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CASE REPORT

Novel Mutations in the *PC* Gene in Patients with Type B Pyruvate Carboxylase Deficiency

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Abstract We have investigated seven patients with the type B form of pyruvate carboxylase (PC) deficiency. Mutation analysis revealed eight mutations, all novel. In a patient with exon skipping on cDNA analysis, we identified a homozygous mutation located in a potential branch point sequence, the first possible branch point mutation in *PC*. Two patients were homozygous for missense mutations (with normal protein amounts on western blot analysis), and two patients were homozygous for nonsense mutations. In addition, a duplication of one base pair was found in a patient who also harboured a splice site mutation. Another splice site mutation led to the activation of a cryptic splice site, shown by cDNA analysis.

All patients reported until now with at least one missense mutation have had the milder type A form of PC deficiency. We thus report for the first time two patients with homozygous missense mutations with the severe type B deficiency, clinically indistinguishable from other patients with type B form of PC deficiency.

The mutations found here are novel; it is noteworthy that four Turkish patients did not have any mutations in common, despite the rarity of PC deficiency. There is thus no evidence for recurrent mutations in the Turkish or other populations.

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Pyruvate carboxylase (PC) deficiency (MIM #262150) is a rare autosomal recessive disorder with an incidence of around 1 in 250,000. It is caused by deficiency of the pyruvate carboxylase enzyme, which is encoded by *PC*. PC is a mitochondrial matrix enzyme that converts bicarbonate and pyruvate to oxaloacetate, which is used by phosphoenolpyruvate carboxykinase and by the Krebs cycle. The enzyme has important functions in gluconeogenesis, where it is considered the major regulatory enzyme, and it is also involved in lipogenesis and biosynthesis of neurotransmitters. PC forms a homotetramer with each subunit having one molecule of biotin covalently attached. Mutations in genes involved in biotin metabolism may cause a secondary deficiency that can be treated with biotin supplementation, whereas PC deficiency is not responsive to biotin therapy.

The clinical presentation of pyruvate carboxylase deficiency has been divided into three groups, with some overlap between the groups (Robinson 2001; Marin-Valencia et al. 2010). Group A patients present with lactic acidosis between birth and 5 months of age, and survival up to 5 years. The biochemical parameters are generally

normal, except for elevated alanine and proline. The most prominent symptom is psychomotor retardation. Group B patients have a more severe clinical presentation with neonatal onset of lactic acidosis and neurological symptoms, and death within 3 months. The biochemical parameters are severely abnormal, including elevated lactate/pyruvate and acetoacetate/β-hydroxybutyrate ratios, and elevated citrulline, lysine, proline, alanine and blood ammonia. In both forms, hepatomegaly, seizures and failure to thrive may occur. In addition, a few patients with a mild form of PC deficiency, group C, have been reported.

Here we report the clinical and molecular results, including eight novel mutations, in seven patients with the severe type B form of PC deficiency, referred to our laboratory from 1992 to 2011. Pyruvate carboxylase activity was measured in cultured fibroblasts as described (Hansen and Christensen 1980). DNA and total RNA was extracted by standard methods from cultured fibroblasts with compromised PC activity and the RNA was reverse transcribed by SuperScript II (Invitrogen). The cDNA sequence of PC was PCR amplified (GoTaq PCR kit Promega), purified (ExoZap, Finnzymes) and sequenced (Big Dye Terminator V1,1 (Applied Biosystems)). The genomic sequence of PC was assessed in a similar manner (Primers and conditions are available upon request).

Mitochondria were isolated from cultured fibroblasts as previously reported (Schägger et al. 1994). For electrophoresis, 10 μg of protein was run on a 9 % SDS acrylamide gel, which was blotted onto a PVDF membrane. Mitochondrial biotin–containing proteins (pyruvate carboxylase, the α subunit of 3-methylcrotonyl-CoA carboxylase and the α subunit of propionyl-CoA carboxylase) were detected with HRP-conjugated avidin (Sigma) at a 1:10,000 dilution and Supersignal West Pico substrate (Pierce) (Singh et al. 2005). The blot was exposed to film and developed.

Seven patients were identified with severely decreased PC activity (Table 1). The clinical data are shown in Table 1. All patients had the type B form of PC deficiency with an uneventful pregnancy and birth, and a birth weight in the low to normal range. As in other type B patients, the presenting symptom was most often respiratory distress or lactic acidosis, and eventually all patients developed lactic acidosis, and most patients developed respiratory distress. Additional symptoms previously seen in type B patients were also found in some of the patients reported here: seizures, liver affection, hypo- and hypertonia and dysmorphism. Brain imaging showed bilateral cystic changes in four patients; additional findings were cortical atrophy and leukodystrophy. Plasma citrulline was measured in three patients and found to be elevated in all three. Type B patients usually die within the first 3 months; this was also the case for five of the patients reported here, whereas two of the seven patients survived until 5 months of age.

