. (2008)	c.256+2T>G/c.659 T>C (1)	Megaloblastic anemia Low serum cobalamin Fatigue, anorexia Thrombocytopenia Methylmalonic aciduria Systolic murmur	No proteinuria	Spanish	4
Overgaard et al. (2010)	c.290T>C/c.79+1G>A (1)	Megaloblastic anemia Hemolysis Pancytopenia Splenomegaly Discrete icterus	Mild proteinuria	Danish	15
Tanner et al. (2012)	hom c.79+1G>A (2) hom c.137 C>T(1)	Megaloblastic anemia	1 out of 12	Norwegian Turkish	–
	hom c.685 G>A (2)			Turkish	
	c.79+lG>A/del Intron 8 to distal of 3′-end (1)			Western European	
	c.79+1G>A/c.1370 T (1)			Siberian	
	c.79+1G>A/c.673A>C (1)			Italian	
	c.290T>C/?(1)			Finnish	
	c.431_438delAGAAGAAC/			Austrian	
	c.974_975insG (1)				
	c.938C>T/? (1)			Arabic	
	c.469T>C/? (l)			Lebanese	

Boina Abdallah et al. (2012)	hom c. 691C>T (2) hom c. 183_186delGAAT (1)	Failure to thrive Glossitis Gastrointestinal symptoms Pallor Neurological symptoms Hair abnormalities Hyperpigmentation	No proteinuria	French	1.5–6
Chery et al. (2013)	c.290T>C/FUT2 (7) c.435_437delGAA/FUT2 (2)	Thromboembolism Macrocytic anemia Megaloblastic anemia Hyperhomocysteinemia	–	Caucasian-european	0.5–43
Sturm et al. (2013)	hom c.l073+5G>A (4) hom c.1073+5G>A (12)	Macrocytic anemia Syncope ALL diagnosis	No proteinuria	Chaldean	1.5–14

() Indicates number of patients. Dash line represents data not available
ALL acute lymphoblastic leukemia

GIF deficiency should be maintained if children present with megaloblastic anemia since prognosis is good once diagnosed.

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References to Electronic Databases

GeneReviews®: http://www.ncbi.nlm.nih.gov/books/NBK1116/

Human Genome Mutational Database HGMD®: http://www.hgmd.org

Online Mendelian Inheritance in Man: http://omim.org

Pubmed: http://www.ncbi.nlm.nih.gov/pubmed

Synopsis

A case report and literature review of inherited GIF deficiency mutation, a severe but treatable disorder of B_{12} metabolism whose presentation can mimic many disorders.

Compliance with Ethics Guidelines

Conflict of Interest

Amaryllis Cloelia Ferrand, Victoria Mok Siu, Melanie P Napier, Osama Al-Dirbashi, Pranesh Chakraborty, Chitra Prasad, and C Anthony Rupar declare no conflicts of interest.

Informed Consent

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

Declaration

The paper is being submitted as an original article.

This paper has not been previously submitted nor is under consideration for publication in any other journal.

Author Contribution

The paper's submission for publication has been approved by all of the authors.

Amaryllis C. Ferrand: Conception, design and drafting. First Author.

Victoria M. Siu: Conception, design and critical revision.

C Anthony Rupar: Analysis and interpretation of data, critical revision.

Melanie P. Napier: Conception, design and critical revision.

Osama Y. Aldirbashi: Analysis and interpretation of data, critical revision.

Pranesh Chakraborty: Analysis and interpretation of data, critical revision.

Chitra Prasad: Conception, design and drafting. Guarantor.

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CASE REPORT

A Cause of Permanent Ketosis: GLUT-1 Deficiency

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Abstract GLUT-1-deficiency syndrome (GLUT1-DS; OMIM 606777) is a treatable metabolic disorder caused by a mutation of *SLC2A1* gene. The functional deficiency of the GLUT1 protein leads to an impaired glucose transport into the brain, resulting in neurologic disorders.

We report on a 6-month-old boy with preprandial malaises who was treated monthly by a sorcerer because of a permanent acetonemic odor. He subsequently developed pharmaco-resistant seizures with microcephaly and motor abnormalities. Metabolic explorations were unremarkable except for a fasting glucose test which revealed an abnormal increase of blood ketone bodies. At the age of 35 months, GLUT1-DS was diagnosed based on hypoglycorrhachia with a decreased CSF to blood glucose ratio, and subsequent direct sequencing of the *SLC2A1* gene revealed a *de novo* heterozygous mutation, c.349A>T (p.Lys117X) on exon 4. It was noteworthy that the patient adapted to the deficient cerebral glucose transport by permanent ketone body production since early life. Excessive ketone body production in this patient provided an alternative energy substrate for his brain. We suggest a cerebral metabolic adaptation with upregulation of monocarboxylic acid transporter proteins (MCT1) at the blood–brain barrier provoked by neuroglycopenia and allowing ketone body utilization by the brain. This case illustrates that GLUT1-DS should be considered in the differential diagnosis of permanent ketosis.

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Introduction

Glucose-transporter type 1 (GLUT-1) is one of the most important transporters of glucose through the blood–brain barrier (Dwyer et al. 2002), selectively in brain at the capillary endothelium. GLUT-1-deficiency syndrome (GLUT1-DS; OMIM 606777), first described by De Vivo et al. in 1991 (De Vivo et al. 1991), is caused by mutations of the *SLC2A1* gene and results in inadequate glucose delivery to the brain (Seidner et al. 1998). Consecutive disturbed cerebral metabolism explains the main symptoms that are developmental delay with deceleration of head circumference, early-onset and pharmaco-resistant epilepsy, and movement disorders (Wang et al. 2005). Moreover, the phenotype spectrum of GLUT1-DS has considerably expanded over recent years making early diagnosis challenging. We report the surprising case of a toddler having spontaneous permanent ketosis, a clinical and biochemical symptom which could be considered as an indicator for early diagnosis of GLUT1-DS and thereby offering treatment option by ketogenic diet.

Case Report

A 6-month-old boy, who is the first child of French non-consanguineous parents, was treated monthly by a sorcerer, because his mother thought this treatment would help against an acetonemic odor. He was referred to a neurologist

due to preprandial malaises with abnormal eye movements, loss of contact, and hypotonia of the head. Standard biological tests and an electroencephalogram were normal. Treatment by antiepileptic drugs had no success. Head circumference was in the reference range and cerebral MRI was normal at 8 months of age. A control electroencephalogram before eating revealed paroxystic epileptiform discharges and generalized discharge with intermittent light stimulation.

Frequency of the preprandial malaises accelerated during the following months and psychomotor development was retarded. The boy walked at 21 months with frequent falls. Head circumference dropped under the third percentile of age. There was no hepatomegaly. A metabolic work-up including venous blood gas analysis, liver and renal function tests, plasma electrolytes, ammonia, lactate, uric acid, amino and organic acids, carnitine, and acylcarnitines was unremarkable. A 14 h fasting test revealed excessive ketone body production with moderate hypoglycemia at the end (2.61 mmol/L): 3-OH butyrate levels raised to 4,236 µmol/L (normal value: 80–900 µmol/L) and acetoacetate raised to 2,809 µmol/L (normal value 30–400 µmol/L). Amino acid analysis revealed decrease of plasma alanine levels at the end of the test (H0: 316 µmol/L; H14: 47 µmol/L; normal values: 177–333 µmol/L), in favor of effective gluconeogenesis.

At 35 months of age, hypoglycorrhachia was observed (CSF glucose: 2.0 mmol/L, blood glucose: 4.5 mmol/L, CSF/blood glucose ratio: 0.44), suggestive of GLUT1 deficiency. Direct sequencing of the *SLC2A1* gene showed a de novo heterozygous mutation, c.349A>T (p.Lys117X) on exon 4. A ketogenic diet was started at diagnosis and helped to control the seizures.

On clinical assessment at the age of 6 years, his head circumference had regained the second percentile for age with normal height and weight. Motor development was moderately impaired in fine motor movements and he suffered from buccofacial dyspraxia.

With the ketogenic diet improving his clinical condition, and an understanding of the underlying disorder, the acetonemic odor is now accepted by his family.

Discussion

Our case illustrates the presence of spontaneous ketosis in a particular genetic condition: haploinsufficiency of the facilitative glucose transporter to the brain, GLUT1. To our knowledge, spontaneous ketosis has not been yet described in patients with GLUT1-DS (Leen et al. 2010; Pearson et al. 2013).

GLUT1 protein is encoded by the *SLC2A1* gene mapped to chromosome 1 (1p35.31.3) (Mueckler et al. 1985). Most of known *SLC2A1* gene mutations are de novo, although autosomal dominant (Brockmann et al. 2001; Klepper et al. 2001) and autosomal recessive inheritance (Rotstein et al. 2010) can be found in affected families. As far as we know the mutation c.349A>T (p.Lys117X) on exon 4 we describe here has never been reported in the literature. Analysis of the patient's parents confirmed that this mutation occurred de novo.

Haploinsufficiency of GLUT1 protein leads to an impaired glucose transport into the brain. Classically, patients with GLUT1-DS have epilepsy, developmental delay, acquired microcephaly, cognitive impairment and varying degrees of spasticity, ataxia, and dystonia. Non-classic phenotypes have also been identified, which include exclusively movement disorders or neurologic manifestations (Pons et al. 2010; Wang et al. 2005). Our patient presented a typical phenotype at the time of diagnosis, with notably episodic chaotic eye movements (opsoclonus) well described in infants with GLUT1-DS (Pons et al. 2010). The symptoms were surprisingly associated and even preceded by a spontaneous permanent ketosis, revealed by the acetonemic odor, which was the first clinical sign. The mechanism by which spontaneous ketosis occurred in our patient is open to discussion.

Ketone bodies are essential alternative energy substrates to glucose during cerebral maturation and fasting state. In the fasting state, brain glycogen storage is exhausted within minutes. Amino acids and fat cannot be used by the brain for energy production. The delivery of ketone bodies (β-hydroxybutyrate and acetoacetate) from blood to brain requires the proton-coupled monocarboxylic acid transporter proteins (MCTs), principally MCT1. The brain's ability to switch from glucose oxidation toward ketone bodies requires a cerebral metabolic adaptation (Zhang et al. 2013) (Fig. 1a). In GLUTI-DS, ketogenic diet remains the treatment of choice, which provides ketone bodies generated from dietary fatty acid oxidation in the liver. Increasing blood ketone body concentrations lead to an upregulation of MCTs at the blood–brain barrier allowing ketone body utilization by the brain (Vannucci and Simpson 2003). This metabolic adaptation may be implicated in the efficacy of the ketogenic diet in GLUT1-DS, which was observed in our patient (Klepper 2008) (Fig. 1b).

Surprisingly, spontaneous ketogenesis occurred in our patient before introduction of the ketogenic diet and independently of a fasting state. The cerebral metabolic adaptation thus occurred before the clinical manifestations of this disorder. This observation has been observed before in an animal model. Marin-Valencia et al. have shown in a mouse GLUT-1-deficiency model that total blood ketone bodies were markedly increased with normal blood glucose concentrations in a non-fasting state. This implies that GLUT-1-deficiency mice exhibit ketosis that can constitute a metabolic adaptation (Marin-Valencia et al. 2012).

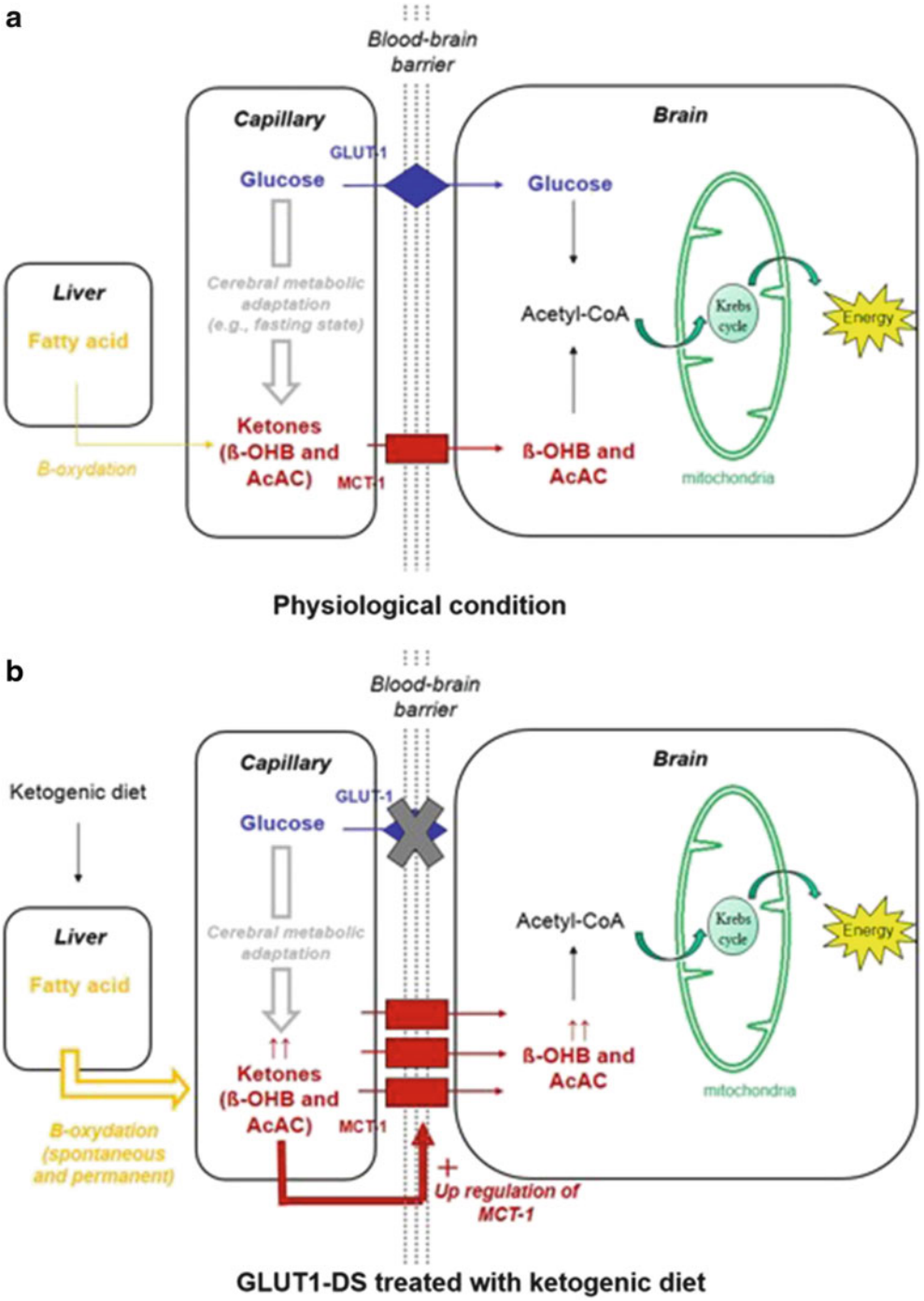

Fig. 1 Cerebral metabolic adaptation in physiological situation (**a**) and in GLUT1-DS treated with ketogenic diet (**b**)

Moreover, the onset of symptoms appeared in the toddler described here during weaning from breast milk. The higher fat content in breast milk might have had a protective role in GLUT1-DS, allowing excessive ketone body production. It has been observed before that suckling rats exhibit a relative ketosis maintained until weaning and facilitated by the high fat composition of maternal milk. The ketotic state allows utilization of non-glucose substrates, as glucose supply is shortened in the immediate postnatal period due to interruption of the permanent materno-fetal glucose supply and an initially low mobilization of glycogen stores and low gluconeogenesis rate (Fung and Devaskar 2006). Furthermore, MCT1 expression has been demonstrated in abundance in brains of rats during this suckling period indicating the potential for regulation of expression of MCT1 by dietary factors (Leino et al. 1999). The potential

for regulation of expression of MCTs by dietary factors implying enhanced transcription and translation is supported by several studies principally based on electron microscopic brain studies using high-resolution immunocytochemical methods (Leino et al. 1999; Canis et al. 2009). Genetic factors modifying the availability of energy substrates as in the case of GLUT1-DS may in this sense influence MCT densities in different tissues in order to maintain cerebral metabolic homeostasis. This adaptation allows utilization of energy substrates in response to their availability that is closed to circulating blood levels and tissue monocarboxylate concentrations (glucose, lactate, pyruvate, and ketone bodies).

The synthesis of ketone bodies is enhanced in several situations: it represents a physiological adaptation to fasting states when glucose supply is shortened. Moreover, it occurs when beta-oxidation from fatty acids is upregulated with impaired utilization of acetyl-CoA in the Krebs cycle. This may be seen in cases of compromised glucose utilization, enhanced gluconeogenesis, or shortening of the Krebs cycle intermediate, oxaloacetate. Oxaloacetate depletion may on the other hand result in impaired gluconeogenesis, thus leading to further activation of fatty acid beta-oxidation. Inherited metabolic disorders resulting in ketotic states therefore include organic acidemias; glycogen storage disorders type 0, III, VI, and IX; mitochondrial respiratory chain disorders; and, if ketone body utilization is affected, ketolysis defects (Sass 2012; Saudubray 2012). Ketone bodies represent an important alternative energy substrate for cerebral metabolism, sparing amino acid utilization for gluconeogenesis. As the brain's demand for energy supply is a central regulating factor of ketone body production, glucose entry to the brain is a key element in the regulation of ketone body synthesis. As demonstrated by the patient described here, differential diagnosis of ketotic states should therefore include GLUT1-DS (Table 1).

Table 1 Differential diagnoses of spontaneous ketosis

Inborn errors of metabolism
– Glycogen storage disease types 0, III, IV, IX
– Gluconeogenesis defects
– Branched chain organic acidemias
– Mitochondrial respiratory chain disorders
– Defects of ketolysis
– GLUT-1-deficiency syndrome
Others
– Fasting states, catabolism
– Diabetes
– Corticosteroids
– Growth hormone deficiency
– Adrenal insufficiency

Conclusion

The patient reported here presented typical features of GLUT1-DS with an original adaptive spontaneous ketosis. Permanent ketotic state may be a key diagnostic element for GLUT1-DS.

Take Home Message

GLUT1-DS can present with spontaneous ketosis, reflecting a cerebral metabolic adaptation, and should be considered in the differential diagnosis of permanent ketosis.

Compliance with Ethics Guidelines

Conflict of Interest

Alexis Chenouard, Sandrine Vuillaumier-Barrot, Nathalie Seta, and Alice Kuster declare that they have no conflict of interest.

Informed Consent

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

Patient investigation and care of the patient and planning: AK, AC.

Patient investigation (molecular analysis): SV-B, NS.

Reporting of the work described in the article: AC, SV-B, NS, AK.

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RESEARCH REPORT

Rare Late-Onset Presentation of Glutaric Aciduria Type I in a 16-Year-Old Woman with a Novel *GCDH* Mutation

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Abstract Glutaric acidemia type I (GA-I) is a treatable autosomal recessive disorder of lysine, hydroxylysine, and tryptophan metabolism caused by glutaryl-CoA dehydrogenase (GCDH) deficiency. Presentation and progression of disease are variable ranging from asymptomatic carrier state to catastrophic encephalopathy. GA-I usually presents before age 18 months, usually triggered by childhood infection, with mild or severe acute encephalopathy, striatal degeneration, and movement disorder, most often acute dystonia. At a presymptomatic stage diagnosis is suggested clinically by macrocephaly, radiologically by widened Sylvian fissures and biochemically by the presence of excess 3-hydroxyglutaric acid and glutaric acid in urine. Treatment consists of lysine-restricted diet and carnitine supplementation, specific diet restrictions, as well as symptomatic and anticatabolic treatment of intercurrent illness. Presymptomatic diagnosis and treatment are essential to prognosis. We report the case of 16-year-old macrocephalic female with late-onset GA-I and unusual paucisymptomatic presentation with fainting after exercise and widespread white matter signal changes at MRI. She was compound heterozygote for a novel mutation (IVS10-2A>G) affecting splicing at *GCDH* and a common missense mutation (c. 1240C>T; p.Arg402Trp, R402W). Interestingly, the site of the novel mutation is the nucleotide position of a common mutation found almost exclusively in patients of Chinese/Taiwanese origin (IVS10-2A>C).

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Introduction

Glutaric aciduria type I (GA-I, OMIM 231670) is a rare but treatable autosomal recessive inborn error of lysine, hydroxylysine, and tryptophan metabolism caused by mutations in the glutaryl-CoA dehydrogenase gene (*GCDH*) resulting in GCDH deficiency (Goodman et al. 1975; Koeller et al. 1995, 2004; Strauss et al. 2003). GCDH is a homotetramer mitochondrial matrix enzyme that catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA and carbon dioxide (Strauss et al. 2003). Clinical presentation with acute encephalopathic crises in infancy or early childhood (typically between 6 and 18 months) often triggered by febrile illness resulting in striatal degeneration and severe dystonic–dyskinetic movement disorder is common, but insidious-onset, late-onset, or slowly progressive cases have also been described (Strauss et al. 2003; Kölker et al. 2011; Kyllerman et al. 1994). In presymptomatic patients, macrocephaly at birth or developing

in the first months of life, wide Sylvian fissures and expansion of anterior frontotemporal CSF spaces are characteristic findings (Strauss et al. 2003; Kölker et al. 2011; Kyllerman et al. 1994; Gitiaux et al. 2008; Oguz et al. 2005). Urine organic acid analysis usually shows increased levels of glutaric acid (GA), 3-hydroxyglutaric acid (3OHGA), glutaryl-CoA, glutarylcarnitine, and glutaconic acids, although glutaric acid and dicarboxylic carnitines can be completely normal in some patients. Increased urinary excretion of glutaric acid may also occur in short-gut syndrome, intestinal infections, and the inborn errors of metabolism glutaric aciduria type 2 (GA-II, also known as multiple acyl-CoA dehydrogenase deficiency, MADD), glutaric aciduria type 3 (GA-III), α-aminoadipic acidemia, and protein lipoylation defects, but these can be differentiated biochemically by a distinct metabolite profile in urinary organic acid analysis and the absence of 3OHGA excretion (Merinero et al. 1995; Pineda et al. 1998; Nyhan et al. 1999; Knerr et al. 2002; Gordon 2006; Navarro-Sastre et al. 2011). Thus increased urinary concentrations of 3OHGA is the most sensitive and specific biochemical marker of GA-I (Al-Dirbashi et al. 2011). The diagnosis is confirmed by GCDH enzyme assay in fibroblasts or leucocytes and molecular genetic analysis (Strauss et al. 2003; Kölker et al. 2011). Prenatal or newborn screening and presymptomatic evaluation of asymptomatic siblings are essential for early diagnosis and treatment and prevention of striatal necrosis and permanent neurological sequelae (Strauss et al. 2003; Kölker et al. 2011). Currently, treatment consists of lysine-restricted diet and carnitine supplementation and prompt symptomatic and anticatabolic treatment of intercurrent illness (Kölker et al. 2006a, 2012).

Since the initial description of GA-I by Goodman et al., more than 450 cases have been documented in the literature (Goodman et al. 1975). Prevalence has been variably estimated between 1:100,000 and 1:30,000 in different studies and populations (Strauss et al. 2003; Kölker et al. 2006a, 2011). Incidence is highest in certain genetically homogeneous communities such as the Old Order Amish of Lancaster County, Pennsylvania (1 in 300–400 newborns), and the aboriginal Ojibway–Cree Indians of Northern Canada (1 in 300 newborns) where common mutations in homozygous state are often found (Morton et al. 1991; Haworth et al. 1991). The *GCDH* gene is located on chromosome 19p13.2, is 7 kb large, contains 11 exons and 10 introns, and codes for a polypeptide product of 438 amino acids, from which the functional GCDH is derived after cleavage of the 44-amino acids N-terminal targeting sequence following import into the mitochondrial matrix (Koeller et al. 1995; Schwartz et al. 1998). More than 150 pathogenic mutations have been identified of which the most frequent mutation in Caucasians is R402W (c.1204C>T) found in up to 10–20% of alleles, while the IVS10-2A>C mutation is found almost exclusively in patients of Chinese/Taiwanese origin (Strauss et al. 2003; Kölker et al. 2006a, 2011; Busquets et al. 2000a; Tang et al. 2000; Shu et al. 2003).

We hereby report the case of a paucisymptomatic 16-year-old Greek female originating from the Aegean island of Lesvos opposite the Minor Asian coast who presented with a fainting episode. Macrocephaly, characteristic neuroimaging findings and urine organic acid analysis suggested the diagnosis of GA-I. Molecular genetic testing confirmed that she was compound heterozygote for the common R402W (c.1204C>T) mutation and the novel mutation IVS10-2A>G (c.1244A>G) at the same nucleotide position as the IVS10-2A>C mutation previously reported exclusively in Far Eastern populations (Busquets et al. 2000a; Tang et al. 2000; Shu et al. 2003).

Case Report

This 16-year-old female patient was admitted to a regional hospital after fainting during physical exercise. She was conceived by IVF with the nonconsanguineous parents as sperm and egg donors and born by cesarean section after 38 weeks of gestation. Psychomotor development was delayed with gait and speech acquired at approximately 2 years of age. She was macrocephalic with occipitofrontal circumference greater than the 98th percentile for chronologic age. She reportedly had mild learning difficulties in writing, spelling, and arithmetic during elementary and secondary school; however she proceeded normally through the state school system, with good grades, despite being considered somewhat "slow" by her mother in relation to her younger sibling. At age 16, she was hospitalized because of nonconvulsive syncope after physical strain. Routine biochemistry, including thyroid status, was normal except for an elevated CRP. Evaluation by Holter electrocardiogram and transthoracic echocardiography was normal. Magnetic resonance imaging (MRI) of the brain revealed periventricular white matter hyperintensities, enlargement of frontotemporopolar CSF spaces, and characteristic widening of Sylvian fissure bilaterally ("bat-wing sign"), while MR angiography was normal. Physical examination revealed macrocephaly, brachydactyly, and mild foot clonus bilaterally. The rest of neurologic and gross neuropsychologic examination were unremarkable. Fundoscopy, EEG, EMNG, and SSEPs were all normal. Diagnosis of glutaric aciduria type I (GA-I) was confirmed by biochemical and molecular genetic studies. Treatment with low protein diet and 3 g/day of carnitine supplementation was initiated and the patient has remained stable without progression of MRI findings at one year follow-up (Kölker et al. 2006a, 2012).

Materials and Methods

Diagnosis of GA-I was supported by blood electrospray-ionization tandem mass spectrometry (ESI-MS/MS) for acylcarnitines and urine gas chromatography–mass spectrometry (GC/MS) for organic acids. Genomic DNA was obtained from fresh blood. PCR conditions and primers were used as previously described for the assessment of mutations in the GCDH gene (Busquets et al. 2000b; Biery et al. 1996). The mutations were detected by Sanger sequencing of all exons of the *GCDH* gene. The numbering of cDNA nucleotides (c.) follows the recommendations of the Nomenclature Working Group and starts with 1 at the first nucleotide of the initiator codon; thus c. numbering values are 36 nucleotides smaller than those used in older publications on GA1 mutations (Antonarakis 1998).

Results

Plasma acylcarnitine and urine organic acid analysis revealed a typical metabolite profile with total carnitine deficiency in plasma, elevated levels of plasma and urinary C5 dicarboxylic carnitine (C5DC, glutarylcarnitine), increased urinary excretion of 3OH glutaric acid (3OHGA), and markedly increased urinary excretion of glutaric acid (GA) (Table 1). Glutaconic acid values were not measured.

Magnetic resonance imaging (MRI) of the brain revealed several findings associated with glutaric aciduria I, such as (1) enlargement of temporopolar CSF spaces (Fig. 1a,i,j), (2) widening of Sylvian fissures ("bat-wing sign") (Fig. 1b, d), (3) widened mesencephalic cistern (Fig. 1c,j,l), (4) periventricular and deep white matter hyperintensities (Fig. 1d–h), and (5) abnormal hyperintensity on T2WI of splenium of corpus callosum (Fig. 1f), dentate nucleus (Fig. 1j), and substantia nigra (Fig. 1k).

Molecular genetic studies with direct sequencing of all exons of the *GCDH* gene detected the common c.1204C>T missense mutation in exon 10 (substitution of Arg by Trp at codon 402, R402W) and the novel c.1244A>G transversion (IVS10-2A>G), causing an alteration in splicing.

Discussion

Glutaric aciduria type I is an autosomal recessive neurometabolic disease that is classified among the *cerebral organic acid disorders*, a subgroup of *organic acid disorders*, where the central nervous system (CNS) is predominantly and severely affected (Strauss et al. 2003; Kölker et al. 2011, 2013). It is an inborn error of lysine, hydroxylysine, and tryptophan metabolism (Strauss et al. 2003; Kölker et al. 2011). Its pathophysiological basis is glutaryl-CoA dehydrogenase deficiency that results in accumulation of organic acids, such as GA and 3OHGA, that share structural and functional similarities with glutamate, in the CNS where they are believed to exert neurotoxic, gliotoxic, and myelinotoxic effects by several pathomechanisms, involving excitotoxic neuronal and oligodendroglial injury, dysregulation of mitochondrial energy production, and oxidative stress (Strauss et al. 2003; Kölker et al. 2008, 2011). Indeed, in GA-I brain GA has been found to exceed plasma and CSF levels by up to two orders of magnitude even in patients with normal or low GA levels in urine, a counterintuitive finding best explained by the "trapping hypothesis" enunciated by Kölker et al. (Strauss et al. 2003; Kölker et al. 2006b, 2011; Merinero et al. 1995; Pineda et al. 1998; Nyhan et al. 1999; Sauer et al. 2006, 2011).

More than 150 pathogenic mutations (missense, nonsense, and intronic variants) in the *GCDH* gene have been so far documented (Strauss et al. 2003; Kölker et al. 2006a, 2011, 2012). The disease exhibits a remarkable clinical variability, but apart from a covert correlation between residual enzymatic activity as determined by the so-called "severe" or "mild" mutations and biochemical phenotype, no other genotypic–phenotypic relationship has been established (Busquets et al. 2000b; Mühlhausen et al. 2003; Funk et al. 2005; Gallagher et al. 2005; Christensen et al. 2004). For example, the missense R402W (p.Arg402Trp) mutation in exon 10, which is the commonest worldwide, accounting for up to 20% of all mutations in different studied populations, when expressed in a prokaryotic system leads to only 3% residual GCDH activity, while the A421V (p.Ala421Val) missense mutation in exon 11, which is prevalent in the Old Order Amish of Pennsylvania, results in 40% residual enzyme activity in the same model (Busquets et al. 2000a, b; Biery et al. 1996). Homozygosity or compound heterozygosity of "severe" mutations is reflected in the typically abnormal urinary organic acid profile, while heterozygosity of at least one "mild" mutation may result in normal or mildly affected metabolic profile (Mühlhausen et al. 2003; Funk et al. 2005; Gallagher et al. 2005; Christensen et al. 1997, 2004). Although according to one meta-analysis ($n = 115$) GA in urine was increased

Table 1 Blood and urine biochemistry results

Metabolite	Result (μmol/L)	Normal (μmol/L)
Free carnitine (C0)	5.90	5.00–75.00
Glutaryl carnitine (C5DC)	1.47	0.00–0.10
Total carnitine	12.19	25.0–75.0
Glutaric acid (GA)	968.1	0.0–3.8
3-Hydroxyglutaric (3OHGA)	36.8	0.0–4.6

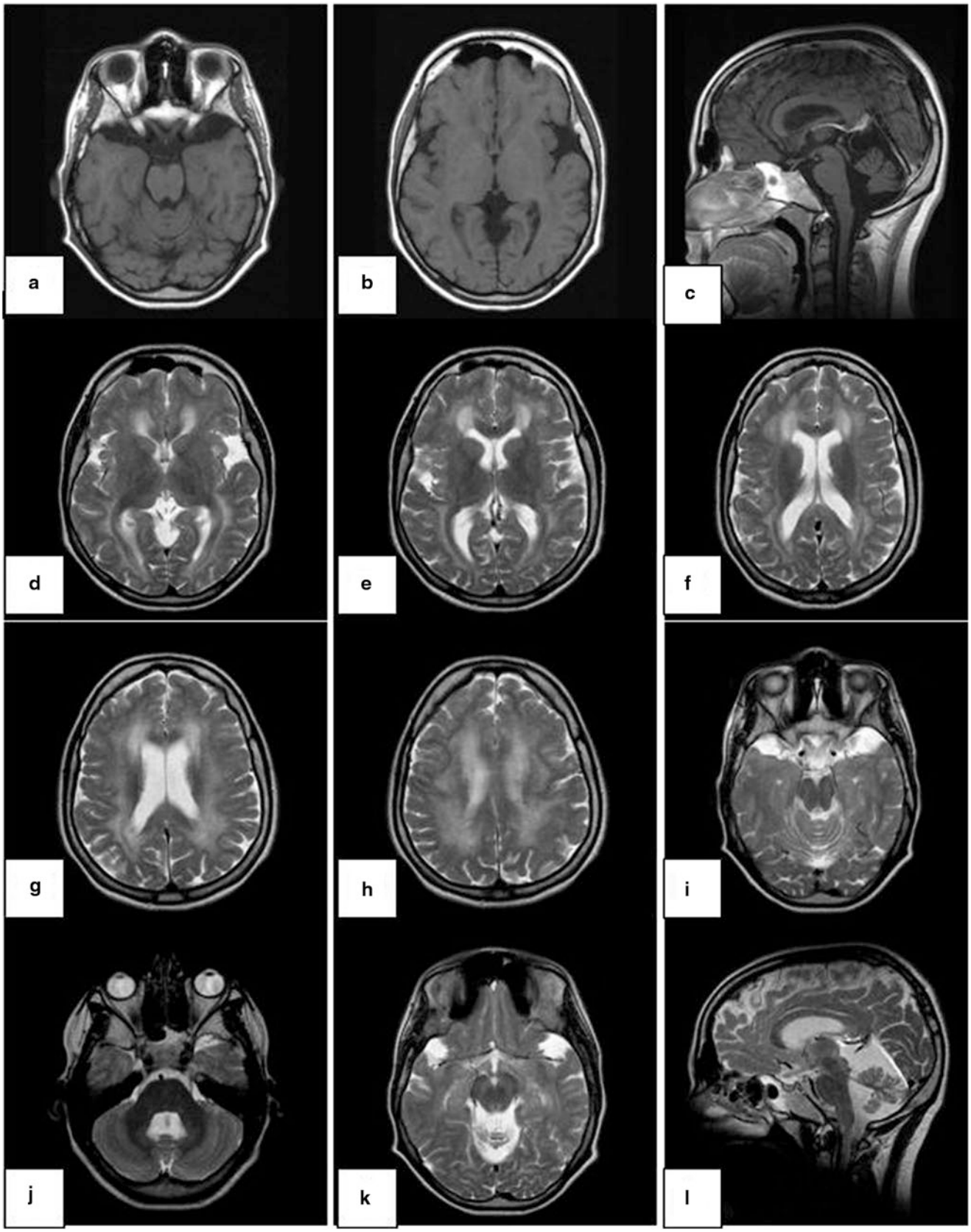

Fig. 1 Magnetic resonance imaging of the brain revealed enlargement of temporopolar CSF spaces (**a**, **i**, **j**), widening of Sylvian fissures ("bat-wing sign") (**b**, **d**), widened mesencephalic cistern (**c**, **j**, **l**), periventricular and deep white matter hyperintensities (**d**–**h**), and abnormal hyperintensity on T2WI of the splenium of corpus callosum (**f**), dentate nucleus (**j**), and substantia nigra (**k**)

in 97% of cases, it has been postulated that patients with GA-I can be categorized biochemically in high and low excretors of GA and 3OHGA and that "severe" mutations, such as R402W (p.Arg402Trp) or A293T (p.Ala293Thr), are most frequent in high excretors, while "mild" mutations such as V400M (p.Val400Met) or R227P (p.Arg227Pro) are only found in low excretors, because compound heterozygosity with at least one "mild" mutation has a rescue effect on both residual GCDH activity and metabolite excretory profile (Busquets et al. 2000b; Biery et al. 1996; Mühlhausen et al. 2003; Funk et al. 2005; Gallagher et al. 2005; Christensen et al. 1997, 2004; Bjugstad et al. 2000). However, despite the characterization of at least two genetically and biochemically distinct patient groups, no correlation has been found between residual GCDH activity or urinary organic acid profile and clinical phenotype and thus between severity of genetic lesion and clinical picture (Busquets et al. 2000b; Bjugstad et al. 2000). This is well exemplified by the often described discordant phenotypes of homozygote siblings or even twins (Zafeiriou et al. 2000).

There is another apparent phenotypic dichotomy that relates to the clinical presentation of GA-I. Typically, GA-I presents in infancy after a precipitating illness with acute metabolic encephalopathy that in a matter of days results in striatal necrosis with stroke-like characteristics and a severe irreversible neurological syndrome variably encompassing axial hypotonia, generalized dystonia and other dyskinetic/hyperkinetic movement disorders, spasticity, developmental regression, seizures, and ultimately dystonic tetraparesis (Strauss et al. 2003; Kölker et al. 2006a, 2011; Kyllerman et al. 1994; Bjugstad et al. 2000). According to a meta-analysis ($n = 115$), GA-I onset before 24 months of age occurs in 87% of cases, with a critical susceptibility time window between 6 and 9 months, when about 25% of infantile acute encephalopathic cases debut and the probability of a poor outcome is highest (Bjugstad et al. 2000). In this study, 8.7% of patients were asymptomatic/paucisymptomatic without disease progression apart maybe from macrocephaly present at birth or developing in the first months of life and neuroimaging findings that remained undetected (Bjugstad et al. 2000). Most of these cases are in fact presymptomatic rather than asymptomatic and encompass at-risk relatives detected by screening or subjects that have not yet had an encephalopathic event but received a diagnosis of GA-I on neuroradiological grounds. In any case, an acute encephalopathic crisis with striatal necrosis is the major prognostic factor of morbidity and mortality in GA-I (Kölker et al. 2011; Bjugstad et al. 2000). However, in a minority of cases, the onset is insidious with gradual disease progression and the absence of an acute encephalopathic episode or major pathological changes of basal ganglia in imaging, but, as a rule, presence of other hallmark GA-I clinical and radiological signs, such as macrocephaly and widening of Sylvian fissures (Strauss et al. 2003; Kölker et al. 2011).

Late-juvenile or adult-onset GA-I is extremely rare. There have been scant reports in the medical literature, and in fact, some cases reported as "adult-onset GA-I" were simply childhood-onset GA-I diagnosed in adulthood (Prevett et al. 1996; Corral I et al. 2001; Bähr et al. 2002; Twomey et al. 2003; Fernandez-Alvarez et al. 2003; Külkens et al. 2005; Sonmez et al. 2007; Harting et al. 2009; Chen J et al. 2011). According to the few reports of truly adult-onset GA-I (onset at the age of 18 years or older), (1) the patients were paucisymptomatic or asymptomatic at the time of diagnosis, (2) the disease followed either an acute encephalopathic or a non-encephalopathic clinical course, and (3) in all cases supratentorial, diffuse, symmetric U-fiber-sparing leukoencephalopathy involving periventricular and deep white matter was seen in cranial MRI. Bähr et al. reported the case of a 19-year-old woman without macrocephaly, who presented with recurrent headaches, mild ophthalmoparesis, and hyperreflexia (Bähr et al. 2002). Fernandez-Alvarez et al. described the case of an adolescent girl without macrocephaly and a history of postural hand tremor that by the age of 19 had developed focal dystonia and orofacial dyskinesia (Fernandez-Alvarez et al. 2003). Külkens et al. reported the interesting case of a 15-year-old boy with macrocephaly, psychomotor retardation, progressive vertigo, and intermittent severe diffuse headaches often induced by physical exercise and relieved by rest (Külkens et al. 2005). Sonmez et al. described the case of a 20-year-old man with a 6-month history of recurrent headaches and hyperactive muscle stretch reflexes (Sonmez et al. 2007). Finally, a Chinese patient has been described with a purported ischemic cerebral stroke in young adulthood, whereby the correct diagnosis of GA-I was later made (Chen et al. 2011). More remarkable was a case reported by Külkens et al. of a 66-year-old man macrocephalic since infancy with a history of severe intermittent headaches since the age of 35 and a complex progressive neuropsychiatric syndrome since the age of 50 encompassing hand tremor, seizures, dementia, and aggressive behavior with acoustic and visual hallucinations (Külkens et al. 2005). In most of these atypical late-onset cases, diagnosis was incidental and suggested by MRI findings and metabolic screening. By contrast, Prevett et al. reported the case of a young lady with mild psychomotor retardation, gait disturbance, and orofacial dyskinesia presenting at 7 years of age, while Corral et al. described two siblings with infantile onset of acute encephalopathy and dystonia at age 9 and 16 months, respectively, that were eventually diagnosed with GA-I in adulthood, at ages 29 and 24, respectively, after an expanded diagnostic procedure with MRI and urine organic acid analysis, but did not

respond to treatment because of advanced disease (Prevett et al. 1996; Corral et al. 2001).

White matter involvement is common in GA-I with an incidence of 56% (Strauss et al. 2003; Kölker et al. 2011; Bjugstad et al. 2000). MRI findings in GA-I associated white matter disease or leukoencephalopathy are diffuse periventricular and deep white matter signal changes appearing as hypointensities in T1W and hyperintensities in T2W and FLAIR images, with low apparent diffusion coefficient (ADC) values and restricted diffusion in DW images. Less frequently high signal on T2W can involve the corpus callosum or medial lemniscus (Kölker et al. 2011; Oguz et al. 2005). Magnetic resonance spectroscopy (MRS) of affected CNS white or gray matter areas may reveal decreased *N*-acetylaspartate/creatine, slightly increased or normal choline/creatine, and increased myo-inositol/creatine ratios, corresponding to neuroaxonal damage, delayed myelination or demyelination, and astrocytosis, respectively (Oguz et al. 2005). Neuropathology demonstrated extensive spongiform leukoencephalopathy at many sites in white matter at the level of cerebral cortex, centrum semiovale, internal capsule, central tegmental tracts or brachium of inferior colliculus without significant reactive astrocytosis, and marked spongiform myelinopathy in myelinated fascicles of putamen and other nuclei such as thalamus, substantia innominata, or inferior colliculus, while arcuate fibers and cortical grey matter were spared (Funk et al. 2005). White matter involvement in GA-I may be found in both early-onset acute encephalopathic and late-onset or progressive degenerative clinical subtypes and may precede striatal damage or be an incidental neuroradiological finding during screening of presymptomatic relatives (Strauss et al. 2003; Kölker et al. 2011; Funk et al. 2005). Leukoencephalopathy is not an age-specific or clinical subtype-specific manifestation in GA-I, as most children with the disease have white matter abnormalities, but it seems to be correlated with disease duration (Bähr et al. 2002). This conclusion is supported by studies of *Gcdh*-deficient mice, where the biochemical phenotype was similar to GA-I patients but there was no striatal pathology and no development of dystonia (Koeller et al. 2004). Yet, in this mouse model spongiform myelinopathy, found in white matter or cortex, increased with age and was similar to the spongiform leukoencephalopathy of GA-I patients (Koeller et al. 2004).

Leukoencephalopathy, albeit of different etiopathogenesis, extent and clinical characteristics, is an important feature of most cerebral organic acid disorders, including glutaric aciduria type I, aspartoacylase deficiency (or Canavan disease), L-2-hydroxyglutaric aciduria, and D-2-hydroxyglutaric aciduria (Kölker et al. 2006b, 2013; Sauer et al. 2006, 2011). Notably, macrocephaly is also a frequent and prominent sign of the first three (Strauss et al. 2003; Kölker et al. 2011). It has been hypothesized that accumulation of organic acids with neurotoxic and myelinotoxic potential might hamper CNS myelination, causing hypomyelination, dysmyelination, delayed myelination, or demyelination, during a critical period of development of the nervous system (Kölker et al. 2006b, 2008, 2011, 2013; Sauer et al. 2006, 2011). This is believed to be mediated by several direct or indirect mechanisms affecting immature and mature oligodendrocytes or neurons, such as excitotoxicity via AMPA/kainate and NMDA receptors, impairment of mitochondrial function and oxidative phosphorylation, generation of reactive oxygen species, deregulation of glutamergic and GABAergic neurotransmission, and cytokine-induced amplification of cellular damage (Strauss et al. 2003; Kölker et al. 2006b, 2008, 2011, 2013; Sauer et al. 2006, 2011; Funk et al. 2005).

The late-onset case of GA-I presented here is remarkable for the following reasons: Few well-documented late-onset GA-I case reports have been published heretofore (Bähr et al. 2002; Twomey et al. 2003; Fernandez-Alvarez et al. 2003; Külkens et al. 2005; Sonmez et al. 2007; Harting et al. 2009; Chen et al. 2011). Our patient was asymptomatic at 16 years of age, apart from macrocephaly and mild psychomotor delay since infancy, and the diagnosis was made incidentally after a fainting episode during physical exercise without encephalopathy. Despite the paucisymptomatic status of this patient, MRI showed white matter disease. Molecular genetic analysis of the *GCDH* revealed compound heterozygosity for the common R402W (p.Arg402Trp) mutation and a *novel* mutation, IVS10-2A>G, at a nucleotide position previously known to be mutated (IVS10-2A>C) exclusively in patients of Chinese/Taiwanese origin (Busquets et al. 2000a; Tang et al. 2000; Shu et al. 2003). Based on the results of urine organic acid analysis, the patient being a high excretor of GA and 3OHGA, we could only deduce a genotype–biochemical phenotype correlation, and we hypothesize that the novel pathogenic mutation in the *GCDH* gene described in this case report (c.1244A>G transversion; IVS10-2A>G) does not alleviate the effect of the common R402W mutation on residual GCDH activity and metabolite excretory profile. The novel mutation described expands the spectrum of mutations causally linked to the disease and asserts the importance of splicing defects in disease pathogenesis.

Given the fact that GA-I is a treatable inborn metabolic disorder, the clinical case reported herein emphasizes the importance of including GA-I in the differential diagnosis of leukoencephalopathy discovered in adolescence and early adulthood, especially if combined with macrocephaly and characteristic MRI findings. It also serves to highlight that thorough biochemical evaluation with chromatographic analysis of urine organic acids in asymptomatic or paucisymptomatic children or adolescents with macrocephaly

will prevent diagnostic delay in GA-I and may prove vital for prognosis (Heringer et al. 2010). Although our case escaped the severe dystonic encephalopathy associated with GA-I, this is a rare occurrence. Thus, early diagnosis of GA-I and prompt implementation of appropriate dietary, therapeutic, and preventive measures can dramatically improve prognosis for this treatable inborn error of metabolism (Heringer et al. 2010).

Compliance with Ethics Guidelines

Conflict of Interest

All the authors of this chapter declare that there are no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the patient included in this study. Proof that informed consent has been obtained is available upon request.

Animal Studies

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

Author Contributorship Statement

Matthew J Fraidakis: conception and design, analysis, and interpretation of data, writing/drafting of the manuscript, final approval of article.

Chrissa Liadinioti: data acquisition.

Argyris Dinopoulos: data acquisition, analysis, and interpretation of data.

Matilda Papathanassiou: data acquisition, analysis, and interpretation of data.

Judit Garcia-Villoria: data acquisition, analysis, and interpretation of data.

Antonia Ribes: data acquisition, analysis, and interpretation of data.