The mutation analysis revealed eight different mutations, all novel (Table 2). Four of the seven patients were homozygous for *PC* mutations, and three were compound heterozygous.

cDNA analysis of patient 1 showed activation of a cryptic splice site at position c.903+8_9 leading to retention of the first seven nucleotides of intron 8 and thus a change of the correct reading frame and a premature stop codon (Fig. 1a). Analysis of genomic DNA revealed the heterozygous presence of a splice site mutation, c.903+1G>A, at the exon 8 donor site. The cDNA analysis of patient 1 also displayed complete skipping of exon 12. Subsequent genomic analysis of the coding sequence, including ~ 600 bp upstream of exon 12, exon 12 and the entire intron 12 (amplified as one fragment), did not reveal any pathogenic mutations. The patient was, however, heterozygous for a known SNP in intron 11, c.1369-529g>a (rs2077432), arguing against a genomic deletion of exon 12. We therefore suspect that the exon 12 skipping is most likely due to an intronic mutation further upstream in the large intron 11 (intron size: ~11 kb).

In patient 2, only trace amounts of PC cDNA could be generated, but direct genomic sequencing showed compound heterozygous presence of a c.3436dupG mutation, leading to a frameshift, and c.3288+1G>A, expected to compromise correct splicing. Both mutations probably activate the nonsense-mediated mRNA decay (NMD) pathway.

Patients 3 and 4 were homozygous for two different nonsense mutations, c.1240C>T (p.Gln414X) in exon 9 and c.370C>T in exon 3 (p.Arg124X), respectively.

Patient 5 was homozygous for a missense mutation in exon 4, c.615G>C (p.Arg205Ser). p.Arg205 is highly conserved among species (Fig. 1b) and changes a charged arginine to an uncharged serine. It is located in the biotin carboxylation domain of the protein, where two other missense mutations have been found (Monnot et al. 2009).

In patient 6, a homozygous missense mutation in exon 16, c.2606G>A (p.Gly869Asp), was found. The affected amino acid residue is located in the carboxyl transferase domain of the protein, and the mutation changes a highly conserved nonpolar glycine to a polar aspartic acid. The absence of other mutations, the severely decreased activity of PC and the normal amounts of PC protein on western blot makes it highly likely that both p.Arg205Ser and p.Gly869Asp are pathogenic.

In patient 7, cDNA analysis encompassing exons 8 to 14 revealed two abnormal fragments of 684 bp and 897 bp (Fig. 1a), but no product of the expected normal size (829 bp). The 684 bp fragment corresponded to a skipping of exon 12, whereas the 897 bp fragment showed retention of the last 68 bp of intron 11, most likely due to an activation of a cryptic splice acceptor site at position

Table 1 Clinical and laboratory data of seven patients with type B PC deficiency

	1	2	3	4	5	6	7
Gender	M	M	F	F	M	F	M
Consanguinity	–	–	+	+	+	?	+
Ethnic origin	Danish	Somalian	Turkish	Turkish	Turkish	Turkish	Turkish
Pregnancy and birth	N.a.		Uneventful	Polyhydramnios	Uneventful	Ablatio placenta	Uneventful
GA at birth	N.a.	38 weeks	Term	41 weeks	34 weeks	Term	37 weeks
BW	N.a.	3,428 g	2,400 g	2,260 g	2,500 g	2,500 g	2,100 g
Age at onset	Neonatal	Neonatal	Neonatal	Neonatal	Neonatal	Neonatal	Neonatal
Presenting symptom	Seizures	Respiratory distress	Hypotonia	Hypothermia	Respiratory distress	Lactic acidosis	Lactic ketoacidosis
Additional symptoms	Psychomotor retardation	Hypoglycemia, liver affection	Apnoe, hypoxia, hypotonia, seizures	Lactic acidosis, respiratory distress, hypertonia	N.a.	Hypoxia, hypotonia, respiratory distress, facial dysmorphism	Facial dysmorphism, seizures
Brain imaging	N.d.	US: multicystic changes bilaterally	US: minimal ventricular dilatation	US: bilateral cysts	MRI: severe cortical atrophy	MRI: intraventricular retrocerebral subdural hemorrhagia, bilateral periventricular cavitations, brainstem hypoplasia	MRI: leukodystrophy, corpus callosum atrophy, globus pallidus hyperintensity, subependymal cysts
Death age	5 months	2 months	18 days	8 days	21 days	5 months	3 days
PC activity	0.02	0.00	0.15	0.00	0.04	0.00	0.07
Plasma citrulline	N.d.	127	N.d.	240	N.d.	230	N.d.
Lactate	8	22	8	20	7	7.7	10