Roser Pons: conception and design, analysis, and interpretation of data, final approval of article.

Leonidas Stefanis: conception and design, analysis, and interpretation of data, final approval of article.

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RESEARCH REPORT

The Biological Clock and the Molecular Basis of Lysosomal Storage Diseases

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Abstract The lysosomal storage disorders encompass nearly fifty diseases provoked by lack or deficiency of enzymes essential for the breakdown of complex molecules and hallmarked by accumulation in the lysosomes of metabolic residues. Histochemistry and cytochemistry studies evidenced patterns of circadian variation of the lysosomal marker enzymes, suggesting that lysosomal function oscillates rhythmically during the 24-h day. The circadian rhythmicity of cellular processes is driven by the biological clock ticking through transcriptional/translational feedback loops hardwired by circadian genes and proteins. Malfunction of the molecular clockwork may provoke severe deregulation of downstream gene expression regulating a complex array of cellular functions leading to anatomical and functional changes. In this review we highlight that all the genes mutated in lysosomal storage disorders encode circadian transcripts suggesting a direct participation of the biological clock in the pathophysiological mechanisms underlying cellular and tissue derangements hallmarking these hereditary diseases. The 24-h periodicity of oscillation of gene transcription and translation could lead in physiological conditions to circadian rhythmicity of fluctuation of enzyme levels and activity, so that gene transfer could be envisaged to reproduce 24-h periodicity of variation of enzymatic dynamics and circadian rhythmicity could have an impact on the schedule of enzyme replacement therapy.

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Introduction

The lysosomal storage disorders (LSDs) comprise about fifty diseases that are caused by the absence or insufficiency of particular enzymes necessary for the breakdown of complex molecules and are hallmarked by amassing in the lysosomes of metabolic residues (Futerman and van Meer 2004; Ballabio and Gieselmann 2009). Lysosomes are cytoplasmic organelles found in nearly every eukaryotic cell, which take part crucially in fundamental aspects of cellular homeostasis such as membrane repair, autophagy, endocytosis, and protein metabolism. LSDs are inherited prevalently in an autosomal recessive manner and only in a minor part as X-linked recessive disorders; even though each of them affects a modest fraction of the population, as a group LSDs represent an important challenge for the health-care systems (Meikle et al. 1999). The genetic

modifications responsible of the LSDs alter the correct working of the lysosomes through the shortage of enzymes catalyzing hydrolysis of molecular substrates, hydrolase activators, or transporters or hinder the vesicular transport in the endosomal/lysosomal system causing lysosomal storage of substrates specific for each disorder type (Journet et al. 2002; Platt and Walkley 2004; Bagshaw et al. 2005; Lübke et al. 2009). Based on these premises, the LSDs are classified in defects in glycan degradation, defects in lipid degradation, defects in protein degradation, defects in lysosomal transporters, and defects in lysosomal trafficking. The lysosomal accumulation leads to formation of large intracellular vacuoles and perturbs the proper functioning of the cell, causing functional and anatomic derangements at the tissue level in numerous organ systems that are responsible of LSDs' clinical manifestations (Bellettato and Scarpa 2010). The biological processes underlying cell, tissue, and organ system function show fluctuations that may be rhythmic, and when the periodicity corresponds to approximately 24 h, the rhythm is defined circadian (from the Latin words *circa*, about, and *diem*, a day) (Mazzoccoli et al. 2011a). Subcellular organelles, such as endoplasmic reticulum structures, show nycthemeral fluctuations in the relative amounts and regional differences in their distribution (Chedid and Nair 1972). Accordingly, histochemistry and cytochemistry studies evidenced patterns of circadian variation of the lysosomal marker enzymes, suggesting that lysosomal function oscillates rhythmically during the 24-h day (Bhattacharya and von Mayersbach 1976; Uchiyama et al. 1981; Uchiyama and von Mayersbach 1981).

In this review article we meant to suggest a possible involvement of the biological clock in the physiopathology of lysosomal storage diseases. We have reviewed the scientific literature on this issue and reported information from comparison of publicly available databases to corroborate our proposal.

The Circadian Clock Circuitry

Circadian rhythmicity characterizes physiological phenomena of all living beings and is driven in mammals by the circadian timing system, comprising central and peripheral oscillators (Hastings et al. 2003; Dibner et al. 2010; Mazzoccoli et al. 2011b, 2012a, b). The central oscillators are located in the hypothalamus and are represented by the neurons of the suprachiasmatic nuclei, which are influenced by photic stimuli perceived by the retinal ganglion cells and conveyed by the retino-hypothalamic tracts (Kalsbeek et al. 2006; Challet 2007). The alternation of light and darkness related to Earth's rotation around its axis entrains the ticking of the central oscillators in the SCN, which in turn drives autonomous and self-sustained oscillators in the peripheral tissues through neural pathways (autonomic nervous system fibers) and humoral mediators (cortisol, melatonin) (Mazzoccoli 2011; Cailotto et al. 2009). At the cellular level the oscillation of biological functions is driven by molecular clockworks ticking through transcriptional-translational feedback loops operated by a set of genes called clock genes, which encode transcripts oscillating with circadian rhythmicity (Ko and Takahashi 2006; Lowrey and Takahashi 2011). The positive limb of the loop is started by the transcription factors Clock (or its paralog Npas2) and Arntl, also called Bmal1 (or its homologue Arntl2/Bmal2), which heterodimerize and activate the transcription of the genes Period (Per 1–3) and Cryptochrome (Cry1-2) (Nagoshi et al. 2004). The circadian proteins Per 1–3 and Cry 1–2 heterodimerize, pass back into the nucleus, and hinder the transcriptional activity of Clock/Arntl heterodimer, closing the loop in approximately 24 h (Duguay and Cermakian 2009). The PER and CRY proteins are posttranslationally modified by different processes, represented by phosphorylation, sumoylation, ubiquitylation, acetylation, and deacetylation, which influence their activity and degradation (Eide et al. 2002; Cardone et al. 2005). Phosphorylation is operated by casein kinase (CK)Iδ and CKIε and glycogen synthase kinase (GSK) 3-β, which target circadian proteins for degradation and regulate their nuclear translocation (Agostino et al. 2009; Sahar et al. 2010). A particular role is played by AMP-dependent kinase (AMPK) whose activity is influenced by ATP/AMP ratio and works also as a nutrient sensor (Fulco and Sartorelli 2008; Cantó and Auwerx 2009; Lamia et al. 2009). Acetylation is operated by Clock that has histone-acetyltransferase activity (Doi et al. 2006), whereas deacetylation is operated by SIRT1, an NAD+-dependent protein deacetylase necessary for high-magnitude circadian transcription of several core clock genes (Asher et al. 2008). The activity of SIRT1 depends on the availability of its cofactor, NAD+, whose synthesis is regulated by NAMPT, encoded by a clock-controlled gene that steers the salvage pathway responsible for circadian oscillation of NAD+ levels and ultimately of SIRT1 activity gauging the energy status of the cell and driving mitochondrial oxidative metabolism (Rutter et al. 2001; Ramsey et al. 2009; Nakahata et al. 2008, 2009; Peek et al. 2013). Besides, Clock/Arntl heterodimer activates the expression of the nuclear receptors Rev-erbα (encoded by NR1D1 gene) and Rorα (encoded by Rora gene), which drive in that order negatively and positively the rhythmic transcription of Bmal1, through competition for binding to a response element on the promoter of the Bmal1 gene (Preitner et al. 2002; Burris 2008; Bugge et al. 2012; Mazzoccoli et al. 2012c; Cho et al. 2012; Jetten et al. 2013). A gene necessary for circadian clock function in *Drosophila melanogaster* is Timeless (Tim), which is maintained in mammals and interplaying with its partner.

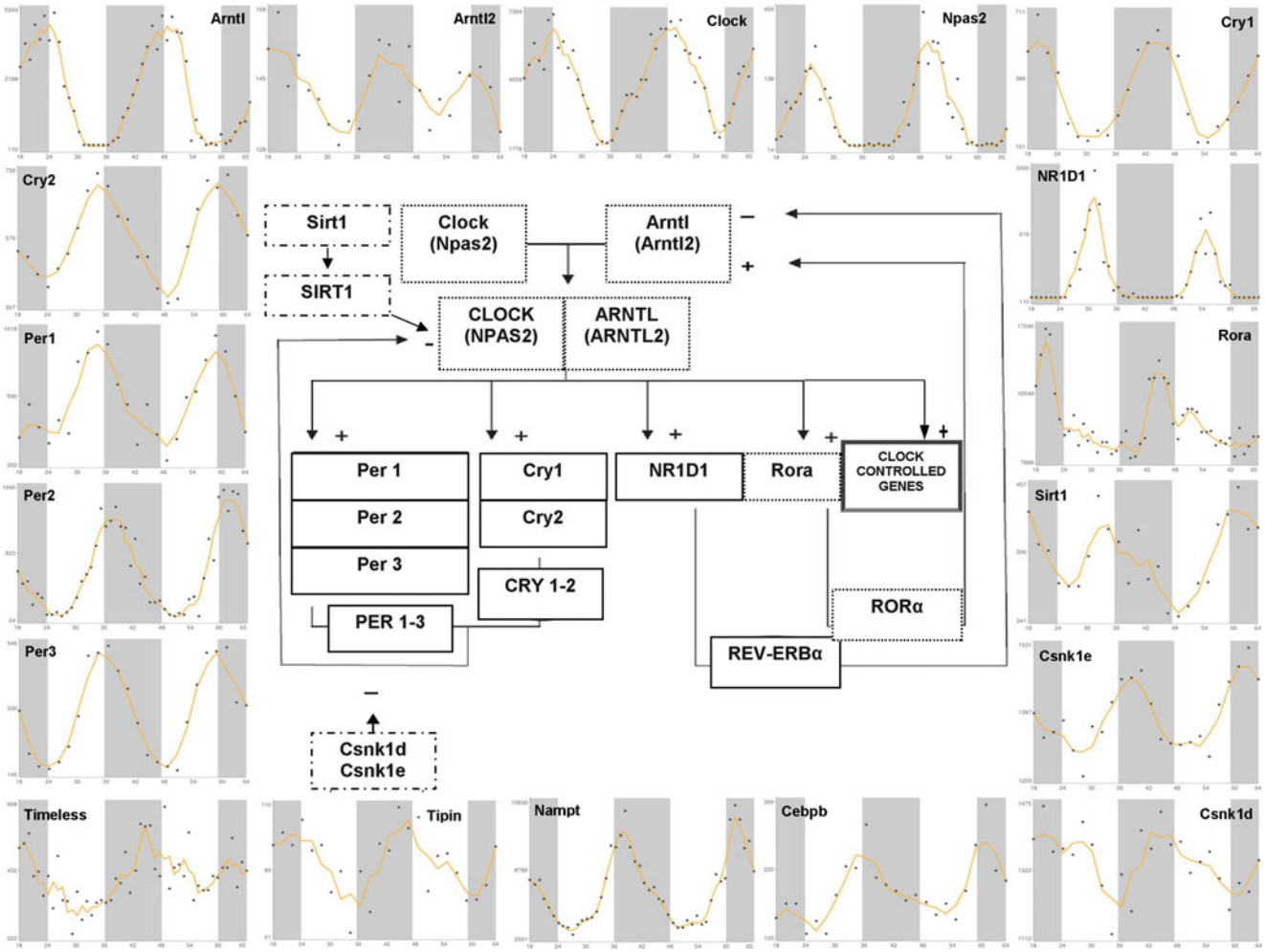

Fig. 1 *x–y* plots showing the time-qualified profile of expression of core clock genes and clock-controlled genes (*source*: CircaDB, web addresses http://circadb.org, http://github.com/itmat/circadb, http://bioinf.itmat.upenn.edu/circa) and a scheme rendering the interactions among the cogwheels of the molecular clockwork. *Continuous line boxes* indicate transcriptional repressors such as PER, CRY, and REV-ERBα; *dotted line boxes* indicate transcriptional activators such as CLOCK, BMAL1, and RORα; *dashed-dotted line boxes* indicate posttranslational modifications catalyzed by SIRT1 and casein kinases such as CSNK1D and CSNK1E. On the *x* axis is represented time in hours, on the *y* axis are represented the mRNA relative expression levels in arbitrary units

TIMELESS-interacting protein (TIPIN) plays a role to regulate DNA replication processes under both normal and stress conditions. TIMELESS and TIPIN are important for ataxia telangiectasia and Rad3-related (ATR)-checkpoint kinase (Chk)1 and ataxia telangiectasia mutated (ATM)-checkpoint kinase (Chk)2-mediated signaling and S-phase arrest (Unsal-Kaçmaz et al. 2007; Smith et al. 2009; Yang et al. 2010; Kemp et al. 2010) (Fig. 1).

The Biological Clock in Physiology and Pathology

The molecular clockwork drives the expression of thousands of genes, so that gene expression profiling studies performed with different algorithms and sampling frequency evidenced that approximately 5–20% of the transcriptome displays circadian rhythmicity (Asher and Schibler 2011). These genes are defined clock-controlled genes (CCGs) and steer cell processes, such as proliferation, differentiation, cell cycle, apoptosis, autophagy, and DNA damage response (Bozek et al. 2009). Tissue-specific output genes driven by the biological clock are responsible for various metabolic and homeostatic processes and distinctively control organ functions, synchronizing them to circadian environmental cues and making them available when mainly required at definite times during the 24-h day (Mazzoccoli et al. 2012d). In this way, the circadian outlines of metabolic gene expression may properly fit the swap between catabolic and anabolic phases corresponding with cycles of sleep/rest/fasting and wake/activity/feeding, respectively (Bass and Takahashi 2010; Bass 2012). This process is regulated by nuclear receptors expressed with 24-h periodicity in metabolically active tissues (liver, white

and brown adipose tissues) (Yang et al; 2006), which recruit cofactors, coactivators, and corepressors and together with other processes, such as rhythmic histone methylation/demethylation, drive circadian waves of chromatin remodeling and epigenetic modifications impinging on transcriptional events and connect the molecular clockwork to metabolism integrating energy flux with varying physiological requirements during daytime and nighttime (Alenghat et al. 2008; Asher et al. 2010; Yin et al. 2010; Grimaldi et al. 2010; Berrabah et al. 2011; Di Tacchio et al. 2011; Dufour et al. 2011; Feng et al. 2011; Valekunja et al. 2013). The alteration of proper synchronization among physiological, behavioral, transcriptional, translational, and posttranslational modification rhythms underlies the pathological basis of metabolic, inflammatory, degenerative, and neoplastic diseases (Takahashi et al. 2008; Mazzoccoli et al. 2010; Maury et al. 2010; Anderson et al. 2013; Bonny et al. 2013; Vinciguerra et al., 2013; Tevy et al. 2013; De Cata et al. 2014; Mazzoccoli et al. 2014).

The Biological Clock and the LSDs: Toward and Beyond Lysosomes

Several experimental efforts using different methodologies have been carried out to identity lysosomal genes, and the categorization of the genes encoding structural, transport, and enzymatic proteins included in the lysosome allows a better understanding of the biology of this subcellular organelle. The Human Lysosome Gene Database (hLGDB) makes available a complete and reachable census of the 435 human genes encompassed in the lysosomal system (Brozzi et al. 2013) (Supplementary Table 1). We matched the genes stored in this database with those obtained by time-qualified genome-scale RNA profiling performed to identify and compare circadian transcripts from mouse liver (Hughes et al. 2009). A set of 2,892 sequence symbols (protein-coding genes, retained introns, pseudogenes, lincRNA, etc.) was refined through conversion of the symbols by using the Mouse Genome Informatics (v 5.17) resource (http://www.informatics.jax.org/batch) and obtaining current and old symbols, Ensembl Gene IDs, corresponding gene names, and feature type information. Among the 435 lysosomal genes listed in the hLGDB human database, 365 genes resulted expressed with circadian rhythmicity (http://circadb.org), and 70 did not show any 24-h periodicity (Fig. 2). On these premises, approximately 80% of the lysosomal genes turn out to oscillate with circadian rhythmicity and arguably are adequate to drive the nycthemeral changes of enzymatic activity and functional processes in the lysosome. Besides, a process tightly linked to lysosomal function is represented by autophagy (Schultz et al. 2011), which shows fluctuations with a pattern of circadian rhythmicity driven directly by the molecular clockwork through the transcription factor CEPB/β, whose expression is controlled by transcriptional activity of Bmal1 through binding at E-box sequences in its promoter, and in turn CEPB/β steers the circadian expression of genes encoding autophagy-related proteins (Ma et al. 2011; Ma and Lin 2012). Another mechanism involved in the pathogenesis of LSDs is represented by endoplasmic reticulum (ER) stress and unfolded protein response (UPR), an adaptive reaction universally preserved to handle the accumulation of unfolded proteins in this subcellular compartment, which eventually leads to apoptosis to preserve the organism in the case it is not sufficient. The amassing of unfolded proteins in the ER turns on IRE1a, PERK, and ATF6 pathways, causing the nuclear translocation of the transcription factors XBP1, ATF4, and ATF6, respectively, which induce the expression of genes encoding proteins involved in peptide folding and degradation to minimize the accumulation of unfolded proteins. UPR shows rhythmic oscillations with ultradian periodicity of approximately 12 h, and UPR-regulated genes are hallmarked by a rhythmic expression according to an ensuing 12-h period dependent on a functional circadian clock. This rhythmic activation of UPR-regulated genes seems to be the consequence of the activation of the IRE1a-XBP1 pathway, which is activated according to the same 12-h period rhythm (Cretenet et al. 2010). Secretory and transmembrane proteins fold in the ER into their native conformations and undergo posttranslational modifications, but alteration of these processes causes accumulation of misfolded proteins in the ER lumen and triggers the UPR. According to studies demonstrating UPR activation in fibroblasts from a large range of LSDs, this mechanism was recently advocated as a frequent mediator of apoptosis in LSDs. Accumulation of unfolded proteins can take place in response to changes in the ER environment, including nutrient starvation and reducing agents. UPR is also related to exhaustion of ER calcium stores and in LSDs has been found alteration of calcium homeostasis, suggesting that this pathway could be engaged in the pathological mechanisms set in motion in the diseases linked to lysosomal accumulation of unmetabolized substrates as well (Vitner et al. 2010). Besides, even if lysosome engulfment is the central derangement in LSDs, defective activity of lysosomal proteins prompts a number of pathogenic cascades, among which a leader role is played by improper activation of inflammation and immune response that may perpetuate inducing a chronic reaction (Vitner et al. 2010). These processes are linked to transcriptional circuits tightly controlled and temporally driven by the biological clock, so that inflammatory signaling pathways and immune-mediated responses are characterized by circadian rhythmicity of activity, rendered by nycthemeral variations of levels of humoral factors and

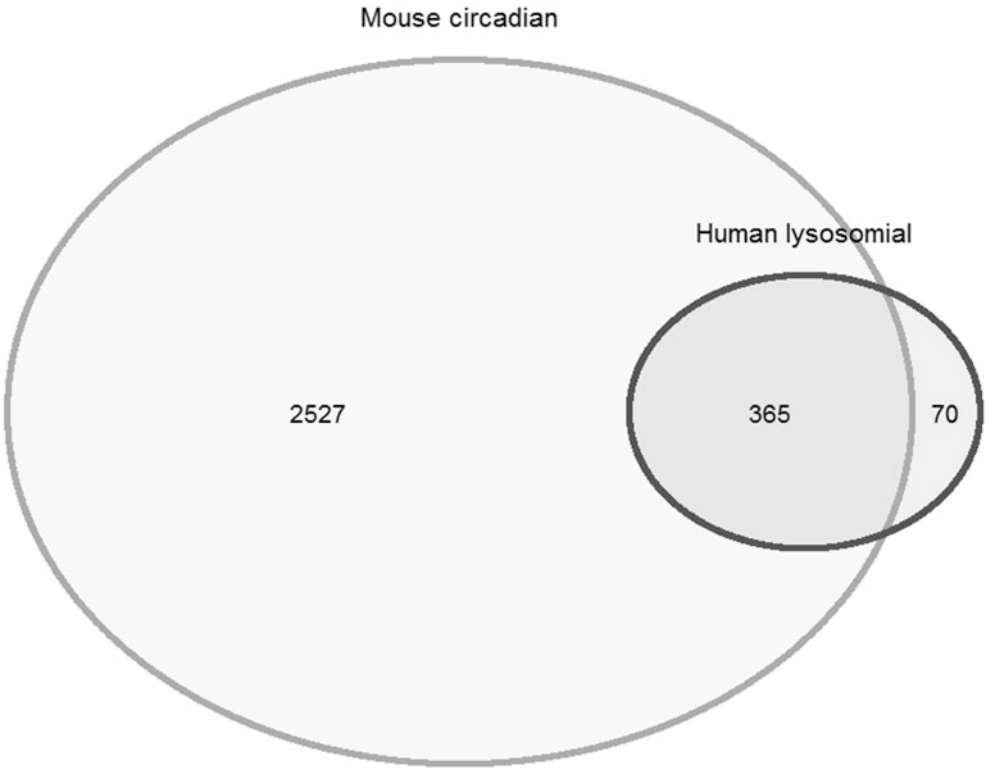

Fig. 2 Comparison between 435 human lysosomal genes against 2,892 circadian protein-coding genes of *Mus musculus*. The mouse genes derive from refinement of a set of sequence symbols (protein-coding genes, retained introns, pseudogenes, lincRNA, etc., doi:10.1371/journal.pgen.1000442.s012). Refinement was done by converting the symbols by using the Mouse Genome Informatics (v 5.17) resource (http://www.informatics.jax.org/batch) and obtaining current and old symbols, Ensembl Gene IDs, corresponding gene names and feature type information

cellular effectors, as well as phagocytic, complement, lysozyme, and peroxidase activity in innate immunity, and antibody and cytokine production, leukocyte trafficking, proliferation, and apoptosis in adaptive immunity (Cermakian et al. 2013; Vinciguerra et al. 2013, 2014).

The Molecular Clockwork and Circadian Rhythmicity in LSDs

The hereditary diseases related to anomalous storage of molecules and intermediary metabolites in the lysosome are characterized by alteration of anatomical integrity and physiological function of many tissues and organ systems in the body underlying the clinical manifestations hallmarking the LSDs. Considering that the biological clock drives processes that are crucial for the maintenance of body homeostasis, it is tempting to speculate on the involvement of the circadian clock circuitry in the pathophysiological mechanism underlying these diseases. In line with this hypothesis, a severe deregulation of expression of clock genes and clock-controlled genes has been evidenced in a study that evaluated by whole transcriptome analysis through next-generation sequencing the expression of circadian genes in normal primary human fibroblasts and compared it to the circadian transcriptome of fibroblasts obtained from Hunter syndrome patients before and 24 h/144 h after iduronate-2-sulfatase treatment in vitro and evaluated also by qRT-PCR the time-related expression of core clock genes before and after 24 h of iduronate-2-sulfatase treatment upon synchronization by serum shock (Mazzoccoli et al. 2013). The expression of several core clock genes and clock-controlled genes was distorted and showed dynamic modifications 24 and 144 h after iduronate-2-sulfatase treatment. Besides, a semantic hypergraph-based analysis highlighted five gene clusters significantly associated to important biological processes or pathways and five genes, AHR, HIF1A, CRY1, ITGA5, and EIF2B3, proven to be central players in these pathways. The results imply a decisive contribution of deregulation of the clock gene machinery in the pathophysiological mechanisms underlying the derangement of cellular processes as well as the alteration of tissue function and anatomical integrity that hallmark the organ systems involved in the patients affected by Hunter syndrome (Mazzoccoli et al. 2013). More importantly, only circadian genes when mutated appear to be responsible of

the altered lysosomal function and resulting anatomical and functional derangements that hallmark the LSDs. The enzyme iduronate-2-sulfatase is encoded by the *IDS* gene, whose expression oscillates with circadian rhythmicity, and this pattern of time-related variation features also the expression of the genes encoding the enzymes whose deficiency is responsible of the other LSDs known at present (source CircaDB, a data set of time course expression experiments from mice and humans deposited as publicly available microarray studies and highlighting circadian gene expression cycles, web addresses http://github.com/itmat/circadb, http://bioinf.itmat.upenn.edu/circa) (Pizarro et al. 2013). As listed in Table 1 and shown in Figs. 3 and 4, all the genes encoding lysosomal enzymes implicated in LSDs are customarily expressed with circadian rhythmicity, with the exception of *GNPTG*, encoding N-acetylglucosamine-1-phosphotransferase γ-subunit, whose mutation causes mucolipidosis III gamma (I-cell) and that is expressed rhythmically with a periodicity of 39 h. Accordingly, among the others the deregulation of circadian genes underlies the physiopathology of Niemann-Pick types A and B disease, caused by mutation of *SGMS2* gene encoding sphingomyelin synthase 2, necessary for the transfer of phosphocholine from phosphatidylcholine onto ceramide to produce sphingomyelin, a major component of cell and Golgi membranes, as well as Niemann-Pick type C disease, caused by mutation of *NPC1* gene, encoding Niemann-Pick C1, necessary for the intracellular trafficking of cholesterol from the late endosome to the trans-Golgi network (Panda et al. 2002; Hughes et al. 2009).