N.d. Not done, *N.a.* Not available, *US* Ultrasound, *MRI* Magnetic resonance imaging

PC activity was measured in cultured fibroblasts in pkat/mg protein (ref 11.0 ± 3.0). Plasma citrulline was measured in μmol/l (ref < 47). Lactate was the highest plasma lactate measured in mmol/l

Table 2 Mutations in seven patients with PC deficiency

Patient	Nucleotide change	Amino acid change	Mutation type	PC protein on western blot analysis
1	c.903+1G>A/?	Exon skipping/Exon skipping	Splice site/?	Absent
2	c.3288+1G>A/c.3436dupG	Exon skipping/?p.Glu1146GlyfsX26	Splice site/Frameshift	Absent
3	c.1240C>T/c.1240C>T	p.Gln414X/p.Gln414X	Nonsense	Absent
4	c.370C>T/c.370C>T	p.Arg124X/p.Arg124X	Nonsense	Absent
5	c.615G>C/c.61G>C	p.Arg205Ser/p.Arg205Ser	Missense	Normal
6	c.2606G>A/c.2606G>A	p.Gly869Asp/p.Gly869Asp	Missense	Normal
7	c.1369-29a>g/c.1369-29a>g	Intron retention and exon skipping	Branch point	Absent

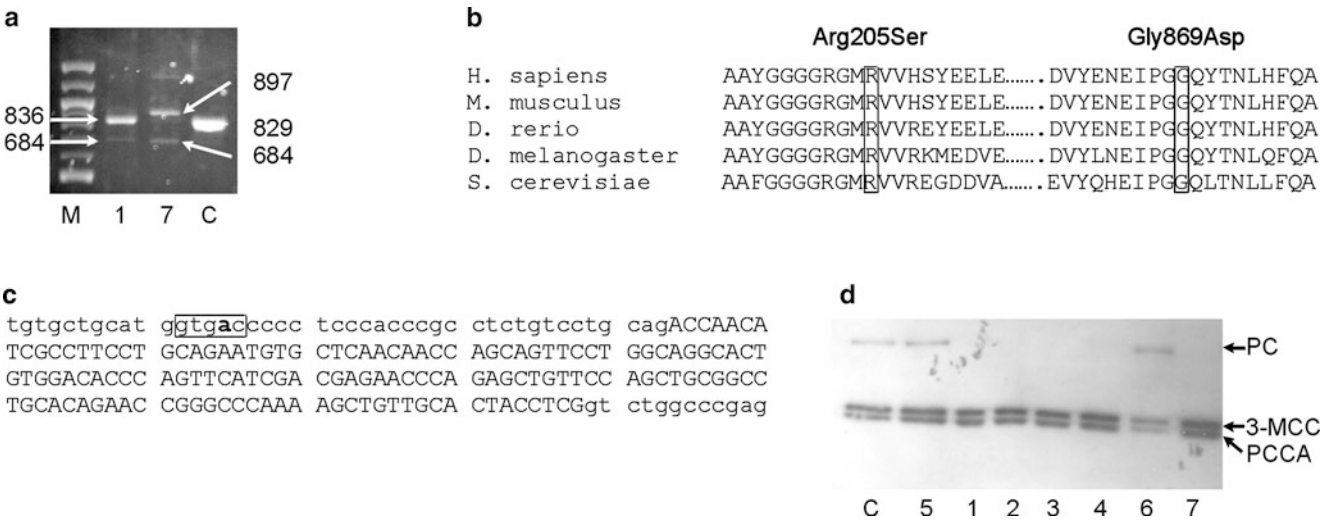

Fig. 1 Analysis of PC protein and sequence analysis. (**a**) cDNA analysis of an exon 8–14 fragment. The normal size fragment of 829 bp is seen in the control (C), whereas a fragment of 684 bp with skipping of exon 12 is seen in patients 1 and 7. Patient 1 also has a band of 836 bp, which corresponds to a fragment with retention of the first 7 nucleotides of intron 8, caused by a splice site mutation, c.903+1G>A. The band of 897 bp in patient 7 represents a fragment with retention of the last 68 bp of intron 11. The intensity of the cDNA fragments in patients 1 and 7 is likely a consequence of activated NMD pathway. (**b**) Alignment of *PC* showing the conservation of the missense mutations found in patients 5 (p.Arg205Ser) and 6 (p.Gly869Asp). (**c**) The genomic sequence of exon 12 and the flanking ~40 bp of intron 11. The c.1369-29A>G mutation (in *bold*) is located in a potential branch point sequence (*boxed*). (**d**) Western blot analysis of protein from fibroblast mitochondria with HRP-conjugated avidin. C: control. 1–7: patients 1–7. The α subunit of 3-methylcrotonyl-CoA carboxylase (3-MCC) and the α subunit of propionyl-CoA carboxylase were used as loading controls

c.1369–69_70. By genomic analysis of exon 12 and ~600 bp of intron 11 we identified a homozygous substitution in intron 11, c.1369-29A>G. The mutation affects a highly conserved adenine in a stretch of five nucleotides (GTG**A**C) with strong resemblance to the consensus branch point sequence Py_{79} T_{75} N A_{92} Py_{75} (79 % of nucleotides are pyrimidine, 75 % thymine, any nucleotide, 92 % adenine, 75 % pyrimidine), except for the first nucleotide, which is a purine (guanine). The possible branch point sequence is located 32–28 bp upstream of the splice acceptor site (consensus 34–21 bp) of exon 12 (Fig. 1c) (Gao et al. 2008).

As expected, western blot analysis showed absence of PC protein in the five patients who had nonsense, frameshift or splicing mutations, whereas normal amounts were found in patients 5 and 6 with missense mutations (Fig. 1d).