The alteration of the circadian clock circuitry may be responsible also of changes of behavioral cycles of sleep/wake, rest activity, and fasting/feeding often evidenced in the patients affected by LSDs. A high prevalence of sleep disorders has been reported in patients affected by type III mucopolysaccharidosis, defined with the eponym Sanfilippo syndrome and representing the most frequent mucopolysaccharidosis, leading to neurodegeneration with habitually severe sleep and behavioral disturbance. Patients affected by Sanfilippo syndrome are characterized by alteration in the circadian rhythm of melatonin rendered by lower urinary concentrations of its metabolite 6-sulfatoxymelatonin at night and higher concentrations in the morning when compared to controls. Based on these reports, therapies aimed at circadian resynchronization such as behavioral treatment, light therapy, or melatonin administration rather than conventional hypnotics have been proposed (Fraser et al. 2002; Guerrero et al. 2006). Accordingly, a beneficial effect of melatonin administration was evidenced in a randomized, double-blind, placebo-controlled, parallel study conducted in a cohort of children with neurodevelopmental disorders and sleep impairment represented by difficulties in initiating and maintaining sleep (impossibility to fall asleep within 1 h of lights out or showing less than 6 h of continuous sleep). Compared to placebo, therapy with melatonin at escalating doses (from a starting dose of 0.5 mg through 2 mg and 6 mg to a maximal dose of 12 mg during the first month of treatment, at the end of which the child was maintained on the attained dose) improved sleep-onset latency and total nocturnal sleep time in a statistically significant way, although the increase of total nocturnal sleep time was not clinically significant (Appleton et al. 2012), which is a major downside of this approach and an example that biological pathways and statistical significance do not necessarily translate into tangible clinical benefit for the patient.

Conclusion

Genetically encoded oscillators maneuvered by transcriptional/translational feedback loops hardwired by circadian genes and proteins drive time-related variations of biological processes. The regular succession of intracellular phenomena is ordered by the biological oscillator ticking in every cell and steering the harmonization of crucial pathways and the compartmentalization in the temporal dimension of poorly compatible biochemical processes. Failure of time-of-day specific transcription of clock genes and clock-controlled genes caused by changes of working of the clock gene machinery may provoke severe deregulation of downstream gene expression regulating a complex array of cellular functions, such as molecule biosynthesis, posttranslational modification, processing, transport, conjugation, internalization and degradation, and cell processes such as cell cycle, autophagy, apoptosis, and DNA damage response. These changes cause perturbation of cellular homeostasis, cell dysfunction, and biochemical and structural derangements that may lead to cell death and tissue dysfunction. The key role played by the molecular clockwork in the control of lysosome function and the involvement of clock-controlled genes encoding circadian transcripts in the pathogenesis of LSDs suggest a direct involvement of the biological clock in the pathophysiological mechanisms underlying cellular and tissue derangements hallmarking these hereditary diseases, and that gene transfer or a proper timetable of enzyme replacement therapy could address the physiological fluctuations driven by the biological clock and appropriately outline circadian rhythmicity.

Table 1 Circadian genes involved in lysosomal storage disorders

Protein defect	Gene	Disease	OMIM	Chromosomal localization
Defects in glycan degradation				
Defects in glycoprotein degradation				
α-Sialidase	*NEU1*	Sialidosis	608272	6p21.33
Cathepsin A	*CTSA*	Galactosialidosis	256540	20q13.12
α-Mannosidase	*MAN2B*	α-Mannosidosis	248500	19p13.2
β-Mannosidase	*MANBA*	β-Mannosidosis	248510	4q24
Glycosylasparaginase	*AGA*	Aspartylglucosaminuria	208400	4q34.3
α-Fucosidase	*FUCA1*	Fucosidosis	230000	1p36.11
N-Acetyl-α-glucosaminidase	*NAGA*	Schindler disease, type III	609421	22q13.2
Defects in glycolipid degradation				
β-Galactosidase	*GLB1*	GM1-gangliosidosis / MPS IVB	230500	3p22.33
β-Hexosaminidase α-subunit	*HEXA*	GM2-gangliosidosis (Tay-Sachs disease)	606869	15q23
β-Hexosaminidase β-subunit	*HEXB*	GM2-gangliosidosis (Sandhoff disease)	606873	5q13.3
GM2 activator protein	*GM2A*	GM2 gangliosidosis	272750	5q33.1
Glucocerebrosidase	*GBA/ GBA2*	Gaucher disease	606463	1q22
Saposins A, B, C, D	*PSAP*	Combined sphingolipid activator protein deficiency	176801	10q22.1
Defects in sulfatide degradation				
Arylsulfatase A	*ARSA*	Metachromatic leukodystrophy	607574	22q13.33
Saposin B	*PSAP*	Metachromatic leukodystrophy due to saposin B deficiency	249900	10q22.1
Formyl-glycin-generating enzyme	*SUMF1*	Multiple sulfatase deficiency	607939	3p26.1
β-Galactosylceramidase	*GALC*	Globoid cell leukodystrophy (Krabbe disease)	606890	14q31.3
Defects in globotriaosylceramide degradation				
α-Galactosidase A	*GLA*	Fabry disease	301500	Xq22.1
Defects in degradation of glycosaminoglycan (mucopolysaccharidoses, MPS)				
Degradation of heparan sulfate				
α-Iduronidase	*IDUA*	MPS I (Hurler, Scheie)	607015	4p16.3
Iduronate sulfatase	*IDS*	MPS II (Hunter)	309900	Xq28
Heparan N-sulfatase	*SGSH*	MPS IIIa (Sanfilippo A)	252900	17q25.3
N-acetyl glucosaminidase	*NAGLU*	MPS IIIb (Sanfilippo B)	252920	17q21.2
Acetyl-CoA transferase	*HGSNAT*	MPS IIIc (Sanfilippo C)	252930	8p11.21
N-acetyl glucosamine 6-sulfatase	*GNS*	MPS IIId (Sanfilippo D)	252940	12q14.3
β-glucuronidase	*GUSB*	MPS VII (Sly)	253220	7q11. 21
Defects in degradation of other mucopolysaccharides				
N-Acetylgalactosamine 4-sulfatase	*ARSB*	MPS VI (Maroteaux-Lamy syndrome)	253200	5q14.1
Galactose 6-sulfatase	*GALNS*	MPS IVA (Morquio A)	253000	16q24.3
Hyaluronidase	*HYAL1*	MPS IX	601492	3p21.31
Defects in glycogen degradation				
α-Glucosidase	*GAA*	Pompe disease	232300	17q25.3
Defects in lipid degradation				
Defects in sphingomyelin degradation				
Acid sphingomyelinase	*SMPD1*	Niemann-Pick type A	257200	11p15.4
Acid sphingomyelinase	*SMPD1*	Niemann-Pick type B (E and F)	607616	11p15.4
Acid ceramidase	*ASAH1*	Farber lipogranulomatosis	228000	8p22

(continued)

Table 1 (continued)

Protein defect	Gene	Disease	OMIM	Chromosomal localization
Defects in triglycerides and cholesteryl ester degradation				
Acid lipase	*LIPA*	Wolman/cholesteryl ester storage disease	278000	10q23.31
Defects in protein degradation				
Cathepsin K	*CTSK*	Pycnodysostosis	601105	1q21.3
Tripeptidyl peptidase	*TPP1*	Ceroid lipofuscinosis 2	607998	11q15.4
Palmitoyl-protein thioesterase	*PPT1*	Ceroid lipofuscinosis, neuronal, 1	600722	1p34.2
Defects in lysosomal transporters				
Cystinosin (cystin transport)	*CTNS*	Cystinosis	606272	17p13.2
Sialin (sialic acid transport)	*SLC17A5*	Salla disease	604322	6q13
Defects in lysosomal trafficking proteins				
UDP-*N*-acetylglucosamine Phosphotransferase γ-subunit	***GNPTG***	**Mucolipidosis III gamma (I-cell)**	**607838**	**16p13.3**
Mucolipin-1(cation channel)	*MCOLN1*	Mucolipidosis IV	605248	19p13.2
Lysosome-associated membrane protein 2	*LAMP2*	Danon	309060	Xq24
Sphingomyelinase	*NPC1*/ *NPC2*	Niemann-Pick type C1 and D	607623	11q11.2
CLN3 protein (battenin)	*CLN3*	Ceroid lipofuscinosis, neuronal, 3	204200	16p11.2
Protein CLN6	*CLN6*	Ceroid lipofuscinosis, neuronal, 6	601780	15q23
Protein CLN8	*CLN8*	Ceroid lipofuscinosis, neuronal, 8	607837	8p23.3
Lysosomal trafficking regulator	*LYST* (*CHS1*)	Chediak-Higashi	214500	1q42.3
Myosin VA	*MYO5A*	Griscelli Type 1	214450	15q21.2
RAB27	*RAB27A*	Griscelli Type 2	607624	15q21.3
Melanophilin	*MLPH*	Griscelli Type 3	609227	2q37.3
HPS1	*HPS1*	Hermansky-Pudlak 1	203300	10q24.2
AP3 β-subunit	*AP3B1*	Hermansky-Pudlak 2	608233	5q14.1

In italic are indicated circadian transcripts, in bold is indicated a transcript characterized by 39 h periodicity
Source: CircaDB, web addresses http://circadb.org, http://github.com/itmat/circadb, http://bioinf.itmat.upenn.edu/circa

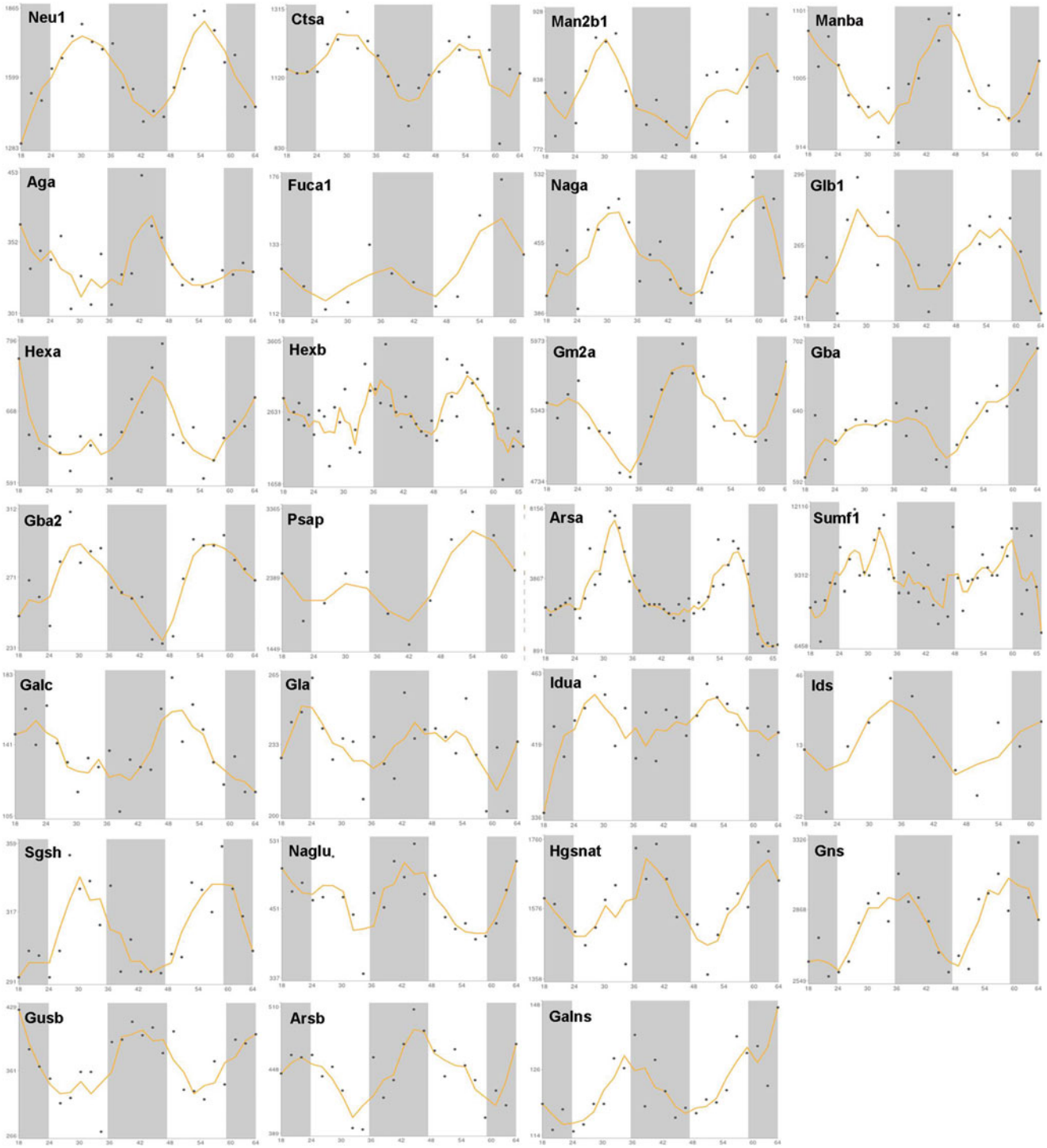

Fig. 3 *x*–*y* plots showing the time-qualified profile of expression of clock-controlled genes whose mutation causes LSDs (*source*: CircaDB, web addresses http://circadb.org, http://github.com/itmat/circadb, http://bioinf.itmat.upenn.edu/circa). On the *x* axis is represented time in hours, and on the *y* axis are represented the mRNA relative expression levels in arbitrary units

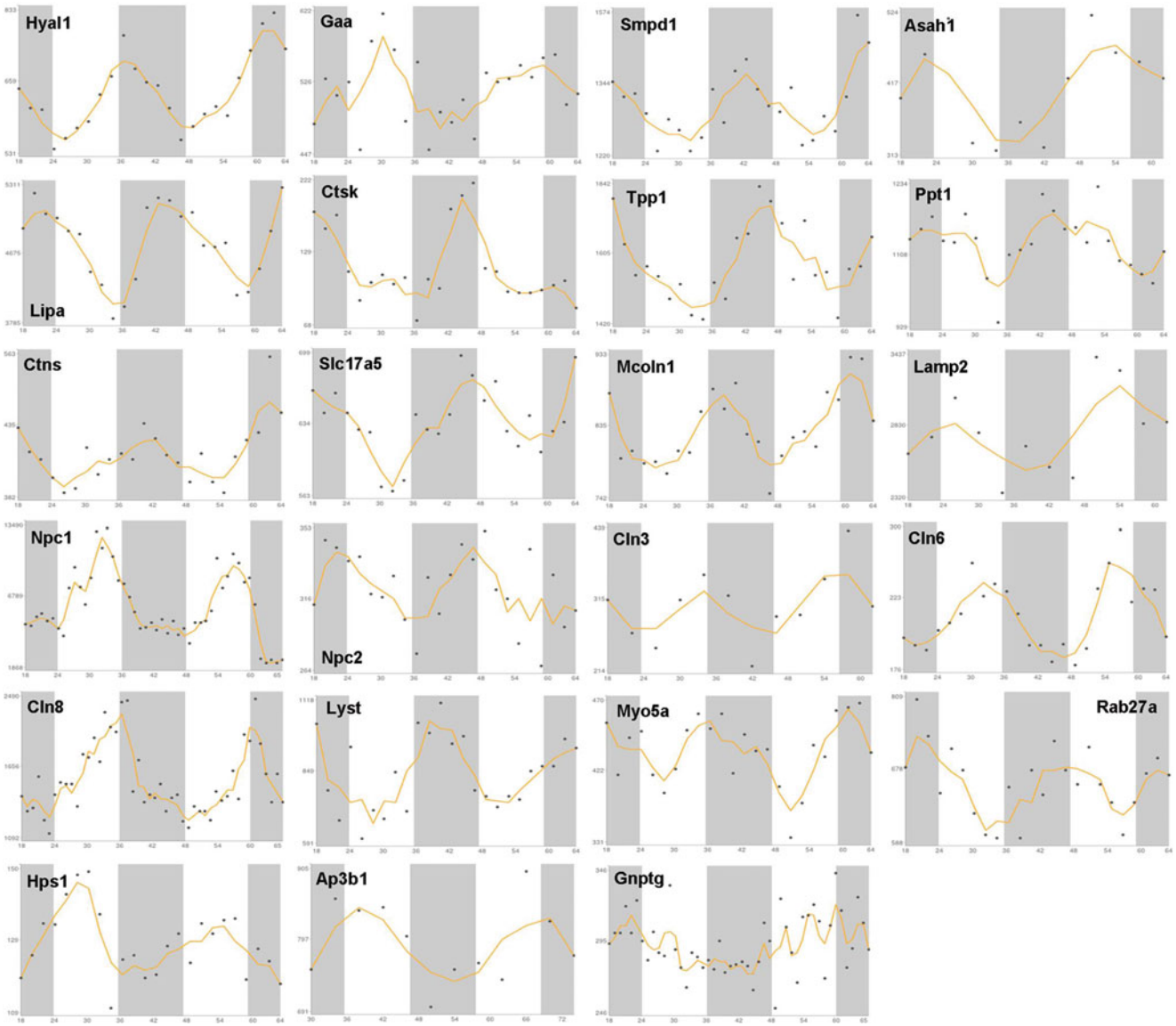

Fig. 4 *x–y* plots showing the time-qualified profile of expression of clock-controlled genes whose mutation causes LSDs (*source*: CircaDB, web addresses http://circadb.org, http://github.com/itmat/circadb, http://bioinf.itmat.upenn.edu/circa). On the *x* axis is represented time in hours, and on the *y* axis are represented the mRNA relative expression levels in arbitrary units

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Take-Home Message (Synopsis)

The lysosomal storage disorders are caused by mutation of genes whose expression is driven with 24-h periodicity by the biological clock, and the circadian pathways impact the pathophysiological mechanisms, implying the involvement of the temporal dimension in the pathogenesis of these hereditary diseases.

Compliance with Ethics Guidelines

Conflict of Interest Statement

Gianluigi Mazzoccoli, Tommaso Mazza, Manlio Vinciguerra, Stefano Castellana, and Maurizio Scarpa declare that there are no conflicts of interest with respect to the authorship and/or publication of this article.

Informed Consent

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

GM conceived the purpose of the review and wrote the article, MV and MS wrote the article, and SC and TM performed bioinformatics analysis and represented scheme and figures.

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RESEARCH REPORT

Severe Impairment of Regulatory T-Cells and Th1-Lymphocyte Polarization in Patients with Gaucher Disease

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Abstract We investigated peripheral blood T-lymphocyte subpopulations and intracellular expression of IFN-γ, IL-4, IL-10, and IL-13, by whole blood flow cytometry, in 22 type I Gaucher disease (GD) patients. Results were compared with those of 19 sex- and age-matched controls. Patients with GD exhibited decreased frequencies and absolute numbers of CD3+/CD4+ helper T lymphocytes ($40.8 \pm 9.8\%$ vs. $49.4 \pm 5.7\%$, $p = 0.002$, and 0.77 ± 0.33 vs. $1.04 \pm 0.28 \times 10^9/\mu L$, $p = 0.011$), as well as increased frequencies of CD3+CD8+ suppressor T lymphocytes ($23.8 \pm 8.0\%$ vs. $18.4 \pm 3.8\%$, $p = 0.010$), resulting in a significantly decreased CD4/CD8 cell ratio ($p < 0.001$). Moreover, they had significantly increased percentages of IFNγ-producing both CD4+ and CD8+ T cells ($p = 0.0003$ and $p = 0.023$, respectively), implying a TH-1 polarization pattern. Finally, patients with GD had decreased percentages and absolute numbers of CD4+CD25^{dim} T lymphocytes ($p = 0.033$ and $p = 0.007$, respectively), of CD4+CD25^{high} T lymphocytes ($p = 0.039$ and $p = 0.016$, respectively), and of CD4+CD25^{high} FOXP3+ regulatory T cells ($p = 0.036$ and $p = 0.019$, respectively). Our results demonstrate that patients with GD have a significant numerical impairment of T-helper lymphocytes and a constitutive TH1 direction pattern of activation of both CD4+ and CD8+ cells, associated with a significant decrease of T-regs. Ineffective T-cell control may explain the chronic inflammatory reaction and the increased incidence of lymphoid malignancies, which have been repeatedly reported among patients with GD.

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Introduction

GD is the most common lysosomal storage disorder, resulting from an abnormal structure and/or impaired function of the enzyme glucocerebrosidase, which leads to inadequate cleavage of glucosylceramide to glucose and ceramide. Consequently, undigested glucosylceramide is accumulated in the macrophageal lysosomes of the reticuloendothelial system, inducing them the characteristic morphology of Gaucher cells and producing the main clinical features of the disease.

There are three clinical variables of GD: the most common non-neuronopathic type 1, the early neuronopathic type 2, and type 3 or the late neuronopathic variant. Besides neurological degeneration, encountered in type 2 and 3 variants, other common clinical features of GD include hepatosplenomegaly of various severity, bone disease, and an unspecific chronic proinflammatory status (Shoenfeld et al. 1982), whose pathogenesis is as yet obscure.

Clinical and experimental studies have shown that GD patients exhibit various immunological abnormalities, such as increased susceptibility to infections (Aker et al. 1993; Maródi et al. 1995), numerical dendritic cell defects (Micheva et al. 2006), and an increased incidence of B-lymphoproliferative disorders and solid tumors (Arends et al. 2013). Laboratory findings include elevated levels of acute-phase proteins (Rogowski et al. 2005) and serum immunoglobulin levels (de Fost et al. 2008), frequent occurrence of monoclonal gammopathy (Arends et al. 2013), detection of various autoantibodies in patients' serum (Shoenfeld et al. 1995), and increased plasma levels of proinflammatory cytokines, including IL-1β, IL-6, IL-8, IL-10, TNF-α, and TGF-β (Allen et al. 1997; Barak et al. 1999; Michelakakis et al. 1996; Deegan et al. 2005; Pérez Calvo et al. 2000). These findings imply that chronic inflammatory status possibly reflects a chronic stimulation of the immune system.

T lymphocytes are the key cells, involved in all immune processes, and they play a pivotal role in immune homeostasis. Nevertheless, little is known about their status and role in GD. Among few studies, Balreira et al. (2005) demonstrated that patients, either under enzyme replacement therapy (ERT) or not, have decreased proportion of CD4+ cells and increased proportion of CD8+ cells. Lacerda et al. (1999), however, found that both CD4+ and CD8+ absolute numbers were decreased in patients with bone manifestations. Moreover, Burstein et al. (1987) reported decreased number of NKT cells, whereas Braudeau et al. (2013) demonstrated that untreated patients exhibit low NK, $T_{\gamma\delta 2}$, and naive CD4+ cells and high CD4+ memory T-cell counts.