This paper adds eight additional mutations to the mutation spectrum in the type B form of PC deficiency. Only a few mutations have been reported previously in patients with the type B form of PC deficiency: four frameshift mutations, an intron retention mutation, a splice site mutation and two missense mutations (Monnot et al. 2009; Carbone et al. 2002; Wexler et al. 1998). All patients reported until now with at least one missense mutation have had the milder type A form of PC deficiency, probably due to an, albeit low, residual activity. We thus report for the first time two patients with homozygous missense mutations with the severe type B deficiency, clinically indistinguishable from the other patients with type B form of PC deficiency.

In addition, we report for the first time a possible branch point mutation in *PC*. The branch point is an element that, together with the polypyrimidine tract, the 5′ and 3′ splice sites and exonic/intronic splicing enhancers/silencers, is essential for correct pre-mRNA splicing, and branch point mutations typically lead to exon skipping, intron retention or the use of a cryptic 3′ splice site, in accordance with our findings of exon skipping and intron retention.

All the mutations found here are novel; it is noteworthy that the four Turkish patients did not have any mutations in common, despite the rarity of PC deficiency. A founder mutation has been found in Canadian Indians (Carbone et al. 1998), but the mutation has not been reported in other populations, where different mutations are found with all families having their private mutation(s). There is thus no evidence for recurrent or founder mutations in other populations, including the Turkish population.

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RESEARCH REPORT

Novel Mutations in the Glucocerebrosidase Gene of Brazilian Patients with Gaucher Disease

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Abstract Gaucher disease (GD) is an autosomal recessive disorder resulting from glucocerebrosidase (GC) deficiency due to mutations in the gene (*GBA*) coding for this enzyme. We have developed a strategy for analyzing the entire *GBA* coding region and applied this strategy to 48 unrelated Brazilian patients with GD. We used long-range PCR, genotyping based on the Taqman® assay, nested PCR, and direct DNA sequencing to define changes in the gene. We report here seven novel mutations that are likely to be harmful: S125N (c.491G>A), F213L (c.756T>G), P245T (c.850C>A), W378C (c.1251G>C), D399H (c.1312G>C), 982-983insTGC (c.980_982dupTGC), and IVS10+1G>T (c.1505+1G>T). The last alteration was found as a complex allele together with a L461P mutation. We also identified 24 different mutations previously reported by others. G377S was the third most frequent mutation among the patients included in this study, after N370S and L444P. Therefore, this mutation needs be included in preliminary screens of Brazilian GD patients. The identification of mutant *GBA* alleles is crucial for increasing knowledge of the *GBA* mutation spectrum and for better understanding of the molecular basis of GD.