We therefore aimed to investigate aspects of T-cell immunity in patients with GD, including enumeration of PB lymphocyte subpopulations and T-regs and their activation pattern and possible contribution to disease phenotype.

Patients, Materials, and Methods

Subjects

Twenty-two patients with type 1 GD (12 females, 10 males, median age 40 years, range 16–67) were studied. Results were compared with those of 19 healthy controls (10 females, 9 males, median age 41 years, range 26–70). Blood was obtained during a scheduled appointment for patients' evaluation. All estimations were performed in the absence of any acute or chronic infection or any other known, active inflammatory condition. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients and controls.

Diagnosis of GD had been confirmed by the detection of low beta-glucosidase activity in peripheral blood (PB) lymphocytes and mutations in the GBA gene. The dominant mutation was N370S, which was identified in 20 patients, of whom 7 had both alleles mutated (homozygous state). At the time of this workup, five patients had detectable bone lesions (mainly osteopenia), 6 had been splenectomized, and another one had manifested nodular-sclerosing Hodgkin's lymphoma, but was in sustained complete remission for 5 years, following high-dose chemotherapy and autologous stem-cell transplantation (Symeonidis et al. 2011). Seventeen patients were receiving ERT, 3 on substrate reduction treatment (SRT), and 2 were not receiving any treatment.

Sample Collection: Labeling of Lymphocyte Surface Markers

For each experiment, 7 mL of PB was collected, of which 4 mL in a heparinized tube and 3 mL in an EDTA tube. The following monoclonal antibodies were used: CD3-FITC (clone UCHT1), CD4-PC5 (clone 13B8.2), CD8-ECD (clone SFCI21Thy2D3), and HLA-DR-PE (clone L243) or CD25-PE (BD Biosciences, San Jose, USA). The following lymphocyte subsets were identified: CD3+, CD3+CD4+, CD3+CD8+, CD4+CD8+, CD4+CD25+, CD4+HLA-DR+, CD8+CD25+, and CD8+HLA-DR+. At least 5×10^4 cells were analyzed per sample by a Coulter FC5000 flow cytometer. Data was processed using the Kaluza software. Determination of absolute counts of the various lymphocyte subsets was estimated from the whole WBC counts, conducted the same day of the experiments, using a Sysmex -1800 automatic cell counter.

T-Regs Analysis

For T-reg labeling, the eBioscience (San Diego, USA) kit was used, which contained CD4-FITC (clone RPA-T4), CD25-PE (clone BC96) for cell surface, and Foxp3-PE-Cy5 (clone PCH101) for nuclear and cytoplasmic staining.

The analysis was performed by a Coulter Epics-XL-MCL flow cytometer. The lymphocytic population was initially defined in a forward (FSC) and a side scatter (SSC) gate. In a dot plot CD4 versus CD25, the CD4+CD25+ subset was determined, based on the exclusion of the CD4-CD25- and CD4+CD25- subsets. Subsequently, two gates of equal size were created, dividing the CD4+CD25+ in two subsets: $CD4+CD25^{dim}$ και $CD4+CD25^{high}$. The $CD4+CD25^{high}$ cells were defined as T-regs. The intracellular expression of FOXP3 per T-reg cell was determined by a

histogram plot. The intracellular expression of FOXP3 in the CD4+CD25- cells was used as an internal control. At least 1×10^5 cells were analyzed, and data were processed using the *Flowing Software* analysis software.

Intracellular Labeling of Cytokines

For estimation of the intracellular expression of IFN-γ, IL-13, IL-4, and IL-10 in CD4+ cells and IFN-γ in CD8+ cells, a slightly modified previously described technique was followed (Karakantza et al. 2004), based on the stimulation of lymphocytes with PMA/ionomycin, in the presence of BFA, for 5 h at 37°C. The following antibodies were used: CD3-ECD (clone UCHT1), CD4-FITC (clone Leu3a-3b, BDIS, Beckman Coulter, Miami, USA), CD8-FITC (clone B9.11, Beckman Coulter), anti-IFNγ-PE (clone 45.15), anti-IL-4-PE (Beckman Coulter Immunotech), anti-IL-10-PE (Beckman Coulter Immunotech), and anti-IL-13-PE (clone JES10-5A2, BD Pharmingen). The monoclonal antibody CD69-PC5 (clone TP1.55.3, Beckman Coulter) was used as a marker of early activation. At least 5×10^4 cells were analyzed, and data were processed using the Kaluza software.

Subject Grouping and Statistical Analysis

Healthy control subjects were nominated group A. Patients as a whole consisted group B. Patients were then divided in three subgroups: those without osteopenia and/or splenectomy ($n = 11$, group C), patients with osteopenia and an intact spleen ($n = 5$, group D), and splenectomized patients irrespective of osteopenia ($n = 6$, group E). For the assessment of intracellular cytokine expression, since the number of patients was rather small ($n = 15$, all under ERT), patients were divided in 2 groups: those without osteopenia or splenectomy ($n = 9$, group III) and those with osteopenia and/or splenectomy ($n = 6$, of whom 4 splenectomized: group IV).

Statistical analysis was performed following logarithmic transformation. Results were analyzed using student's *t*-test. Data are described using mean ± standard deviation, which were determined according to the raw form (normal) of data and not the logarithmic. For statistical analysis, GraphPad Prism software was used.

Results

Total Lymphocytes and Total T lymphocytes

No statistically significant difference in the WBC count between patients and controls, as well as in the total lymphocyte count (TLC) between patient group B or group C and controls, was observed. However, group D patients had significantly lower TLC ($p = 0.048$), whereas group E patients had significantly higher TLC ($p = 0.006$).

The total T-cell population (CD3+ cells) was not significantly different between patient group B or D and controls. CD3+ cells were significantly lower in patient group C and higher with marginal significance in group E ($p = 0.023$ and $p = 0.052$, respectively, Table 1).

Major T-Lymphocyte Subpopulations

Patients as a whole (group B) had significantly decreased both percentage and absolute CD4+ T-lymphocyte count ($p = 0.002$ and $p = 0.011$, respectively) compared to controls. The same was true for patient group C ($p = 0.013$ and $p < 0.001$, respectively). Patient group D exhibited lower absolute CD4+ cell count alone ($p = 0.022$), whereas splenectomized patients had significantly decreased percentage of CD4+ cells ($p < 0.001$). Moreover, patient groups B and D exhibited significantly increased percentages, but not absolute counts of CD8+ T lymphocytes, compared to controls ($p = 0.010$ and $p = 0.018$, respectively). Splenectomized patients had significantly increased both percentage and absolute CD8 + T lymphocytes ($p = 0.001$ and $p < 0.001$, respectively). The CD4/CD8 ratio was significantly lower in all patient groups compared to controls (Table 1).

B Lymphocytes

The absolute CD20+ lymphocyte count was significantly lower in the osteopathic patient group compared to controls ($p = 0.015$), whereas splenectomized patients had higher absolute CD20+ lymphocyte counts ($p = 0.035$). The CD20+CD5+ cells (autoreactive B lymphocytes) did not differ significantly between the controls and any patient group (data not shown).

Activated T lymphocytes

The presence of activated T lymphocytes was determined by the CD25 and HLA-DR surface antigen expression. Patients as a whole exhibited significantly higher frequencies of CD8 +HLA-DR+ cells compared to controls ($p = 0.019$), but HLA-DR expression on CD8+ cells was not different, as was also absolute CD8+HLA-DR+ cell count. HLA-DR expression on both CD4+ and CD8+ cells was higher in splenectomized patients ($p = 0.089$ and $p < 0.001$, respectively). The same was observed for the absolute CD4 +HLA-DR+ lymphocyte count ($p = 0.003$) and the percentage and the absolute CD8+HLA-DR+ cell count ($p < 0.001$ and $p < 0.001$, respectively). HLA-DR expression on CD4+ cells did not differ significantly between

Table 1 Basic lymphocyte subpopulations and regulatory T-cells subsets in patients and controls

(Sub)population	Controls $N = 19$ (mean ± SD)	PT group B $N = 22$ (mean ± SD)	PT group C $N = 11$ (mean ± SD)	PT group D $N = 5$ (mean ± SD)	PT group E $N = 6$ (mean ± SD)	p (Gr B) (Log-trans.)	p (Gr C) (Log-trans.)	p (Gr D) (Log-trans.)	p (Gr E) (Log-trans.)
WBC count ($\times 10^9$/L)	7.15 ± 1.31	6.13 ± 2.42	5.95 ± 2.39	4.32 ±1.15	7.95 ± 1.95	0.036	0.029	<0.001	n.s.
Abs. lymphoc. count × (10^9/L)	2.07 ± 0.49	2.05 ± 0.98	1.73 ± 0.80	1.59 ± 0.58	3.05 ± 0.84	n.s.	0.059	0.048	0.006
CD3+ (%)	71.8 ± 6.21	70.3 ± 11.3	70.3 ± 13.1	74.7 ± 6.00	66.6 ± 9.70	n.s.	n.s.	n.s.	n.s.
CD3+ (abs. count $\times 10^9$/L)	1.51 ± 0.39	1.40 ± 0.58	1.15 ± 0.37	1.21 ± 0.50	2.00 ± 0.53	n.s.	0.023	n.s.	0.052
CD3+CD4+ (%)	49.4 ± 5.72	40.8 ± 9.78	41.8 ± 11.0	43.7 ± 6.1	36.6 ± 8.3	0.002	0.013	n.s.	<0.001
CD3+CD4+ (abs. count $\times 10^9$/L)	1.04 ± 0.28	0.77 ± 0.33	0.67 ± 0.21	0.72 ± 0.34	1.08 ± 0.29	0.011	<0.001	0.022	n.s.
CD3+CD8+ (%)	18.4 ± 3.76	23.8 ± 7.99	21.2 ± 7.83	24.8 ± 6.32	27.6 ± 7.84	0.010	n.s.	0.018	0.001
CD3+CD8+ (abs. count $\times 10^9$/L)	0.38 ± 0.11	0.48 ± 0.32	0.35 ± 0.12	0.40 ± 0.18	0.85 ± 0.40	n.s.	n.s.	n.s.	<0.001
CD4+/CD8+ ratio	2.85 ± 0.80	1.91 ± 0.76	2.18 ± 0.86	1.88 ± 0.48	1.45 ± 0.49	<0.001	0.018	0.006	<0.001
CD20+ (%)	12.3 ± 3.18	13.1 ± 8.34	11.3 ± 4.12	10.9 ± 3.51	18.4 ± 13.5	n.s.	n.s.	n.s.	n.s.
CD20+ (abs. count $\times 10^9$/L)	0.23 ± 0.11	0.26 ± 0.18	0.20 ± 0.10	0.14 ± 0.04	0.45 ± 0.23	n.s.	n.s.	0.015	0.035
CD4+DR+ (%)	2.29 ± 0.69	2.67 ± 2.46	1.70 ± 1.23	3.23 ± 2.50	4.00 ± 3.50	n.s.	0.032	n.s.	n.s.
CD4+DR+ (abs. count $\times 10^6$/L)	48.1 ± 15.1	54.3 ± 50.8	28.5 ± 15.7	51.9 ± 50.8	103.7 ± 56.4	n.s.	0.003	n.s.	0.003
CD4+CD25+ (% as CD4+ subset)	4.36 ± 1.03	6.62 ± 6.17	3.97 ± 1.30	7.10 ± 4.98	11.1 ± 9.10	n.s.	n.s.	n.s.	0.002
CD4+CD25+ (%)	1.01 ± 0.39	0.60 ± 0.36	0.59 ± 0.36	0.71 ± 0.22	0.42 ± 0.28	0.027	0.020	n.s.	0.005
CD4+CD25+ (abs. count $\times 10^6$/L)	20.4 ± 9.1	10.7 ± 6.9	9.6 ± 7.09	9.3 ± 2.97	11.3 ± 5.96	0.007	0.009	n.s.	0.016
CD8+DR+ (%)	1.92 ± 0.67	3.69 ± 2.92	2.41 ± 1.46	3.17 ± 2.31	6.38 ± 3.55	0.019	n.s.	n.s.	<0.001
CD8+DR+ (abs. count $\times 10^6$/L)	41.3 ± 17.2	87.7 ± 111	40.5 ± 24.7	56.5 ± 54.1	201 ± 155	n.s.	n.s.	n.s.	<0.001
CD8+DR+ (% as CD8+ subset)	11.1 ± 4.13	15.3 ± 8.56	11.9 ± 6.40	14.9 ± 7.51	23.1 ± 8.26	n.s.	n.s.	n.s.	0.009
CD4+CD25+^{dim} (%)	0.78 ± 0.34	0.50 ± 0.30	0.50 ± 0.36	0.60 ± 0.20	0.35 ± 0.23	0.033	0.085	n.s.	0.074
CD4+CD25+^{dim} (abs. count $\times 10^6$/L)	17.1 ± 7.7	7.7 ± 6.3	8.7 ± 6.8	7.9 ± 2.7	9.3 ± 4.9	0.007	0.033	n.s.	n.s.
CD4+ CD25+^{dim} (% as CD4+ subset)	2.29 ± 0.74	1.51 ± 0.74	1.48 ± 0.79	1.84 ± 0.56	1.16 ± 0.60	0.014	0.027	n.s.	0.042
CD4+CD25+^{dim} FOXP3+ (%)	0.43 ± 0.23	0.24 ± 0.17	0.26 ± 0.21	0.22 ± 0.04	0.21 ± 0.16	0.021	0.030	0.041	n.s.
CD4+CD25+^{dim}FOXP3+ (abs. count $\times 10^6$/L)	9.79 ± 5.47	4.75 ± 4.01	4.88 ± 4.58	3.07 ± 1.13	5.65 ± 3.58	0.008	0.064	0.045	n.s.
CD4+CD25+^{high} (%)	0.15 ± 0.06	0.10 ± 0.05	0.11 ± 0.06	0.11 ± 0.04	0.07 ± 0.05	0.039	n.s.	n.s.	0.029
CD4+CD25+^{high} (abs. count)	3.10 ± 1.46	2.09 ± 1.28	1.92 ± 1.36	1.40 ± 0.41	1.98 ± 1.22	0.016	0.038	0.042	n.s.
CD4+CD25+^{high}FOXP3+ (%)	0.14 ± 0.05	0.08 ± 0.04	0.09 ± 0.05	0.08 ± 0.03	0.06 ± 0.04	0.036	0.030	0.054	0.028
CD4+CD25+^{high}FOXP3+ (abs. count)	2.90 ± 1.28	1.57 ± 1.12	1.68 ± 1.31	1.07 ± 0.31	1.55 ± 0.89	0.019	0.028	0.031	n.s.
FOXP3+ (on CD4+CD25+^{dim} %)	56.9 ± 17.5	50.2 ± 19.4	50.5 ± 19.2	39.1 ± 9.90	57.6 ± 7.46	n.s.	n.s.	n.s.	n.s.
FOXP3+ (on CD4+CD25+^{high}, %)	90.2 ± 6.8	81.9 ± 11.8	83.9 ± 8.7	79.5 ± 16.4	78.9 ± 2.1	0.031	0.083	n.s.	0.021

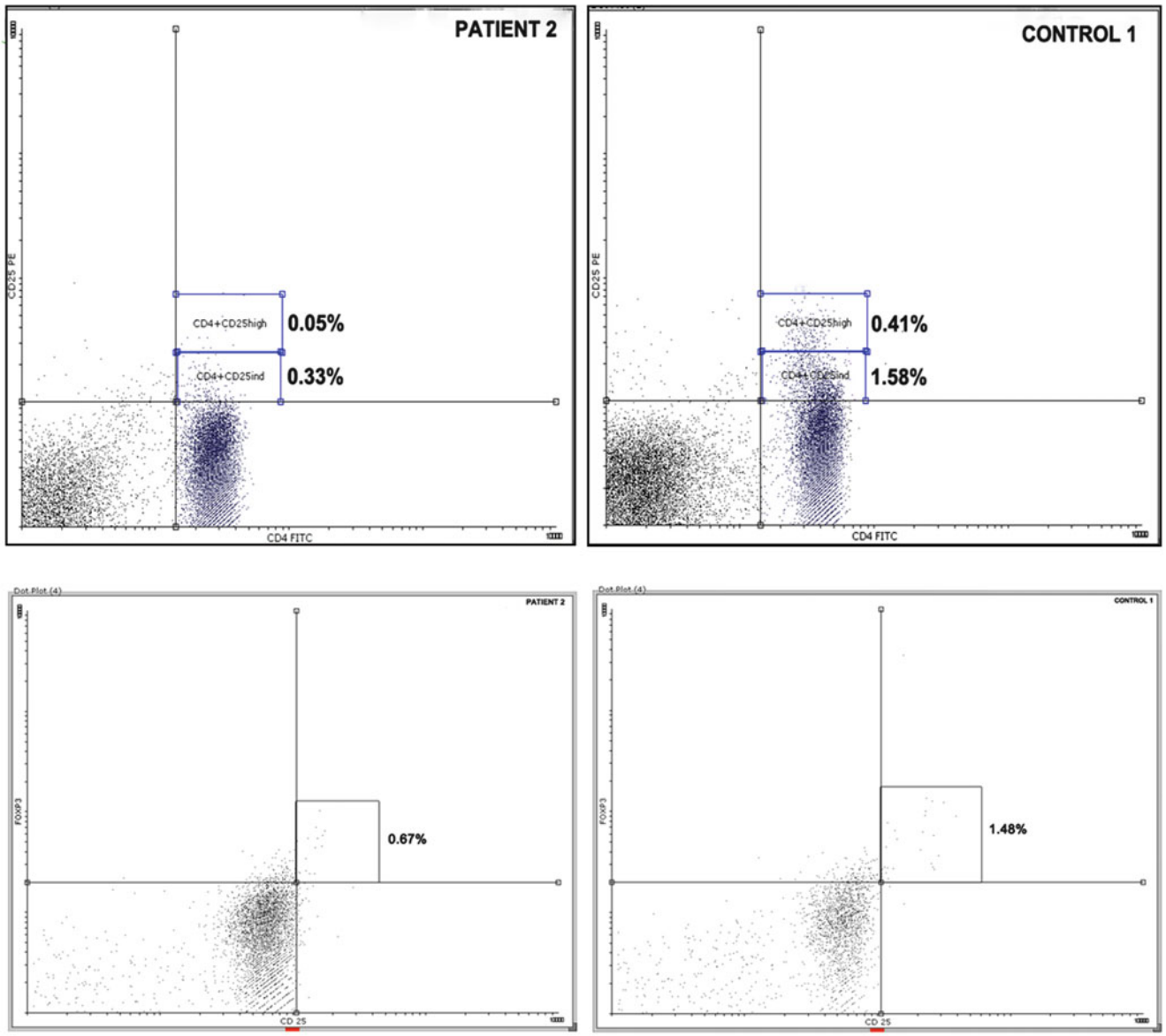

Fig. 1 Comparative results for the CD4+CD25^{dim}+, CD4+CD25^{high}+, and CD25+FOXP3+ cell population of a typical patient and a healthy control subject. These cell populations were significantly reduced in the patient group compared to those of the healthy controls

patients and controls. Surprisingly, non- osteopathic patients had significantly decreased CD4+HLA-DR+ cells, but HLA-DR expression on CD4+ cells was similar. This could be attributed to the lower percentage of CD4+ cells exhibited in these patients (Table 1).

CD25 Expression on CD4+ Cells and T-Regs

CD25 is an early activation T-cell marker and also a marker of T-regs. The CD4+CD25^{dim} cell subset represents the compartment of activated CD4+ T lymphocytes. Patient groups B and C exhibited decreased both percentages ($p = 0.033$ and $p = 0.085$, respectively) and absolute CD4+CD25^{dim} cell counts ($p = 0.007$ and $p = 0.033$, respectively) compared to controls. These results were also confirmed by the significantly reduced expression of CD25^{dim} on CD4+ cells in patient groups B, C, and E compared to controls.

The CD4+CD25^{high} subpopulation is considered representative of T-regs. This population was significantly reduced, as percentage in patient groups B ($p = 0.039$) and E ($p = 0.029$) and as absolute count in patient group B ($p = 0.016$), group C ($p = 0.038$), and group D ($p = 0.042$), compared to controls (Fig. 1). The same was also found for the CD4+CD25^{high}FOXP3+ cell subset. Patient group E also exhibited significantly lower

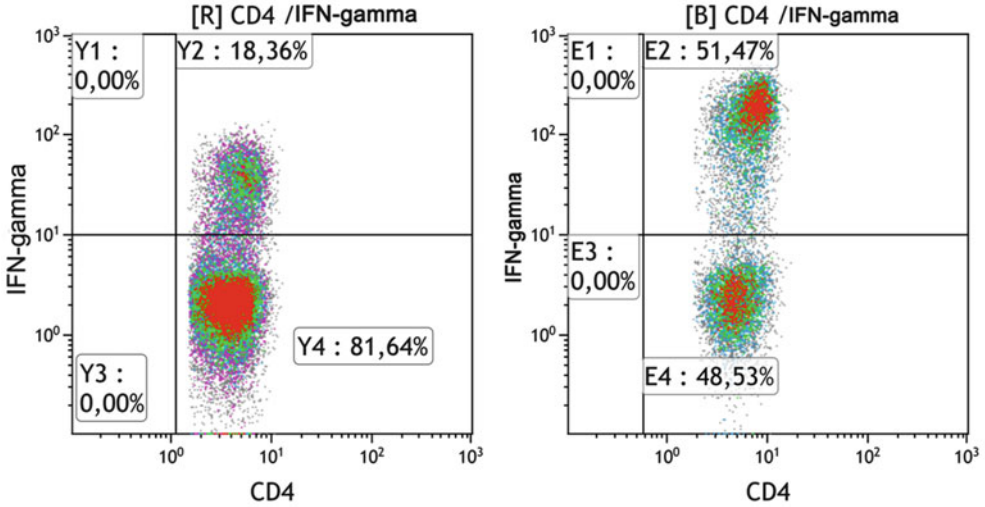

Fig. 2 Typical scattergram of IFN-γ-producing CD4+ populations, following PMA/ionomycin stimulation of a patient with Gaucher disease (*left*) and a healthy control subject (*right*)

Table 2 Intracellular cytokine expression following mitogenic stimulation

	Control Gr I	PT Gr II	PT Gr III	PT Gr IV	*p* value (log transformed)		
% on CD4+ or CD8+ cells	$N = 10$ (mean ± SD)	$N = 15$ (mean ± SD)	$N = 9$ (mean ± SD)	$N = 6$ (mean ± SD)	I vs. II	I vs. III	I vs. IV
CD4+IFNγ+	22.2 ± 7.10	39.3 ± 12.0	34.4 ± 11.5	46.8 ± 8.4	<0.001	0.016	<0.001
MFI of IFNγ on CD4+ cells	3004 ± 53.2	3144 ± 122.7	3122 ± 136	3177 ± 89.3	0.005	0.036	<0.001
CD8+IFNγ+	46.0 ± 14.2	65.3 ± 22.8	53.4 ± 21.4	83.2 ± 8.9	0.023	n.s.	<0.001
MFI of IFNγ on CD8+ cells	3044 ± 110	3064 ± 54.5	3059 ± 46.4	3071 ± 64.2	n.s.	n.s.	n.s.
CD4+IL13+	1.21 ± 0.95	1.26 ± 0.79	0.91 ± 0.75	1.62 ± 0.69	n.s.	n.s.	n.s.
CD4+IL4+	0.84 ± 0.32	1.03 ± 0.54	1.05 ± 0.54	1.13 ± 0.54	n.s.	n.s.	n.s.
CD4+IL10+	1.03 ± 0.49	1.15 ± 0.71	1.21 ± 0.84	1.14 ± 0.41	n.s.	n.s.	n.s.

percentages of CD4+CD25^{high} and CD4+CD25^{high}FOXP3+ cells ($p = 0.029$ and $p = 0.028$, respectively), but not absolute counts of these populations. The proportion of FOXP3 expressing cells among the CD4+CD25+^{high}cells was significantly reduced in patient groups B and E compared to controls (Table 1).

The CD8+CD25+ subpopulation exhibited substantial variation among patients, and no significant difference between any patient group and controls was found (data not shown).

NK Cells

We examined the various NK- and of NKT-cell subpopulations, as defined by CD3, CD16, and CD56 surface antigen expression. We found that groups B and C patients had absolute NK-cell counts, and CD3-CD56^{dim} and CD3-CD56^{dim}CD16+ subpopulations almost had 2-fold lower, and group D had 3-fold lower compared to controls, yet these differences were not statistically significant. Group D patients exhibited significantly lower absolute CD3-CD56+^{high} and CD3-CD56+^{high}CD16+ cell counts ($p = 0.036$ and $p < 0.001$, respectively, data not shown).

Intracellular Expression of Type 1 and Type 2 Cytokines

In all patient groups, stimulation with PMA/ionomycin resulted in a significant increase of IFNγ-producing CD4+ T cells compared to controls. The same was true for IFNγ-producing CD8+ T cells, in patient groups II and IV (Fig. 2). Also, the MFI of IFNγ on CD4+ cells (but not on CD8+ cells) was significantly higher for all patient groups. In contrast, the percentage of IL13-, IL4-, and IL10-producing CD4+ T cells, following mitogenic stimulation, did not differ significantly between any patient group and controls, implying a strong TH1 direction of lymphocyte activation. Intracellular cytokine expression in the unstimulated CD4+ and CD8+ cells was almost zero in both groups (Table 2).

Discussion

This study confirmed that type 1 GD patients exhibit mainly quantitative, but also functional abnormalities of the adaptive immunity. The CD4+ cell compartment is the most commonly and almost universally affected population, resulting in a significant proportional and absolute count decrease of T-helper cells. Impairment of T-helper cell function appears to be a constitutive feature of GD, since it is not affected by disease severity, effectiveness of treatment, previous splenectomy, or other features. Low CD4+ cell count is a hallmark of chronic inflammatory diseases, such as HIV infection (Catalfamo et al. 2011) and chronic parasitic infestations (Kalinkovich et al. 1998), and may result from an increased rate of apoptosis in the CD4+ cell compartment, something already described in chronic immune stimulation-associated diseases and conditions (Jelley-Gibbs et al. 2005).

Our study also showed that in patients with GD (excluding osteopathic patients), the CD8+ cell subset has an increased proportional representation. We did not find disturbed absolute CD8+ cell counts, either in patients with or in those without bone involvement. This finding argues with what other studies have reported, namely, that GD patients with bone involvement have reduced absolute CD8 + cell counts (Lacerda et al. 1999). This discrepancy might be attributed to the relatively small number of osteopathic patients included in our study ($n = 5$).

To estimate the direction of lymphocyte activation, we investigated the intracellular cytokine expression, following mitogenic stimulation. We found that IFNγ-producing helper T cells were significantly higher among patients than controls, although helper T cells producing the Th2 cytokines IL-4 and IL-13 and the immunoregulatory cytokine IL-10 were not significantly different. The same was also true for the IFNγ-producing CD8+ cells, except of the non-osteopathic patient group. Therefore, it appears that patients with type 1 GD have a TH1 polarization of their CD4+ cells and an increased activity of IFNγ-producing cytotoxic CD8+ cells. Similar findings have been observed in other diseases and conditions, associated with chronic immune stimulation (Smolenska et al. 2012; Berner et al. 2000). The high frequencies of IFNγ-producing T cells could result from the increased expression of CD1d and MHC class II molecules (LeibundGut-Landmann et al. 2004) that has been reported on the monocytes of these patients (Balreira et al 2005). Additionally, patients with GD produce more IFNγ per CD4+ cell compared to controls, as it has been shown by the MFI values. This may indicate that the Th1 CD4+ cells also demonstrate alterations in the IFNγ production at the cellular level, and this issue would be interesting to be further investigated.

To our knowledge, increased IFNγ expression in the T cells of patients with GD has not yet been reported. However, upregulation of the IFNγ gene expression in CBE-treated mouse microglia (Hong et al. 2006) or increased serum IFNγ levels in GBA-deficient mice has been reported (Pandey et al. 2012; Liu et al. 2012). Therefore, the cause of increased presence of TH1 IFNγ-producing cells, possibly resulting in increased serum IFNγ levels of patients with GD, needs to be explored.

On the other hand, excluding CD8+ cells of splenectomized patients, neither CD4+ nor CD8+ lymphocytes displayed significantly increased HLA-DR expression. This was rather unexpected, since TH1 polarization should lead to HLA-DR+ phenotype of T cells (Volk et al. 1986). A possible explanation might be that almost all studied patients were under ERT, which could have resulted in downregulation of the HLA-DR expression, as this has been reported, following treatment of chronic inflammatory conditions (Wilson et al. 1994; Bass et al. 1992). To ascertain this, further study of HLA-DR expression before and after therapy in a larger group of patients is designed by our group.

The expression of another activation marker, CD25, was found reduced on the CD4+ T cells of all patient groups, with the exception of osteopathic patients. CD25 is the α-chain of the IL-2 receptor that promotes T-helper cell proliferation. In vitro studies have shown that activated CD4+ lymphocytes exhibit increased CD25 expression, which is released into the culture medium and is detected as sIL-2R (Brusko et al. 2009). It has been reported that type 1 GD patients have increased serum levels of sIL-2R (Barak et al. 1999). Thus, we could assume that the reduced CD25 expression in CD4+ cells of GD patients implies an activation and proliferation state of the CD4+ cells.

Low NK-cell counts have been reported in patients with GD. In our study, although the cytotoxic CD3-CD56+^{dim} NK-cell subset was almost 2-fold lower, it was not significantly reduced compared to controls. However, we found a significant decrease in the absolute immunoregulatory CD3-CD56+^{high} cell count among patients with osteopenia, i.e., in patients, more severely affected by GD. An anticipated exception was observed in previously splenectomized patients, for whom it is well known that exhibit increased NK-cell subpopulations. Among splenectomized GD patients the CD3-CD56+^{high} cell population was not significantly different, compared to controls Reduced NK-cell function may, at least partially, explain the increased sensitivity to infections and neoplasias, which has been reported in patients with GD.

We have shown for the first time that patients with GD exhibit significantly decreased T-regs. Numerical T-reg impairment was a global phenomenon in our patient population, and it was not affected by age, disease severity, and years of treatment. This finding implies that there is a significant deficiency in the immunoregulatory ability of

GD patients, since the low number of T-regs cannot effectively control the overactive T-helper cell compartment (Sakaguchi et al. 2009). Therefore, the quantitative deficiency of T-regs might contribute to the establishment of the TH1 polarization (Shevach et al. 2001).

Taken together, we have demonstrated a heterogeneous immunophenotypic profile among patients with type 1 GD, depending on whether they have bone disease/osteopenia or previous splenectomy. Non-osteopathic patients did not exhibit higher IFNγ-producing CD8+ cells, in contrast to the other patient groups. We have confirmed that splenectomized patients have higher T-, B-, CD4+HLA-DR+, and CD8+HLA-DR+ lymphocyte counts and NK cells compared to controls. These variations might influence the course of the disease, and it is unclear whether they can be reversed by an effective treatment program.

There are some limitations in our study. First, we included low numbers of osteopathic and splenectomized patients. It is therefore important to confirm these data in a larger patient cohort exhibiting these features. Second, we were unable to investigate the impact of treatment on the immune abnormalities, since almost all patients were under treatment long before their evaluation, and they were well responders. We have studied only two treatment-naïve patients, who did not show significant differences compared to the remaining. Third, although we have shown numerical impairment of T-regs, we did not investigate their functionality. This could be accessed through the measurement of suppressive activity of purified T-regs in vitro.

In conclusion, our study has shown that patients with GD exhibit an important deregulation of the immune system, characterized by a profile of uncontrolled immune response (reversal of the CD4/CD8 cell ratio, low regulatory T cells, and increased IFNγ-producing T cells) (Belkaid and Rouse 2005), and this possibly contributes to the establishment of low-grade chronic inflammatory status that has been observed in these patients.

Acknowledgments Argiris Symeonidis designed and conducted the study. Christos Sotiropoulos performed the research as part of his PhD thesis work. George Theodorou and Eugenia Verigou contributed to the flow cytometric evaluation of regulatory T cells and NK cells. Constantina Repa and Theodore Marinakis contributed to the design of the study and provided clinical data on patient information and follow-up. Elena Solomou and Marina Karakantza supervise lab work and supported the appropriate analysis of the data.

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Take-Home Message

Patients with Gaucher disease have severe numerical impairment of their regulatory T-cell counts and exhibit a strong TH1 direction of lymphocyte activation.

Compliance with Ethics Guidelines

Conflict of Interest

Christos Sotiropoulos, George Theodorou, Constantina Repa, Theodoros Marinakis, Eugenia Verigou, Elena Solomou, Marina Karakantza, and Argiris Symeonidis have no conflict of interest to disclose in relation to this piece of scientific work.

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 and controls for being included in this study.

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CASE REPORT

Infants with Tyrosinemia Type 1: Should phenylalanine be supplemented?

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Abstract Tyrosinemia type 1 (HT1) is an inborn error of tyrosine catabolism caused by fumarylacetoacetase deficiency. Biochemically, this results in accumulation of toxic metabolites including succinylacetone. Clinically, HT1 is characterized by severe liver, kidney, and neurological problems. Treatment with NTBC and dietary restriction of tyrosine and phenylalanine have strongly improved outcome, but impaired neurocognitive development has been reported. Whether impaired neurocognitive outcome results from high blood tyrosine or low blood phenylalanine concentrations is currently unknown. In this report, two HT1 newborns, diagnosed by neonatal screening, are presented. The first patient showed low phenylalanine concentrations, growth retardation, neurological impairments, and skin problems, clearly improving after institution of phenylalanine supplementation (~30 mg/kg/day) at age 6 months, while both blood phenylalanine and tyrosine concentrations increased. In the second patient, phenylalanine supplementation (~20 mg/kg/day) was initiated as soon as low phenylalanine concentrations were observed at age 19 days. On this regimen, blood phenylalanine concentrations increased, and hypophenylalaninemia was less frequently observed than in the first patient, whereas blood tyrosine concentrations tended to increase. Clinically, no growth, neurological, or skin problems have been observed. The combination of knowledge obtained from these cases suggests that hypophenylalaninemia rather than hypertyrosinemia during the first months of life may impair neurocognitive development in young HT1 infants. Phenylalanine supplementation should really be considered in HT1 patients with consistently low blood phenylalanine concentrations during the first months of life. However, the minimal phenylalanine concentrations acceptable and the optimal phenylalanine supplementation regimen require further investigation.

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Introduction

Clinically, tyrosinemia type I (HT1; OMIM 276600, fumarylacetoacetase deficiency) is characterized by liver failure, hepatocellular carcinoma, renal tubulopathy, and porphyria-like syndrome (de Laet et al. 2013). Implementation in neonatal blood spot screening (NBS) and treatment with 2-(2-nitro-4-trifluoromethyl-benzyl)-1,3-cyclohexanedione (NTBC) to prevent accumulation of toxic metabolites by inhibiting tyrosine catabolism upstream from the primary enzymatic defect has largely improved clinical outcome (Larochelle et al. 2012; Mayorandan et al. 2014).

Unfortunately, however, neurocognitive deficits are observed, which may be related to HT1 itself, NTBC treatment, increased tyrosine concentrations, or low phenylalanine concentrations (De Laet et al. 2011; Masurel-Paulet et al. 2008; Thimm et al. 2012; Bendadi

et al. 2014). NTBC increases tyrosine concentrations. Therefore, tyrosine intake is limited by severe natural protein restriction and supplementation of a synthetic amino acid mixture devoid of tyrosine and phenylalanine, as tyrosine is primarily derived from hydroxylation of dietary phenylalanine. This results in both high tyrosine and low phenylalanine concentrations (De Laet et al. 2011; Daly et al. 2012; Wilson et al. 2000), with dietary treatment being primarily monitored based on tyrosine concentrations. In phenylketonuria, too strict dietary treatment, resulting in "low" phenylalanine concentrations, has shown to be related to impaired growth and development (Teissier et al. 2012; Rouse 1966; Smith et al. 1990; Pode-Shakked et al. 2013). In tyrosinemia type II, high tyrosine concentrations are associated with mental retardation. Together, this raises the question whether current dietary treatment creates a tyrosine toxicity or a phenylalanine deficiency that may impair growth and brain development (De Laet et al. 2011; Daly et al. 2012; Wilson et al. 2000) and whether phenylalanine supplementation is beneficial or only increases tyrosine concentrations (Daly et al. 2012; Wilson et al. 2000).

This study reports on blood phenylalanine and tyrosine concentrations as well as on growth and development of two HT1 infants detected by NBS, who received phenylalanine supplementation because of very low phenylalanine concentrations during the first months of life.

Patients and Methods

Patient 1 and patient 2 are both children of healthy, Dutch, non-consanguineous parents with an uneventful fetal development and a normal birth process. HT1 was detected by NBS. Treatment was started with NTBC (1 mg/kg) and dietary restriction of phenylalanine and tyrosine, aiming at a total protein intake of 2.5 g/kg. In below, both patients are described until phenylalanine supplementation was introduced.

Patient 1 (girl) was diagnosed in 2009 (NBS, SA 4.7 μmol/L; DNA, homozygous for FAH c.554-1G>T) at day 7. High tyrosine levels above the upper target of 400 μmol/L (de Laet et al. 2013) led to further decrease of natural protein intake to a minimum of 0.5 g/kg/day (Fig. 1a, b). On this regimen, blood tyrosine concentrations decreased. Also, blood phenylalanine concentrations decreased, while blood concentrations of other essential amino acids remained well within the normal range (valine, 167–373 μmol/L; isoleucine, 39–121 μmol/L; leucine, 82–220 μmol/L; methionine, 19–42 μmol/L; histidine, 72–108 μmol/L; threonine, 138–473 μmol/L; tryptophan, 56–93 μmol/L; and lysine, 146–277 μmol/L). During the subsequent months, she first developed "eczema" (Fig. 2). Later, impaired growth was observed, while BMI was undisturbed (Fig. 1a, b). Thereafter, she showed increasing myoclonus periorally and in the extremities. Electroencephalographic and myographic recordings at day 100 were abnormal demonstrating cortical myoclonus. Development of motor milestones seemed to be impaired despite adjustments by either increasing or decreasing the natural protein intake and tyrosine concentrations varying from some 215–609 μmol/L. In this period, prealbumin decreased from 0.19 g/L at day 9 to values ranging from 0.12 to 0.15 g/L. Because of persisting low blood phenylalanine concentrations (<20 μmol/L), phenylalanine supplementation was started at day 191.

Patient 2 (boy) was diagnosed in 2013 (NBS, SA 5.1 μmol/L; DNA, FAH c.554-1G>T/c.674T>G) at day 7. Because of low blood phenylalanine concentrations (<20 μmol/L) and the previous experiences with patient 1, phenylalanine supplementation was started at day 19 (Fig. 1c), without clinical signs suggesting phenylalanine deficiency. At day 11, a prealbumin concentration of 0.17 g/L was measured.

NTBC Treatment and Protein Intake

For both patients, NTBC treatment was regularly adjusted based on body weight, targeting at a dose of 1 mg/kg/day.

Both patients received breast milk combined with an amino acid-based tyrosine-free and phenylalanine-free powdered infant formula (Tyr Anamix Infant, Nutricia Advanced Medical Nutrition) during the first 3 months (patient 1) and during the first 5 months (patient 2). Thereafter, breast milk was replaced by a regular infant formula. Additional feedings started at around 6 months (patient 1) and 5 months (patient 2).

Phenylalanine Supplementation

In patient 1, at day 191, ~30 mg/kg/day phenylalanine supplementation was started, which was increased to ~40 mg/kg/day at day 209 and reduced to ~20 mg/kg/day at day 247 (Fig. 1a, b).

In patient 2, phenylalanine supplementation of ~20 mg/kg/day was started at day 19. Due to low blood phenylalanine concentrations (5–48 μmol/L), phenylalanine supplementation was increased to ~25 and ~30 mg/kg/day at day 85 and 99, respectively. Thereafter, dosage has frequently been adjusted (ranging from ~10 to ~30 mg/kg/day), especially based on blood phenylalanine concentrations (Fig. 1c).

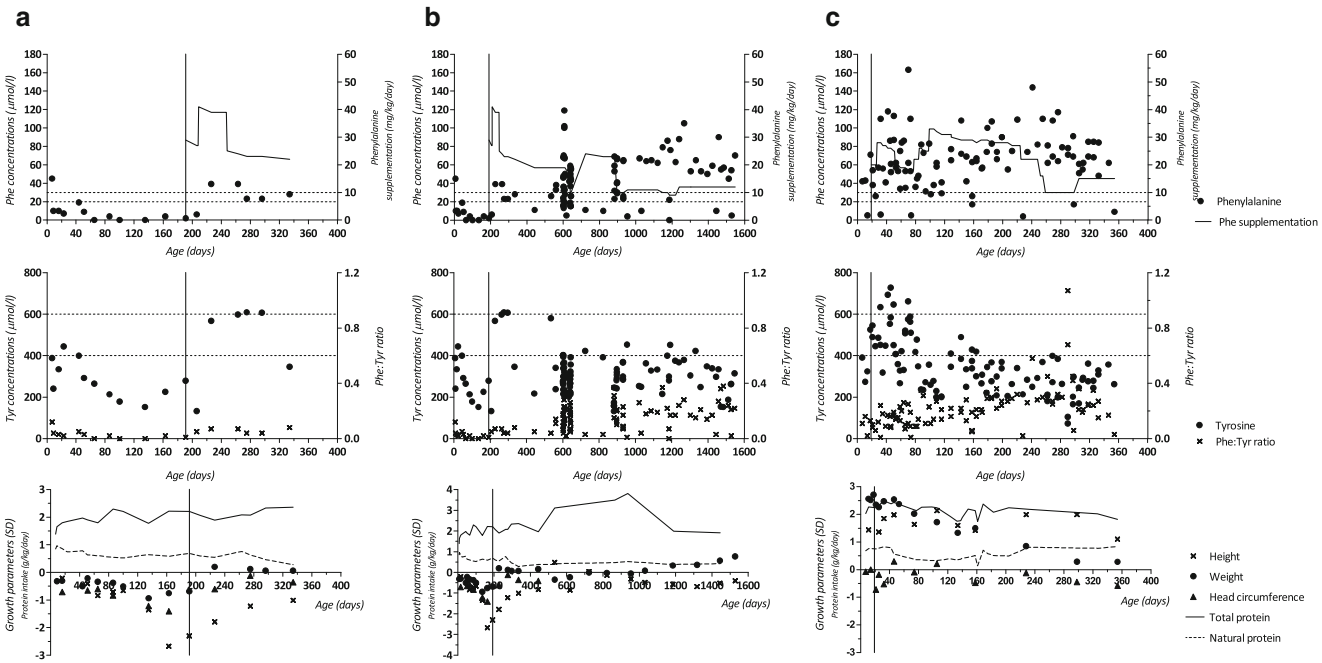

Fig. 1 Phenylalanine concentrations and supplementation (*upper*), tyrosine concentrations and ratios of blood phenylalanine-tyrosine concentrations (*middle*), and growth parameters as well as protein intake (*lower*) in patient 1 during the first year (**a**) and the first 4 years of life (**b**) and in patient 2 during the first year of life (**c**). The vertical line indicates the start of phenylalanine supplementation

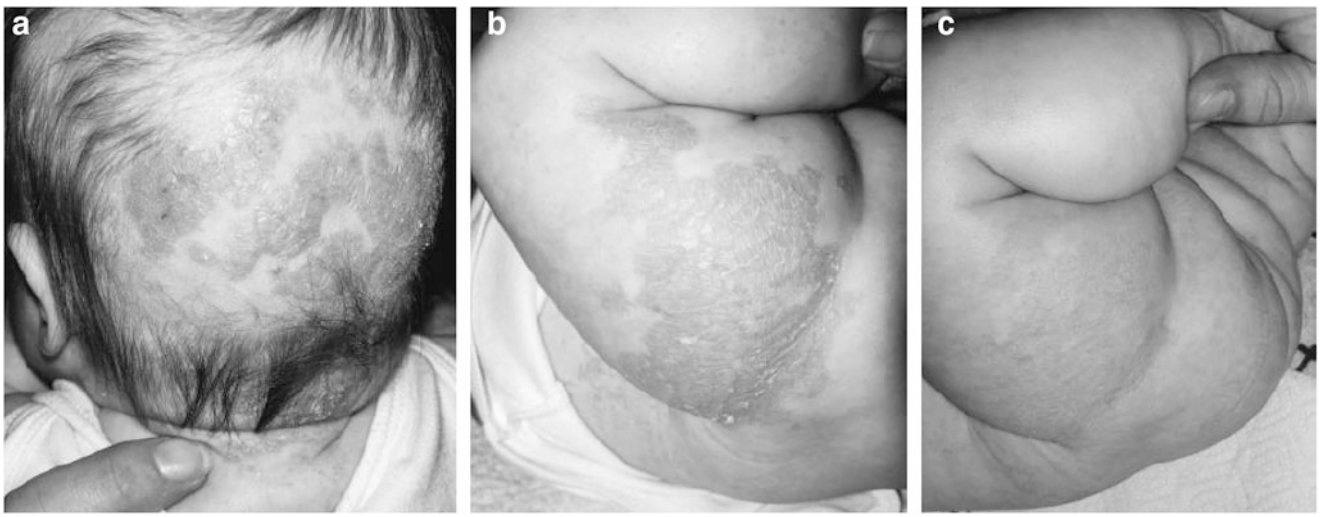

Fig. 2 Patient 1 – eczema-like skin eruptions developed at 5 months of age on the whole body, as depicted on the back of the head (**a**) and buttock (**b**). After 3 days of phenylalanine supplementation, the skin problem had largely been improved (**c**)

Biochemical Analyses

Phenylalanine and tyrosine concentrations were measured in plasma samples as well as in dried blood spots on filter paper. Plasma samples were drawn at the outpatient clinic, not taking into account the time of blood sampling. Actual measurement was performed with standard techniques on a Biochrom 20 or 30 analyzer (Pharmacia Biotech, Cambridge, UK). Blood spots were taken at home at variable times of the day. In dried blood spots, phenylalanine and tyrosine were quantified using high-performance liquid chromatography tandem mass spectrometry. If both plasma and dried blood spot analyses were available from a single time point, results from plasma samples were used.

Assessment of Developmental Milestones

Assessment of developmental milestones was performed at regular intervals at the child health center according to Dutch guidelines. In The Netherlands, developmental milestones are evaluated in children from 4 weeks to 5 years of age according to the Van Wiechen neuro-developmental assessment (Schlesinger-Was 1985). This assessment is based on milestones and consists of five domains: gross motor skills, fine motor skills, personality, social behavior, and communication skills.