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Introduction

Gaucher disease (GD) is the most common lysosomal storage disorder and results from an inborn deficiency of the enzyme glucocerebrosidase (GC; EC 3.2.1.45; also known as acid β-glucosidase) (Beutler and Grabowski 2001). This enzyme is responsible for glycosphingolipid glucocerebroside (glucosylceramide) degradation. Enzyme deficiency leads to the accumulation of undegraded substrate, mainly within cells of the monocyte/macrophage lineage, and this is responsible for the clinical manifestations of the disease. Three types of GD are distinguished based on neurological involvement. GD is an autosomal recessive disorder caused by mutations in the glucocerebrosidase gene or, rarely, by mutations in the GC activator protein, saposin C (Beutler and Grabowski 2001).

The gene encoding GC (*GBA*; GenBank accession # J03059) is located on chromosome 1q21 and spans 7.6 kb of genomic DNA divided into 11 exons. In addition to the functional gene, a highly homologous pseudogene sequence (*GBAP*; GenBank accession # J03060) is located 16 kb downstream (Horowitz et al. 1989; Winfield et al. 1997). The gene and pseudogene are in the same orientation and have

96 % exonic sequence homology (Horowitz et al. 1989; Sidransky 2004). To date, more than 350 mutations have been reported in *GBA*. These include missense and nonsense mutations, small insertions or deletions that lead to frameshifts or in-frame alterations, splice junction mutations, and complex alleles carrying two or more mutations (Hruska et al. 2008). The frequencies of specific mutant alleles vary in different populations. In patients of Ashkenazi Jewish ancestry, four mutations account for nearly 90 % of the disease alleles. These mutations are N370S (c.1226A>G), L444P (c.1448T>C), 84insG (c.84dupG), and IVS2+1G>A (c.115+1G>A). Among non-Jewish patients, these mutations account for about 50–60 % of GD-associated mutations and there is a broad spectrum of other mutations (Grabowski and Horowitz 1997; Alfonso et al. 2007).

The diagnosis of GD is established by measuring GC activity in leukocytes from peripheral blood and/or fibroblasts from skin biopsies (Beutler and Grabowski 2001). Molecular analysis complements this biochemical assay. Our group has been involved in the biochemical diagnosis of Brazilian patients with GD for the last 20 years (Michelin et al. 2005). We have also introduced screening for the common mutations (N370S, L444P, 84insG, and IVS2+1G>A). As expected, this approach is able to detect roughly half the mutant alleles in our sample population, and just over 60 % of patient genotypes. Hence, a more comprehensive scheme is desirable in order to be able to identify the remaining mutant alleles.

We have therefore designed a strategy for analyzing the entire *GBA* coding region and applied this approach to 48 unrelated Brazilian patients with GD among 128 patients referred to our laboratory and previously confirmed by biochemical analysis. We describe here seven novel mutations associated with GD, as well as other rare *GBA* mutations.

Materials and Methods

Patients

In this study, we examined 48 unrelated non-Jewish GD patients from different regions of Brazil. There were 24 males and 24 females. Ages at diagnosis, when available, ranged from 2 months to 57 years. The inclusion criteria were (1) low GC activity in leukocytes and/or fibroblasts (Michelin et al. 2005) and (2) at least one unidentified disease-causing allele following screening for the common mutations (N370S, L444P, 84insG, and IVS2+1G>A). The study was approved by our hospital ethics committee.

In order to confirm that we were dealing with meaningful sequence alterations, we also sequenced *GBA* in DNA from 104 (208 alleles) healthy Brazilian subjects (52 males and 52 females) to rule out the possibility that some of the novel variants identified were simply polymorphisms.

Sample Collection and DNA Isolation

Blood samples (5 mL) were collected in EDTA, and genomic DNA was isolated from peripheral blood leukocytes as described (Miller et al. 1988) and kept at –20 °C. DNA was quantified with a fluorescence-based kit (Quant-It^{TM} dsDNA BR Assay kit; Invitrogen, Carlsbad, CA, USA) in a Qubit^{TM} fluorometer (Invitrogen).

Amplification of the Entire *GBA* Gene

Long-range polymerase chain reaction (PCR) was used to selectively amplify the functional *GBA* gene using primers GBALF (5′ CGACTTTACAAACCTCCCTG 3′) and GBALR (5′ CCAGATCCTATCTGTGCTGG 3′); this generated a fragment of 7765 bp. The long-range PCR reaction was performed in final volumes of 25 μL containing 12.5 ng genomic DNA, 200 μM of each dNTP, 0.2 μM of each primer (forward and reverse), 60 mM of Tris-SO_4 (pH 9.1), 18 mM of $(NH_4)_2SO_4$, 1.7 mM of $MgSO_4$, and 1 μL of Elongase® Enzyme Mix (Invitrogen, Carlsbad, CA, USA). Cycling conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and elongation at 68 °C for 8 min, with final extension at 68 °C for 10 min.

Screening of Common Mutations

Mutations N370S and L444P were screened by genotyping based on TaqMan® PCR (Applied Biosystems, Foster City, CA, USA). Primers and probes were designed with Primer Express® software version 3.0 (Applied Biosystems), and the primer sequences are shown in Table 1. PCR reactions were performed in final volume of 12 μL containing 0.5 μL of the long-range PCR product, 0.3 μL of specific TaqMan assay medium and 6 μL of 2x PCR Genotyping Master Mix (Applied Biosystems). Amplification included an initial step at 50 °C for 2 min (activation of the AmpErase UNG function), AmpliTaq® Gold activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing extension at 60°C for 1 min. The allelic discrimination step was performed at 60°C for 1 min. PCR products were analyzed by allelic discrimination plot with Sequence Detection System software version 1.2.1 in an ABI PRISM® 7500 Sequence Detector System (Applied Biosystems).

Mutation 84insG was screened by Amplification Refractory Mutation System-PCR (ARMS-PCR). Two PCR reactions were performed for each sample using different specific primers to discriminate between wild-type and mutant alleles. Primers GAU-84GGWRT (5′ GCATCATGG-CTGGCAGCCTCACAGGACTGC 3′) and GAU-2R

Table 1 Sequences of the primers and probes used to identify frequent mutations

Mutation	Primer sequence (5′ > 3′)	Probe sequence (5′ > 3′)
N370S	Forward	Normal
	GCCTTTGTCTCTTTGCCTTTGTC	TTACCCTAGAACCTCCTG – VIC
	Reverse	Mutant
	CCAGCCGACCACATGGTA	ACCCTAGAGCCTCCTG – FAM
L444P	Forward	Normal
	CTGAGGGCTCCCAGAGAGT	CTGCGTCCAGGTCGT – VIC
	Reverse	Mutant
	GCCATCGGGATGCATCAGT	TGCGTCCGGGTCGT – FAM

(5′ GCCCAGGCAACAGAGTAAGACTCTGTTTCA 3′) were used to amplify the wild-type allele, and GAU-84GGMTF (5′ GCATCATGGCTGGCAGCCTCACAG-GACTGG 3′) and GAU-2R for the mutant allele. These reactions generated a fragment of 255 bp when the allele sequence was complementary to the primer sequence. Each PCR reaction was performed in a total volume of 25 μL, containing 200 ng of genomic DNA, 200 μM of each dNTP, 1 μM of each primer (forward and reverse), 20 mM of Tris (pH 8.4), 50 mM of KCl, 2.5 mM of $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. Amplification conditions were 96 °C for 5 min, followed by 13 cycles of 96 °C for 45 s, then 45 s at 70 °C −0.5 °C/cycle, and 72 °C for 45 s, then 22 cycles of 96 °C for 45 s, 64 °C for 45 s, and 72 °C for 45 s, with final extension at 72 °C for 5 min. Amplified products were resolved by electrophoresis on 2 % (w/v) agarose gels and visualized under UV light.

Mutation IVS2+1G>A was screened by a PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) strategy using primers GAU-84GGWRT and GAU-2R (sequences shown above) that generated a fragment of 255 bp. The PCR reaction was performed in a total volume of 25 μL, containing 200 ng of genomic DNA, 200 μM of each dNTP, 1 μM of each primer (forward and reverse), 20 mM of Tris (pH 8.4), 50 mM of KCl, 2.5 mM of $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. The amplification protocol was initial denaturation at 96 °C for 5 min, 35 cycles of denaturation at 96 °C for 45 s, annealing at 68 °C for 45 s, and extension at 72 °C for 45 s, followed by final extension at 72 °C for 10 min. Ten μL of PCR product was digested in a total volume of 15 μL containing 20 mM of Tris-acetate, 50 mM of potassium acetate, 10 mM of magnesium acetate, 1 mM of dithiothreitol (pH 7.9), and 1 U of *Hph*I (New England Biolabs, Ipswich, MA, USA). The reaction was then placed at 37 °C and left overnight, after which digestion products were resolved by electrophoresis on 3 % (w/v) agarose gels and visualized under UV light. Digestion of the PCR product of the normal allele generates fragments of 141, 72, and 42 bp, while digestion of the PCR product of the mutant allele produces fragments of 213 and 42 bp, because the IVS2+1G>A mutation removes an *Hph*I site.