Assessment of Spontaneous Motor Repertoire

The most reliable method to evaluate the integrity of the central nervous system of young infants is to assess the quality of their spontaneous motor repertoire (Einspieler and Prechtl 2005). Around the age of 3 months, the infant's motor repertoire is characterized by so-called fidgety movements (FMs) that are highly predictive of neurological outcome (Prechtl et al. 1997). From patient 2, video recordings were made during an outpatient visit at the age of 10 weeks and 1 day and at the age of 14 weeks and 1 day. The recordings were made during a period of active wakefulness with the infant lying supine and lasted 10 min (Einspieler and Prechtl 2005). Certified author MMH analyzed the recordings off-line according to Prechtl's method (Einspieler et al. 1997) and blinded for the outcome. First, the quality of FMs was labeled as normal, abnormal (exaggerated speed, amplitude, and jerkiness), or absent (no FMs observed). Additionally, a more detailed analysis was performed based on the motor optimality score (MOS) by using the Motor Optimality List (Bruggink et al. 2009). The MOS is composed of FM quality (12 points if normal, 4 points if abnormal, and 1 point if absent) plus 4 other items including age adequacy, normality of movement patterns, normality of postural patterns, and quality of the concurrent motor repertoire (4 points if normal, 2 points if nonoptimal, and 1 point if abnormal). The MOS may range from 5 (low optimality) to 28 (high optimality).

Neurocognitive Assessment

Neurocognitive assessment was performed by a qualified clinical psychologist. Verbal and nonverbal IQ were assessed separately. The SON-R (2.5–7) assessment was used for measurement of nonverbal IQ (Tellegen et al. 1998). Verbal performance was assessed using the General Language Index of the WPPSI-III-NL (Hurks et al. 2013).

Results

Clinical Outcome

In patient 1, subsequently to the institution of phenylalanine supplementation at day 191, all clinical parameters improved. Within some days after initiation of phenylalanine supplementation, eczema disappeared (Fig. 2). This was soon followed by resolution of cortical myoclonus as well as improvement of growth parameters (Fig. 1a, b). In patient 2, skin, growth, and development of motor milestones have all been normal.

Developmental Milestones

In patient 1, at 1 month of age, developmental milestones were all reached. At 3, 9, 12, and 15 months of age, fine motor skills, personality, social behavior, and communication skills were normal. However, development of gross motor skills seemed to be impaired without the tendency of progression. In patient 2, developmental milestones were normal at all ages at which developmental milestones were measured, being at age 1, 2, 3, 6, 9, and 12 months.

Neurocognitive Development

In patient 1, neuropsychological assessment was performed at age 4 years and 1 month. IQ score according to the nonverbal SON-R (2.5–7) assessment was 77 (80% CI: 63–79). Verbal performance on the General Language Index of the WPPSI-III-NL was 77 (95% CI: 69–93). These scores correspond to a mental development of children at 3 years of age.

Patient 2 was too young for a detailed neuropsychological assessment to be applied. Instead, video assessment of spontaneous movements was performed. At both recordings, we observed normal FMs with an MOS of 26. The infant only scored nonoptimal on the item concurrent motor repertoire with the movements having a monotonous and a slightly stiff appearance.

Blood Phenylalanine and Tyrosine Concentrations

Median phenylalanine and tyrosine concentrations as well as the incidence of hypophenylalaninemia (blood phenylalanine <30 μmol/L or <20 μmol/L) and hypertyrosinemia (blood tyrosine >400 μmol/L or >600 μmol/L) before and after initiation of phenylalanine supplementation are presented in Table 1.

Table 1 Phenylalanine and tyrosine concentrations at diagnosis and before and after institution of phenylalanine supplementation

	Patient 1				Patient 2				
Additional Phe/day	Diagnosis	8–364	Before 8–190	After 191–1,547	Diagnosis	8–364	8–190	Before 8–18	After 19–364
Phe (μmol/L); median (range)	45	9 (0–39)	6 (0–19)	43 (0–119)	42	64 (4–163)	58 (5–163)	43 (5–71)	65 (4–163)
Phe <30 (μmol/L)		88% (15/17)	100% (10/10)	33% (32/97)		9% (9/97)	10% (6/61)	33% (1/3)	9% (8/94)
Phe <20 (μmol/L)		71% (12/17)	100% (10/10)	15% (15/97)		6% (6/97)	5% (3/61)	33% (1/3)	5% (5/94)
Tyr (μmol/L); median (range)	388	292 (133–609)	253 (152–444)	298 (132–609)	391	331 (73–728)	384 (200–728)	324 (274–526)	333 (73–728)
Tyr >400 (μmol/L)		24% (4/17)	10% (1/10)	11% (11/97)		31% (30/97)	49% (30/61)	33% (1/3)	31% (29/94)
Tyr >600 (μmol/L)		12% (2/17)	0% (0/10)	2% (2/97)		5% (5/97)	8% (5/61)	0% (0/3)	5% (5/94)
Phe: Tyr ratio; median (range)	0.12	0.03 (0.00–0.08)	0.02 (0.00–0.05)	0.15 (0.00–0.38)	0.11	0.21 (0.01–1.07)	0.16 (0.02–0.35)	0.13 (0.02–0.16)	0.21 (0.01–1.07)

Phe phenylalanine, *Tyr* tyrosine

Incidence of hypophenylalaninemia and hypertyrosinemia are defined as the percentage of blood samples with phenylalanine concentrations <30 μmol/L or <20 μmol/L and tyrosine concentrations >400 μmol/L or >600 μmol/L, respectively

In patient 1, phenylalanine concentrations could not be quantitated in three samples

Overall, during the first 6 and 12 months of life, median blood phenylalanine concentrations were lower, and hypophenylalaninemia was observed more frequently in patient 1 than in patient 2. Median blood tyrosine concentrations, however, were comparable for both patients, and hypertyrosinemia was observed as frequently in both patients during the first 12 months of life. During the first 6 months of life, median blood tyrosine concentrations were even higher, and hypertyrosinemia was observed more frequently in patient 2 than in patient 1. Ratios of blood phenylalanine-tyrosine concentrations during the first 12 months of life tended to increase with increasing age in patient 2, while this increasing trend was not observed in patient 1.

From the day after diagnosis until day 190 (patient 1) and day 18 (patient 2), in both patients, hypophenylalaninemia was observed more frequently than hypertyrosinemia. Especially in patient 2, phenylalanine supplementation increased median blood phenylalanine concentrations and reduced the incidence of hypophenylalaninemia, while also increasing median blood tyrosine concentrations and the incidence of hypertyrosinemia. Irrespective of phenylalanine supplementation, between 40 and 120 days of age, both in patient 1 (without supplementation) and in patient 2 (with supplementation), blood tyrosine concentrations tended to decrease.

Discussion

The most important findings of this report are that (1) hypophenylalaninemia rather than hypertyrosinemia during the first months of life was observed to correlate with impaired growth, cortical myoclonus, neurological deficits, and skin problems and that (2) phenylalanine supplementation resolved these clinical problems despite causing a higher incidence of hypertyrosinemia.

Blood of both patients was collected at variable times of the day. However, blood phenylalanine concentrations in HT1 have previously been reported to show a large diurnal variation, consistently being lower in the afternoon (Daly et al. 2012), while previous reports showed that detection of deficient intake of essential amino acids can best be detected by comparing an overnight fasting and postprandial blood sample (Ozalp et al. 1972). Further studies may therefore best include a differential overnight fasting to postprandial measurement.

High blood tyrosine is often regarded as the cause of neurocognitive problems in HT1 (Thimm et al. 2011, 2012). However, these two cases support the hypothesis that hypophenylalaninemia rather than hypertyrosinemia could be the most important (De Laet et al. 2011; Bendadi et al. 2014). Patient 1 with the lowest incidence of hypertyrosinemia but highest incidence of hypophenylalaninemia showed skin problems, poor growth, and impairment in motor and cognitive development. In contrast, patient 2 with early preventive phenylalanine supplementation showed a higher incidence of hypertyrosinemia, normal skin, growth, developmental milestones, and quality of spontaneous movements. In addition, the start of phenylalanine supplementation in patient 1 immediately improved growth and resolved eczema and cortical myoclonus.

These cases also show that blood phenylalanine concentrations may not immediately increase in response to phenylalanine supplementation as all supplemented phenylalanine is first used for protein synthesis, leaving phenylalanine concentrations in blood unchanged, thereby supporting the idea that phenylalanine had indeed been rate limiting for protein synthesis. Consistent with this idea, concentrations of other essential amino acids (results not shown) as well as tyrosine all decreased after phenylalanine had been initiated, and catch-up growth was observed.

The fact that, before phenylalanine supplementation had been initiated, both height and weight were affected and weight-for-height remained normal supports the idea that a qualitative rather than a quantitative malnutrition had been present. Also, in both patients, prealbumin concentrations had increased after phenylalanine supplementation. This all suggests that low phenylalanine rather than high tyrosine – at least during the first months of life – may impair growth and neurocognitive outcome in HT1 infants.

This idea is supported by the early experiences with treatment of phenylketonuria in which phenylalanine deficiency during the first months of life has been reported to impair growth and intellectual development and to induce eczema (Rouse 1966). Further circumstantial evidence might be that these problems at least had not been reported before the era of NTBC. This might well be due to a lack of focus, as preventing liver failure and hepatocellular carcinoma was the primary and ultimate target of treatment in those days. However, it could also be that developmental delay and skin problems were indeed not present, for not only tyrosine concentrations were high but also blood phenylalanine concentrations were rather high than low in those patients (Couce et al. 2011). Alternatively, NTBC – at its own – may have resulted in these issues as well, but NTBC dose did not vary substantially between and in our patients.

In conclusion, these cases urge to consider the danger of low phenylalanine concentrations with respect to neurocognitive outcome in HT1 patients and to treat accordingly. Further studies including a larger patient population are certainly required. The exact minimal phenylalanine level – as the maximal tyrosine level – is a matter of further investigation. Also, the optimal phenylalanine supplementation regimen to prevent both phenylalanine deficiency and tyrosine toxicity requires additional research.

Take-Home Message

Phenylalanine supplementation should really be considered in HT1 patients with consistently low blood phenylalanine concentrations during the first months of life.

Compliance with Ethical Guidelines

Conflicts of Interest

Esther van Dam has received advisory board fees from Merck Serono.

Margreet van Rijn has received research grants, consultancy fees, and advisory board fees from Merck Serono and Nutricia Research; speaker's honoraria from Merck Serono, Nutricia Research, and Orphan Europe; and expert testimony fees from Merck Serono.

Terry G.J. Derks has received research grants from Sigma Tau and Vitaflo and speaker's honoraria form Nutricia and Vitaflo.

Francjan J. van Spronsen has received research grants, advisory board fees, and/or speaker's honoraria from Merck Serono, Merck (the Netherlands), Nutricia Research, and Vitaflo.

Danique van Vliet, Gineke Liefaard-Venema, Marrit M. Hitzert, Roelineke J. Lunsing, and M. Rebecca Heiner-Fokkema declare that they have no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000. This study was waived from approval from the responsible committee on human experimentation. Informed consent was obtained from all patients for which identifying information is included in this article.

Details of the Contributions of the Authors

Danique van Vliet drafted the initial manuscript, revised the manuscript, and approved the final manuscript as submitted.

Esther van Dam performed the dietary follow-up of the second patient, drafted the initial manuscript, and approved the final manuscript as submitted.

Margreet van Rijn performed the dietary follow-up of both patients, critically reviewed the manuscript, and approved the final manuscript as submitted.

Terry G.J. Derks performed the treatment and monitoring of both patients, cowrote the manuscript, and approved the final manuscript as submitted.

Gineke Venema-Liefaard performed the dietary follow-up of the first patient, critically reviewed the manuscript, and approved the final manuscript as submitted.

Marrit M. Hitzert performed assessment of spontaneous motor repertoire in patient 2, critically reviewed the manuscript, and approved the final manuscript as submitted. Roelineke J. Lunsing performed was responsible for neurological follow-up of both patients, critically reviewed the manuscript, and approved the final manuscript as submitted.

M. Rebecca Heiner-Fokkema was responsible for the biochemical analyses, critically reviewed the manuscript, and approved the final manuscript as submitted.

Francjan J. van Spronsen was responsible for the diagnosis of HT1, performed the treatment and monitoring of both patients, cowrote the manuscript, and approved the final manuscript as submitted.

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RESEARCH REPORT

Neurodevelopmental Profiles of Children with Glutaric Aciduria Type I Diagnosed by Newborn Screening: A Follow-Up Case Series

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Abstract Glutaric aciduria type I (GA-I) is an inherited metabolic disorder that may lead to severe motor disorder and cognitive impairment. GA-I is now included in the newborn screening programme in many countries as early detection allows for prompt treatment and effectively reduces the risk of poor developmental outcome. Information regarding the long-term neurodevelopmental outcome of children with GA-I treated early is sparse.

We recruited children with a confirmed diagnosis of GA-I diagnosed via newborn screening, treated in our centre and >3 years of age ($n = 6$). Children were assessed at two time points using a comprehensive neuropsychological test battery. Four of these had been the subject of a previous report. All participants were male, 3–6 years at the initial assessment and 6–12 years of age at the follow-up assessment.

Fine motor skills were below average in all patients. Speech, which was affected in all four patients reported previously, improved following speech therapy. IQ scores remained generally stable within the normal range. Executive functioning was average to high average in four patients. Behaviour, as assessed through parental questionnaires, was problematic in two patients. Compounding factors included child neglect, family history of autism and multiple admissions to hospital ($n = 1$ in each).

GA-I affects fine motor skills and speech, regardless of early treatment, but not IQ scores. Patients with GA-I should be referred for assessment and appropriate early intervention. Further research is needed to correlate specific neuropsychological deficits with neuroimaging.

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Introduction

Glutaric aciduria type I (GA-I) is a rare inherited neurometabolic disorder due to a deficiency of the enzyme glutaryl–CoA dehydrogenase (Goodman et al. 1975), with an incidence of approximately 1: 100,000 newborns (Lindner et al. 2004; Boneh et al. 2008). Glutaric acid and 3-hydroxyglutaric (3OHGA) acid are produced as a result of the enzyme deficiency and are trapped within the brain due to their inability to cross the blood–brain barrier, resulting in a metabolic toxicity affecting the central nervous system (Kölker et al. 2006a).

Children with GA-I may suffer from irreversible motor disorder and develop severe dystonia and speech

impairment following an intercurrent infection, usually in the first two years of life, and may have varying degrees of intellectual disability and behavioural problems (Kyllerman et al. 2004; Naughten et al. 2004). Neurological damage underlying these functional impairments is thought to be due to early encephalopathic crises that predominantly cause bilateral striatal injury (Kölker et al. 2006b). The clinical outcome of patients diagnosed by newborn screening has improved compared with those diagnosed clinically, since precautionary measures and treatment are initiated early (Hoffmann et al. 1996; Kölker et al. 2007; Boneh et al. 2008). Indeed, patients diagnosed via newborn screening show a lower incidence of striatal injury (Harting et al. 2009) and acute encephalopathic crises (Kölker et al. 2007). However, recent studies suggest that neurological damage can occur in the absence of an encephalopathic crisis due to neurotoxicity in utero or early life and that this may be associated with delayed myelination and brain maturation (Harting et al. 2009). This theory is supported by findings of brain abnormalities and widening of the sylvian fissures and fronto-temporal atrophy in newborns and as early as 33 weeks gestation (Lin et al. 2002; Mellerio et al. 2008), which may resolve over time (Viau et al. 2012).

Children with early brain injury may be at further risk of developing cognitive, social and behavioural problems. Previous reports on neuropsychological outcomes of patients diagnosed clinically found speech and motor impairments (Kyllerman et al. 1994; Kyllerman et al. 2004). Children diagnosed via newborn screening generally have average intelligence, although they may be vulnerable to more subtle deficits in language and speech and fine motor skills (Beauchamp et al. 2009; Viau et al. 2012; Lee et al. 2013). Environmental factors can act as protectors or increase vulnerability (Dennis 2000). This has been referred to as the 'double hazard theory' (Breslau 1990). Whilst some studies have considered factors such as timing of diagnosis and severity of illness (Bjugstad et al. 2000), environmental factors such as socioeconomic background, family functioning and social support are largely ignored. One of the unresolved questions regarding patients with GA-I diagnosed and treated early is their brain vulnerability after age 5 years.

We have previously reported on the outcome of four children (age range 3–6 years) diagnosed with GA-I through newborn screening using a comprehensive neuropsychological assessment battery that focused on identifying specific neurodevelopmental strengths and difficulties (Beauchamp et al. 2009). In order to investigate the putative effects of a possible on-going neurotoxicity of glutaric acid and 3OHGA beyond age 5–6 years, we have reassessed the four children from the previous report and another two other children diagnosed via newborn screening.

Methods

Participants

Children recruited for this study had a confirmed diagnosis of GA-I and have been treated at the Royal Children's Hospital (RCH), Melbourne, since their diagnosis via newborn screening ($n = 6$). All participants were male and 3–6 years at the initial assessment (younger patients were excluded for consistency of testing) and between 6 and 12 years at the follow-up assessment (Table 1). Patient history has been documented in a previous report (Boneh et al. 2008). Of note, patient 4 is from a relatively low SES background and has an earlier history of neglect with a period of foster care that resulted in improved development. He is currently wheelchair bound, requires support with self-care activities and attends a special development school.

Procedure

Ethics approval was granted from the RCH Human Research Ethics Committee (HREC #32218A). Information letters were sent out to the parents of eligible participants, and informed consent was provided by parents. Assessments were conducted at the RCH, Melbourne, Australia. The assessment duration was two to three hours with breaks.

Measures

Children were assessed using a comprehensive, standardised neuropsychological assessment battery. The assessment protocol was modified from the original protocol (Beauchamp et al. 2009) to include measures of executive functioning, attention, social skills and language, in order to provide a more comprehensive assessment of strengths and difficulties. Tests included were the following: *Motor Function*: Movement Assessment Battery for Children (MABC-2) Manual Dexterity (fine motor), Aiming and Catching and Balance (gross motor) (Henderson et al. 2007). *Language*: Clinical Evaluation of Language Fundamentals Version 4 (CELF-4) (Semel et al. 2003) – Concepts and Following Directions (receptive language), Formulating Sentences, Recalling Sentences, Word Structure/Classes (expressive language). *Intelligence*: Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler 1999) and Wechsler Preschool and Primary Scale of Intelligence Third Edition (WPPSI-III) (Wechsler 2002). *Memory*: NEPSY II – Narrative Memory, Memory for Designs (Korkman et al. 2007). *Attention*: NEPSY II Statue, Test of Everyday Attention for Children (TEA-Ch) (Manly et al 1999) – Sky Search Attention score (selective), Score (sustained),

Table 1 Clinical and medical history

ID	Sex	SES decile	Age time1	Age time 2	MRI findings	Speech therapy	Other intervention	Comorbidities	Relevant family history	Hospital admissions
01	M	10	4	10	N/A	Yes	Nil	Speech – diagnosed articulation difficulty		3
02	M	8	3	6	2010 3.5 years Bilateral symmetrical mild malformation of frontal lobe	No	Psychologist – behavioural issues	Speech difficulties, delay in handwriting, behaviour	Sibling diagnosed with autism spectrum disorder	6
03	M	4	5	6	2009 2 years Bilaterally symmetrical abnormality of globus pallidus, substantia nigra and medial lemniscus. Enlarged extra-axial spaces extending inferiorly, anterior to anterior temporal poles	Yes	Early intervention, occupational therapy	Otitis media, poor motor development with intoeing gait, tremor, speech difficulties		30+
04	M	4	6	11	2006 5 years 2003 2 years Extensive periventricular and frontal lobe white matter abnormality. Bilateral putamen abnormality. No evidence of further deterioration over time	Yes	Special development school	Severe movement disorder, previously diagnosed severe language delay, expressive language impairment	Early history of neglect and foster care	5
05	M	10	6	12	N/A	Yes	Nil	Slight tremor, speech – articulation		2
06	M	10	3	8	2005 1 year Increase in extra-axial CSF spaces, widening of sylvian fissures. Myelination normal	No	Nil	Speech difficulties suspected at previous assessment[a]		13

[a] Suspected but not confirmed due to age constraints of testing

Sky Search DT (divided). *Executive Functioning*: NEPSY II – Inhibition (Naming, Inhibition and Switching), Animal Sorting, Behaviour Rating Inventory of Executive Functioning (BRIEF) Parent Rating Form (Gioia et al. 2003). *Behaviour*: Strengths and Difficulties Questionnaire Parent Form (SDQ) (Goodman 1997). *Adaptive Behaviour*: Vineland Adaptive Behaviour Scales Second Edition (VABS) (Sparrow et al. 2005). *Social*: NEPSY II – Affect Recognition, Social Skills Improvement System (SSIS) Parent Rating Form (Gresham and Elliott 2008).

Scores on all tests were compared to standardised age norms. A standard deviation (SD) of two or more below the mean was considered to be 'impaired' and a significant deficit. Scores between 1 and 2 SD below the mean represented below average or 'borderline' skills and were considered a mild deficit with clinical significance (Tabachnick et al. 2000). A clinically meaningful change in scores between time points was operationalised as of >1SD. Interpretation of test scores are as follows: WASI/WPPSI, VABS and SSIS (standard scores (STD): mean = 100, SD = 15); NEPSY, TEA-Ch, MABC-2 and CELF (scaled scores (SS): mean = 10, SD = 3); BRIEF (T-scores: mean = 50, SD = 10); and VABS maladaptive behaviour scale (<17 normal, 18–20 borderline and 21–24 impaired). The SDQ is a screening tool that uses cut-off values for each subscale based on the 80th and 90th percentile to categorise individuals into three levels (normal, borderline or impaired). For measures where subtests provided multiple scores for various components of a skill, the total score was utilised. Socioeconomic status (SES) was determined using the socioeconomic advantage and disadvantage index based on Socioeconomic Index of Areas 2011 census data where a decile of 1 reflects the lowest 10% of disadvantages areas (Australian Bureau of Statistics 2011). Parents completed a background and demographic questionnaire that included developmental history and the use of clinical services. Clinical observation of speech was formally recorded during each assessment and clinical history of speech therapy was documented. Background medical history and previous magnetic resonance imaging (MRI) scans were obtained via medical records and parental report.

Results

Previously, we reported that patients with GA-I showed impaired fine motor skills and some degree of speech deficit for which they were referred for speech therapy (Beauchamp et al. 2009). The assessments revealed that cognitive and behavioural profiles ranged around average for their age in all other patients apart from patient 4, whose tests outcomes were modified by environmental factors, rather than purely a result of GA-I. The medical history of patients 2, 3, 4 and 6 showed varying degrees of bilateral brain pathology based on previous brain imaging obtained clinically (Table 1).

In the current study all patients performed within the impaired range in fine motor skills, except for one who was in the borderline range, indicative of a mild deficit (Table 2). By contrast, gross motor skills were found to be at age-appropriate levels apart from patient 4 who has a motor disorder as mentioned above. Patient 2 was the only one who showed a decrease in fine motor scores between the two time points; other patients' scores did not show any further differences.

Articulation difficulties resolved following speech therapy for those who were previously diagnosed (Beauchamp et al. 2009) (patients 1 and 5). Whilst patient 6 was suspected of having a mild articulation problem at the previous assessment, this was not evident at the follow-up assessment, and expressive language skills were intact. Patient 4, who has a history of language and speech problems, was observed to have significant difficulty in speech production and symptoms of dysarthria which hindered his communication. Language assessment found his receptive language within the average range, whilst expressive language fell in the impaired range (Table 2). Qualitatively, his speech was slow and difficult to comprehend. Given time, he was able to respond to questions although it was apparent that it was laborious for him. Patient 3 had impaired expressive language, as demonstrated on the 'Formulating Sentences' subtest. Whilst he had no previous intervention, he has been referred for speech therapy. Both receptive and expressive language skills were within the average to high average range in patients 1, 5 and 6. Patient 2 had average expressive language skills and receptive language skills in the borderline range, but this may be due to inattention during the task (see below).

IQ scores generally remained stable, with the exception of patient 2 who was found to score more than one standard deviation lower on the current review assessment (95% CI 84–99) and patient 3 who showed more than one standard deviation increase (95% CI 89–104) (Table 2). It should be noted that patient 3's brother, who does not have GA-I, is diagnosed with autism spectrum disorder and that the assessment was done at a time of stressful family issues. Patients 1 and 3 showed a regression in verbal memory scores by one standard deviation from their previous assessment, whilst all other patients' scores remained stable. Interestingly, their scores on the 'Recalling Sentences' language subtest (also reliant on verbal memory) were in the low average range, indicating that this may represent an area of difficulty for both children. No impairments in visual memory were found.

On attention measures, patients 2 and 4 scored within the impaired range. Patient 2 showed reduced selective attention

Table 2 Results of child direct measures

Patient	1		2		3		4		5		6	
Age at testing (years, months)	4–10	10–1	3–4	6–1	5–4	6–11	6–0	11–6	6–11	12–8	3–0	8–8
Motor Skills (*MABC-2*)												
Manual Dexterity (fine)	*6*	**4**	8	**3**	**4**	**3**	**1**	**1**	**4**	**3**	*5*	*5*
Aiming and Catching (gross)	7	12	*6*	8	8	12	<**4**	<**4**	9	12	8	9
Dynamic Balance (gross)	14	8	16	*6*	7	7	<**4**	<**4**	10	11	10	15
Language												
GLI (WPPSI-III)	105		125				**70**		110		119	
CLI (CELF)		103 (10)		90 (8)	*84* (*7*)	*81* (*6*)		**66** (**3**)		123 (15)		117 (13)
Concepts and Following		10		*5*	8	8		10		14		12
Word Structure/Classes		11		12	8	8		**3**		15		14
Recalling Sentences		7		9	*5*	7		**2**		12		14
Formulating Sentences		14		7	8	**4**		**3**		14		11
FSIQ (*WPPSI*, *WASI*)	106	99	112	91	*74*	96	**62**	**65**	124	126	109	124
Memory (*NEPSY*)												
Narrative Memory	9	*5*	10	9	8	*5*	**1**	**3**	15	14	10	11
Memory for Designs	14	13	7	7	*6*	11	8	7	11	12	12	14
Attention (*TEA-Ch*)												
Statue (NEPSY)	13		10		**3**		<**4**		11		14	
Sky Search Attention (selective)		12		**2**		7		**1**		10		11
Score (sustained)		13		7		7		DNC		8		10
Sky Search DT (divided)		15		DNC		8		DNC		13		15
Executive Functioning (*NEPSY*)												
Animal Sorting		10		–		–		1		9		11
Inhibition – Naming		9		*6*		13		DNC		7		10
Inhibition – Inhibiting		14		DNC		7		DNC		11		11
Inhibition – Switching		14		–		–		DNC		12		13
Social Perception (*NEPSY*)												
Affect Recognition		13		7	7	11		*5*		13		15

FSIQ full scale IQ, *GLI* general language index, *CLI* core language index, *DNC* did not complete, (–) not applicable due to age range
Scores in bold indicate the impaired range of at least 2 SD below the mean or less; italicised scores are borderline or 1–2 SD below the mean

on Sky Search, and he did not complete the divided attention task Sky Search DT due to noncompliance. Qualitative observation showed that this child found it difficult to sit for long periods of time and often required instructions to be repeated. Patient 4 demonstrated adequate selective attention by identifying correct targets; however, his fine motor impairments (hand movements required for circling targets) impacted on timing of task completion, thereby reducing his score. He was not able to complete the remaining tasks as he was not able to demonstrate the required ability to count to 15. Patient 3 had borderline results on the selective attention task and on Score, the sustained attention task.

Executive functioning, as measured in a test of response inhibition, was average to high average in patients 1, 3, 5 and 6. These children also performed in the average range on the Animal Sorting task, indicating intact planning and concept formation (patient 3 was not administered this task). Patients 2 and 4 were not able to complete the entire executive function component of the assessment. Parent ratings on the BRIEF questionnaire, tapping everyday executive skills, showed that only patient 2 was rated to have impaired skills in the area of emerging metacognition comprising of working memory, planning and organisation abilities.

Behaviour was assessed primarily through parental questionnaires. For patients 2 and 3, behaviour was rated as impaired (Table 3). Patient 2 was rated highly for externalising symptoms such as hyperactivity and conduct problems on the SDQ and the maladaptive behaviour scale (VABS).

Table 3 Results of parent questionnaires

	1	2	3	4	6
Executive functioning (*BRIEF*)					
Behavioural Regulation Index	38	40	**80**	52	38
Metacognition	33	**83**	**76**	43	35
Global Executive Functioning	34	49	**80**	46	36
Adaptive Behaviour (*VABS*)					
Communication	100	91	83	**69**	138
Daily Living Skills	95	123	77	**40**	127
Socialisation	94	100	92	**57**	119
Motor Skills	–	81	78	–	–
Adaptive Total	96	98	79	**55**	129
Maladaptive Behaviours Index	–	*18*	*20*	*19*	12
Internalising	–	15	**22**	12	13
Externalising	–	*19*	17	17	12
Behaviour (*SDQ*)					
Emotional Symptoms	3	0	**5**	0	0
Conduct Problems	0	**4**	*3*	0	0
Hyperactivity	0	**7**	**9**	0	0
Peer Problems	0	*3*	0	1	0
Behaviour Total	3	**19**	**17**	1	0
Prosocial Behaviour	9	*5*	7	10	10
Social Skills (*SSIS*)					
Social Skills Scale	121	95	**71**	110	123
Problem Behaviour Scale	85	102	**135**	100	84

N.B. BRIEF T-scores ($m = 50$, SD = 10), SSIS standard scores ($m = 100$, SD = 15). Lower scores on BRIEF, SDQ, SSIS problem behaviour and VABS maladaptive behaviour scales indicate better outcome; SDQ prosocial scale, SSIS social skills scale and VABS higher scores indicate better outcome. VABS maladaptive behaviour scaled scores interpreted as follows: <17 normal, 18–20 borderline and 21–24 impaired. Scores in bold indicate impaired range (>2 SD) or BRIEF T-score >65, SSIS impaired social skills = <85, problem behaviours >115 or impaired range for SDQ based on clinical cut-off scores; *italics* indicate borderline range between 1 and 2SD below the mean *No results for patient 5 as parent did not complete forms

Patient 3 was rated as showing a heightened level of emotional symptoms, conduct problems and hyperactivity. He demonstrated impaired skills on the BRIEF Behavioural Regulation index, particularly for emotional control and inhibition. Taken together, these results indicate a clear pattern of behavioural issues. Adaptive behaviour was reduced only in patient 4, who had significant motor impairment that impact on most areas of his daily functioning.

Patients 1, 3, 5 and 6 performed within the average range to high average on a task of social skills; patient 2's scores were in the low average range; and patient 4 was in the borderline range. Consistent with this, parents rated their child's social skills (SDQ and SSIS) (Table 3) within the normal range in these domains with the exception of patient 3 who was rated impaired. It should be noted that he was the only patient who rated as displaying 'autistic behaviours' on the autism spectrum disorder subscale of the SSIS at both time points. Patient 2 was rated in the borderline range on prosocial skills as reported on the SDQ.

Discussion

The aim of this case series was to evaluate neurodevelopmental outcome throughout childhood in patients with GA-I diagnosed through newborn screening and treated early. Using a comprehensive neuropsychological assessment enabled us to identify specific strengths and difficulties that may not have been identified by conducting an IQ assessment alone. In doing this, we have shown that children with GA-I have a profile of impaired fine motor skills and are particularly susceptible to speech and language difficulties. Overall it appears that intellectual functioning is at the expected level in children treated early, unless affected by environmental factors. Of note, although some children's performance declined in particular areas, there was no particular trend of regression over time. Furthermore, speech difficulties were mostly ameliorated in those who had therapeutic intervention since the previous assessment. This highlights the importance of

early assessment and identification of deficits so that intervention may be sought as early as possible and enables children to have the best possible outcome.

Motor skills

Weaknesses in fine motor skills have been previously reported by our group (Beauchamp et al. 2009). In this follow-up assessment, these weaknesses were still evident in all patients. It is important to note that apart from patient 4, patient 2 was the only one to show a clinically significant impact of his fine motor deficit. In his daily life, this manifested as a delay in handwriting skills. In all other patients, fine motor weakness did not have a direct impact on daily lives and was not of concern to parents. The consistency of this finding at both time points indicates that this deficit is an on-going clinical feature of GA-I, likely to be due to an early impact of GA-I on brain development. Early effects of GA-I on the brain have been documented in newborns and as early as 33 weeks gestation (Lin et al. 2002; Mellerio et al. 2008). GA-I causes striatal and fronto-temporal pathology (Harting et al. 2009), areas that are associated with motor skills and motor planning (Groenewegen et al. 2003).

Speech and Language

Previous language assessment found no weaknesses, but most children were found to have some degree of speech difficulty (e.g. dysarthria, articulation difficulties). The more comprehensive language assessment in the current study showed that expressive and receptive language skills were at age-appropriate levels in patients 1, 5 and 6. The observation that speech therapy following the previous assessment leads to improvement highlights the need for all patients with GA-I to have a speech assessment and early intervention where required. Previous research has found a correlation between motor deficits and speech and language impairments in clinically diagnosed children with GA-I (Kyllerman et al. 2004). This relationship has been reported in the general population (Hill 2001), and in children with oral-motor deficits and fine motor impairments (Hill 2001; Newmeyer et al. 2007), and is consistent with the overlap in the neural networks underpinning these skills, which are developing rapidly in early life (Diamond 2000). It is suggested that speech and language problems may be related to a motor planning deficit affecting oral-motor skills (Beauchamp et al. 2009), given that GA-I can affect the striatum (Strauss and Morton 2003; Lagranha et al. 2014). In this context it is of note that all but one child in this case series had some degree of previous speech difficulties (primarily relating to articulation) and most patients had impaired fine motor skills. Another possible explanation is a genetic link, as has been shown between motor impairment and speech–language impairments (Bishop 2002). However, this link was reported to be most evident on tasks assessing accuracy of speech production which were not formally assessed in our patients.

IQ/Memory

IQ scores remained relatively stable, apart from patient 3 who showed an increase in scores and patient 2 who showed a decline of more than one standard deviation. His score still remained within the average range, not indicating any significant disability. A similar decline was also found in his overall language score. This is likely to represent a failure to meet age-appropriate developmental gains rather than further deterioration, although this was difficult to clarify given that raw scores were not comparable due to the use of different tests. Further compounding factors are a family history of autism and testing during a stressful stage in his life, as mentioned above. Since all other patients remained within a similar score range, overall there is no evidence of any trend or deterioration of intellectual functioning in children over time.

There was little change in visual memory between the two time points. The fact that verbal memory was below average in patients 1 and 3, with a small regression in scores since the previous assessment, highlights this as area of difficulty for these children. Memory difficulties have not been found in any previous studies on GA-I patients, although GA-I-deficient mice have been shown to have short- and long-term memory deficits (Busanello et al. 2013). Problems with speech and expressive language may contribute to a poorer verbal memory performance (Korkman et al. 2007). As not all children showed a decline in verbal memory, it cannot be assumed that this is a clinical feature of GA-I.

Attention/Executive Functioning

Children who are treated early show high signal intensity of deep white matter, and it is thought that there is still significant risk of brain insult during the first year of life, possibly due to increased vulnerability of the CNS during periods of intercurrent illness (Harting et al. 2009; Lee et al. 2013). It is proposed that this extra-striatal damage, rather than striatal injury, is the cause of neurodevelopmental issues in those diagnosed early (Busanello et al. 2013). Whilst brain maturation is delayed, it has often caught up by four years of age (Harting et al. 2009).

White matter changes commonly found in GA-I are likely to affect attention and executive function abilities (Harting et al. 2009). This was not assessed in depth during the previous assessment due to age constraints. In the current group, those with average IQ scores had average or above

average levels of attention and executive functioning. Using the more comprehensive tests of selective, sustained and divided attention, two patients were identified as having below expected levels of attention, although patient 4's results were impacted by his lack of fine motor skills and as he did not complete the other subtests, it is difficult to interpret his results. Executive function problems were noted in patients 2 and 3; however, since these skills continue to develop into adolescence and since this group of children is still young, it is difficult to estimate the long-term impact of this result (Anderson 2001). Future studies with larger cohorts and systematic brain imaging will hopefully correlate brain pathology and neurodevelopmental outcomes.

Behaviour and Social Skills

Although most children displayed no behavioural problems, patients 2 and 3 were reported to have difficulties that had developed since the previous assessment (Beauchamp et al. 2009). Externalising behavioural problems have been noted in children with 'intoxication type' metabolic disorders such as GA-I (Simons et al. 2006), and there have been two reports of attention deficit hyperactivity disorder diagnoses in children with GA-I, one in a clinically diagnosed child (Kyllerman et al. 2004) and another diagnosed by newborn screening and treated early (Couce et al. 2013). Furthermore, GA-I mice have been found to have altered dopaminergic functioning, associated with hyperactivity (Busanello et al. 2013). Whether behavioural problems are related to the disorder is unclear as it was not a consistent finding within the group, and secondary environmental or genetic factors may moderate this relationship.

The parents of patient 3 rated their child as having impaired social skills, whilst patient 2 was also rated as lacking prosocial behaviours. This is not surprising given the presence of speech and language problems in these children, which can make it difficult to communicate with peers, leading to fewer social experiences. This may increase the incidence of psychosocial problems in the long term (Glogowska et al. 2006; Snowling et al. 2006). Moreover, it should also be noted that other genetic factors may contribute to the outcome of children with a particular disorder, for example, patient 2 who has a sibling diagnosed with autism. Behaviour and social development are also impacted by environment, a factor that is rarely considered but may be important to consider in the neuropsychological profiles of children with metabolic disorders. For example, early treated children with phenylketonuria from low SES backgrounds were found to have a higher incidence of behavioural problems (Wu et al. 2011). In our current small group, two patients (3 and 4) who had significant social and behavioural problems are from a lower SES background in comparison to other patients. Additional factors contributing to their performance include child neglect and multiple admissions to hospital in early life (Table 1). One should therefore be aware that now that children are treated early they have a better chance of survival and a potentially less severe clinical course, environmental factors may become more relevant and will need additional attention.

Conclusions

The results presented in this case series suggest that GA-I affects fine motor skills and speech, regardless of IQ scores and early treatment. IQ, executive functioning, attention, gross motor skills and visual memory were generally average to above average, whilst performance on language and verbal memory tasks was more variable. Behavioural problems were identified in two patients. Further research that correlates specific neuropsychological deficits (function) with brain imaging (structure) may be helpful in clarifying this relationship. Children with metabolic disorders that affect brain development may be more vulnerable to environmental factors, and this may moderate their outcome. Our observation of improved speech following speech therapy suggests that children with GA-I should be referred for neuropsychological and speech assessments early in life, so that they can be referred for early and appropriate intervention where required.

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Synopsis

Children with glutaric aciduria type I who are diagnosed through newborn screening have reduced fine motor skills and are at risk of speech and language difficulties despite early diagnosis and treatment.

Compliance with Ethics Guidelines

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

Informed Consent

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

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

Details of the Contributions of Individual Authors

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

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

Miriam Beauchamp devised previous study protocol, collected previous data and assisted in reviewing and editing the manuscript.

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

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

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CASE REPORT

Mild Lesch–Nyhan Disease in a Boy with a Null Mutation in *HPRT1*: An Exception to the Known Genotype–Phenotype Correlation

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Abstract Hypoxanthine–guanine phosphoribosyltransferase (HPRT) deficiency results in a continuous spectrum of clinical phenotypes though all include overproduction of uric acid with hyperuricaemia, urate nephrolithiasis and gout. *HPRT1* mutations that result in very low or no HPRT enzyme activities are generally associated with the classic Lesch–Nyhan disease (LND) phenotype with intellectual disability, motor handicap and self-injurious behaviour. Mutations that permit a higher residual HPRT activity are seen in some patients with the milder LND variant phenotypes with varying degrees of cognitive, motor handicap and maladaptive behaviour without recurrent self-injury. We present a boy with a LND variant phenotype due to a deletion of exon 5 of *HPRT1* predicted to fully abolish HPRT activity. Metabolic analysis confirms lack of significant residual enzyme activity. The boy, currently age 10, presented with hyperuricaemia, hypotonia, developmental delay and extrapyramidal and pyramidal involvement. He has never shown any signs of self-injurious or maladaptive behaviour. This boy is one of the rare cases with a suspected null mutation in *HPRT1* that associates with a milder than expected phenotype with lack of self-injurious behaviour.

Key Clinical Message *HPRT1* mutations that result in very low or no hypoxanthine–guanine phosphoribosyltransferase enzyme activities are generally associated with the classic Lesch–Nyhan disease. This report presents one of the rare cases with a null mutation in the *HPRT1* gene that associates with a milder than expected phenotype with lack of self-injurious behaviour.

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Introduction

Lesch–Nyhan disease (LND) is an X-linked monogenic disorder associated with development of gout due to marked overproduction of uric acid (Torres and Puig 2007). At one end of the clinical spectrum are patients with the classic LND phenotype associated with overproduction of uric acid, severe motor handicap resembling dystonic cerebral palsy, intellectual disability and recurrent self-injurious behaviour. At the other end of the spectrum, patients have an overproduction of uric acid without apparent neurological or behavioural deficits. Collectively, the attenuated phenotypes are classified as LND variants and are often distinguished from the classic LND by the lack of self-injurious behaviour although many exhibit maladaptive behaviour (Jinnah et al. 2010). In the vast majority of LND variants, patients have mutations in *HPRT1* that allow some residual activity of hypoxanthine–guanine phosphoribosyltransferase (HPRT), whereas complete loss of enzymatic activity is associated with classic LND (Jinnah et al. 2010; Fu et al. 2013).

Recognition of LND variants is important for understanding the pathogenesis and natural history as well as for diagnosis of all patients with HPRT deficiency. We present a boy hemizygous for a deletion of exon 5 in *HPRT1* resulting in almost complete absence of residual HPRT activity but with no signs of self-injurious or maladaptive behaviour.

Case Report

A 10-year-old boy born to non-consanguineous parents was referred at age seven months due to hypotonia. The mother was diagnosed with phenylketonuria as a newborn and during her pregnancy, serum levels of phenylalanine were generally held below 360 μmol/L as recommended during pregnancy. Family history was otherwise unremarkable. Brain magnetic resonance imaging showed no abnormalities. He had an elevated serum uric acid at 0.78 mmol/L (ref. 0.12–0.32 mmol/L) and the urine uric acid/creatinine ratio was at the upper normal limit (1,500 μmol/mmol creatinine; ref. 260–1,540). Unfortunately, the genetic basis for possible Lesch–Nyhan disease was not investigated at this early age.

Dystonia with repetitive abnormal posturing of the limbs along with hypertonia emerged during the following year. Hyperreflexia limited to the legs, clonus limited to the ankles and a rate-dependent increase in limb tone with presence of catch in all four extremities indicated spasticity. Psychomotor delay became more evident during his second and third year, when it became clear that he was moderately mentally retarded. His gait worsened and by the age of five, he could hardly stand or walk by himself.

At the age of 10, a routine examination revealed elevated serum creatinine at 13.4 μmol/L (1.1–6.4 μmol/L) and blood urea nitrogen at 86 mmol/L (29–56 mmol/L). Serum uric acid was elevated at 0.78 mmol/L (0.12–0.32 mmol/L). A renal ultrasound showed no signs of nephrolithiasis, but both kidneys were enlarged, hyperechogenic and without a clear border between the cortex and medulla. Analysis of purines and pyrimidines in urine revealed elevated excretion of uric acid (1,530 μmol/mmol creatinine; ref. 100–660) and hypoxanthine (55 μmol/mmol creatinine; ref. 2–19) suggestive of LND, which was confirmed by lack of HPRT activity in an erythrocyte lysate (<0.1% of normal control) determined essentially as described elsewhere (Shin-Buehring et al. 1980). We have previously found this level of HPRT activity in boys with classic LND and definite null mutations and consider such activity as assay background. Mutation analysis of *HPRT1* disclosed a 498 base pair deletion (c.385-51_402 + 430del) enclosing the entire exon 5, which encodes six centrally located amino acids. Segregation analysis revealed that the mutation was de novo.

The boy has had no signs of self-injurious behaviour, no impulsive act of aggression such as striking out or spitting and no use of foul or sexually charged language. No habitual fingernail biting, impulsivity, hyperactivity or any signs of an obsessive–compulsive disorder.

Discussion

LND is characterized by motor dysfunction resembling cerebral palsy, cognitive and behavioural disturbances and uric acid overproduction. The most common presenting features are hypotonia and developmental delay during the first year of life (Jinnah et al. 2006). Affected children have delayed milestones and may never walk. Within the first few years, extrapyramidal involvement (e.g. dystonia, choreoathetosis, opisthotonos) and pyramidal involvement (e.g. spasticity, hyperreflexia, extensor plantar reflexes) become evident (Jinnah et al. 2010). Cognitive impairment and behavioural disturbances emerge between ages two and four. Persistent self-injury (biting the fingers, hands, lips and cheeks; banging the head or limbs) is a hallmark of the classic LND. Self-injury typically presents before age four, and though it may be delayed until late teenage years (Jinnah et al. 2010), this is exceptional.

It is generally accepted that null mutations cause classic LND, whereas mutations resulting in some residual activity underlie the LND variant phenotype. Exceptions to this concept do exist. Fu et al. (Fu et al. 2013) summarized six cases with the LND variant phenotype and deletions affecting the coding region of *HPRT1*. These apparent exceptions could, however, be explained by either incomplete phenotypic evolution due to diagnosis at a very early age or due to unusual molecular mechanisms that permit residual enzyme activity (Fu et al. 2013). In addition, three HPRT deficient patients with apparent exon 5 exclusion, due to a splice site mutation, have been described. As a result of the splice defect, these patients were predicted to present an in-frame exon 5 deletion resulting in a protein deleted for six amino acids. Two of the patients were primarily described as LND (Jinnah et al. 2004; Mak et al. 2000) and one presented as a LND variant (Torres et al. 2010), but none of them presented typical self-injurious behaviour (Jinnah et al. 2010; Jinnah et al. 2006). Our patient has a full exonic deletion removing the same six centrally located amino acids which are predicted to be essential for correct function, and the mutation is anticipated to completely disrupt HPRT enzyme function. Confirming this, we found a very low residual activity of HPRT of less than 0.1% of normal mean value. Thus, it appears that both exon 5 deletion and exclusion may result in LND variant phenotype. To what extent this variant phenotype relies on an abnormal in-frame transcript remains unknown, as the exact consequence of the splice site mutations and the gross deletion on mRNA splicing is difficult to predict.

Absence of self-injurious and maladaptive behaviour at the age of 10 is highly unusual given the above

biochemical/molecular findings. Thus, this case represents an enigma as to why our patient presents clinically as a LND variant and not classic LND. In theory, accelerated de novo biosynthesis of inosine/guanosine monophosphate could compensate for the reduced purine recycling in our patient, leading to a milder phenotype than expected. This possibility remains speculative, however, as no studies have investigated whether the de novo pathway can influence the phenotype of LND patients (Fu et al. 2013). In summary, the present case represents one of the rare exceptions to the generally accepted genotype–phenotype correlation in the LND disease spectrum.

Contributors List

Dr. A. Bayat and F. Wibrand had the idea to write the article.

F. Wibrand and M. Christensen were responsible for performing the metabolic investigations.

M. Duno was responsible for the genetic investigations.

Dr. A. Bayat and Dr A. Lund were both responsible for collecting medical records, gathering relevant literature and together writing the first script. All authors were involved in the ongoing revision of the manuscript. All authors contributed to the revision of the final manuscript.

Conflict of Interest

Allan Bayat, Mette Christensen, Flemming Wibrand, Morten Dunø and Allan Lund declare that they have no conflict of interest.

Compliance with Ethics Guidelines

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

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