PCR Amplification and Direct DNA Sequencing

Coding sequences and flanking regions (exons 1 to 11) were amplified by PCR using long-range PCR products as templates. The *GBA* coding region was divided into 10 different amplicons, with exons 10 and 11 analyzed together. PCR reactions were performed in total volumes of 25 μL, containing 0.5 μL of long-range PCR product, 200 μM of each dNTP, 0.2 μM of each primer (forward and reverse), 20 mM of Tris (pH 8.4), 50 mM of KCl, 2.5 mM of $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. The amplification protocol was initial denaturation at 94 °C for 10 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57–62 °C for 30 s, and extension at 72 °C for 30 s, followed by final extension at 72 °C for 10 min. Each PCR product was verified by electrophoresis on a 1.5 % (w/v) agarose gel and visualization under UV light. Specific annealing temperatures and primer sequences are given in Table 2.

Amplicons were purified using 2.5 U of Exonuclease I (USB, Cleveland, OH, USA) and 0.25 U of Shrimp Alkaline Phosphatase (USB, Cleveland, OH, USA). Direct DNA sequencing was performed with a BigDye® Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions, and sequences were analyzed with DNA Sequencing Analysis software v. 5.2 (Applied Biosystems) in an ABI PRISM® 3130*xl* Genetic Analyzer. All identified mutations were confirmed by sequencing an independent DNA sample with forward and reverse primers.

Isolation of Total RNA, cDNA Synthesis, and PCR Amplification

Total RNA was isolated from peripheral blood leukocytes using a LeukoLOCK™ Total RNA Isolation kit (Applied Biosystems).

Table 2 Primers used to amplify and sequence the *GBA* gene

Exons and flanking regions	Primer sequence (5′ > 3′)		$T_{annealing}(^0C)$	Length(bp)
	Forward	Reverse		
1	CCTAGTGCCTATAGCTAAGG	CTGGATTCAAAGAGAGTCTG	57	236
2	GTCCTAATGAATGTGGGAGACC	CTTACTGGAAGGCTACCAAAGG	61	286
3	GTTCAGTCTCTCCTAGCAGATG	GGAAACTCCATGGTGATCAC	61	353
4	GTCCTCCTAGAGGTAAATGGTG	GCAGAGTGAGATTCTGCCTC	61	316
5	GATAAGCAGAGTCCCATACTCTC	CTGTACAAGCAGACCTACCCTAC	62	281
6	CTAATGGCTGAACCGGATG	GGAAGTGGAACTAGGTTGAGG	62	346
7	CAAAGTGCTGGGATTACAGG	CTCTAAGTTTGGGAGCCAGTC	62	404
8	CTAGTTGCATTCTTCCCGTC	GCTTCTGTCAGTCTTTGGTG	63	407
9	CTCCCACATGTGACCCTTAC	CTCGTGGTGTAGAGTGATGTAAG	61	329
10 and 11	GTGGGTGACTTCTTAGATGAGG	CTTTAGTCACAGACAGCGTGTG	62	473

RNA was quantified using a fluorescence-based assay (Quant-It™ RNA Assay kit) in a Qubit™ fluorometer.

RNA samples of 1 μg were used as templates for complementary DNA (cDNA) synthesis. Reverse transcription (RT) was performed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a total volume of 15 μL. Following the RT reaction, PCR was performed using primers GAU-G10S (5′ CTGAACCCCGAAGGAGGACC 3′) and GAU-9/11R (5′ GGTTTTTCTACTCTCATGCA 3′), which generated a product of 988 bp. Five microliters of each RT reaction was used for the PCR in a total volume of 25 μL, containing 200 μM of each dNTP, 0.8 μM of each primer (forward and reverse), 20 mM of Tris (pH 8.4), 50 mM of KCl, 1.5 mM of $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. The amplification protocol was initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. An aliquot of each PCR product was verified by electrophoresis on a 1.0 % (w/v) agarose gel and visualization under UV light. PCR products were purified and sequenced as described above.

Evaluation of Novel Mutations

Amino acid sequences of GC from 12 different species were compared by multiple alignment program in order to determine whether changes identified in their amino acid sequences alter conserved residues of GC. The GC sequences were searched using the protein database from the National Center for Biotechnology Information (NCBI). The amino acid sequences were aligned with ClustalW v. 2.0 using the FASTA format.

The novel mutations in the *GBA* coding region were analyzed using three web-based tools, PolyPhen (http://genetics.bwh.harvard.edu/pph) (Ramensky et al. 2002), SNPs3D (http://www.snps3d.org) (Yue and Moult 2006), and SIFT (http://sift.bii.a-star.edu.sg) (Kumar et al. 2009) in order to assess their potential pathogenicity.

Nomenclature of Mutations

The reference sequences used in our study were NM_000157 and NP_000148. Nucleotide sequences were numbered from the upstream initiator codon ATG. Amino acid numbers are those of the mature proteins after cleavage of the leader sequence. Following the current recommendation that all variants be described at the most basic level, the mutations discussed in the text are referred to by the traditional name of the corresponding allele to facilitate recognition, followed by the cDNA nomenclature in brackets (den Dunnen and Antonarakis 2000).

Results

Screening the whole *GBA* coding sequence in each of the 48 GD patients allowed us to identify 95.8 % of the mutant alleles, including seven novel sequence variants. These novel changes were not found among 208 alleles from normal individuals. Twenty-four rare mutations were also found among the mutant alleles studied. Data for these 31 variants are summarized in Table 3.

The novel alterations consist of five missense changes [S125N (c.491G>A), F213L (c.756T>G), P245T (c.850C>A), W378C (c.1251G>C), and D399H (c.1312G>C)], one in-frame insertion [982-983insTGC (c.980_982dupTGC)], and a splicing mutation found in a complex allele [L461P + IVS10+1G>T]. These findings are described in more detail below.

The first novel sequence alteration is caused by a G to A change at position 491 of the cDNA (exon 5 of *GBA*) and leads to a serine to asparagine substitution at residue 125 of the protein (S125N). This mutation was found in a male

Table 3 Alleles defined by this study. The novel sequence variants are shown in bold

GBA mutations[a]	cDNA nucleotide substitution[b]	Exon	Protein[c]	# of alleles
Substitutions				
R48Q	c.260G>A	3	p.Arg87Gln	1
R120W[d]	c.475C>T	5	p.Arg159Trp	3
M123T	c.485T>C	5	p.Met162Thr	1
S125N	c.491G>A	5	p.Ser164Asn	1
R131C	c.508C>T	5	p.Arg170Cys	1
W179X	c.653G>A	6	p.Trp218X	1
N188S[d]	c.680A>G	6	p.Asn227Ser	1
G202R[d]	c.721G>A	6	p.Gly241Arg	1
F213L	c.756T>G	6	p.Phe252Leu	1
P245T	c.850C>A	7	p.Pro284Thr	1
H311R	c.1049A>G	8	p.His350Arg	1
Y313H	c.1054T>C	8	p.Tyr352His	1
E349K	c.1162G>A	8	p.Glu388Lys	1
R353W	c.1174C>T	8	p.Arg392Trp	1
N370S	c.1226A>G	9	p.Asn409Ser	26
G377S	c.1246G>A	9	p.Gly416Ser	12
W378C	c.1251G>C	9	p.Trp417Cys	6
N396T	c.1304A>T	9	p.Asn435Thr	4
V398I	c.1309G>A	9	p.Val437Ile	6
D399H	c.1312G>C	9	p.Asp438His	1
L444P[d]	c.1448T>C	10	p.Leu483Pro	2
I489T	c.1583T>C	11	p.Ile528Thr	7
R496H	c.1604G>A	11	p.Arg535His	1
Insertions				
84insG	c.84dupG	2	p.Leu29AlafsX18	1
982-983insTGC	c.980_982dupTGC	7	p.Leu327_Pro328insLeu	1
Deletions				
413delC	c.413delC	4	p.Pro138LeufsX62	1
793delC	c.793delC	7	p.Gln265SerfsX5	2
Complex alleles				
E326K + L444P[d]	c.1093G>A c.1448T>C	8 10	p.Glu365Lys p.Leu483Pro	1
M361I + N370S	c.1200G>A c.1226A>G	8 9	p.Met400Ile p.Asn409Ser	1
	c.1448T>C	10	p.Leu483Pro	
Rec*Nci*I	c.1483G>C	10	p.Ala495Pro	3
	c.1497G>C	10	p.Val499Val	
L461P[d] + **IVS10+1G>T**	c.1499T>C c.1505+1G>T	10	p.Leu500Pro p.Lys464_Arg502del	1

[a] *GBA* mutations are named according to www.hgvs.org/mutnomen

[b] Nucleotides are numbered from the A of the first ATG

[c] Amino acid designations ("p.") are based on the primary *GBA* translation product, including the 39-residue signal peptide

[d] These alterations correspond to the normal pseudogene sequence

type 1 GD patient from the south of Brazil who was diagnosed at 56 years of age and carries the N370S mutation on the other chromosome. Abdominal pain, splenomegaly, and chronic bone pain are among the clinical features of this patient.

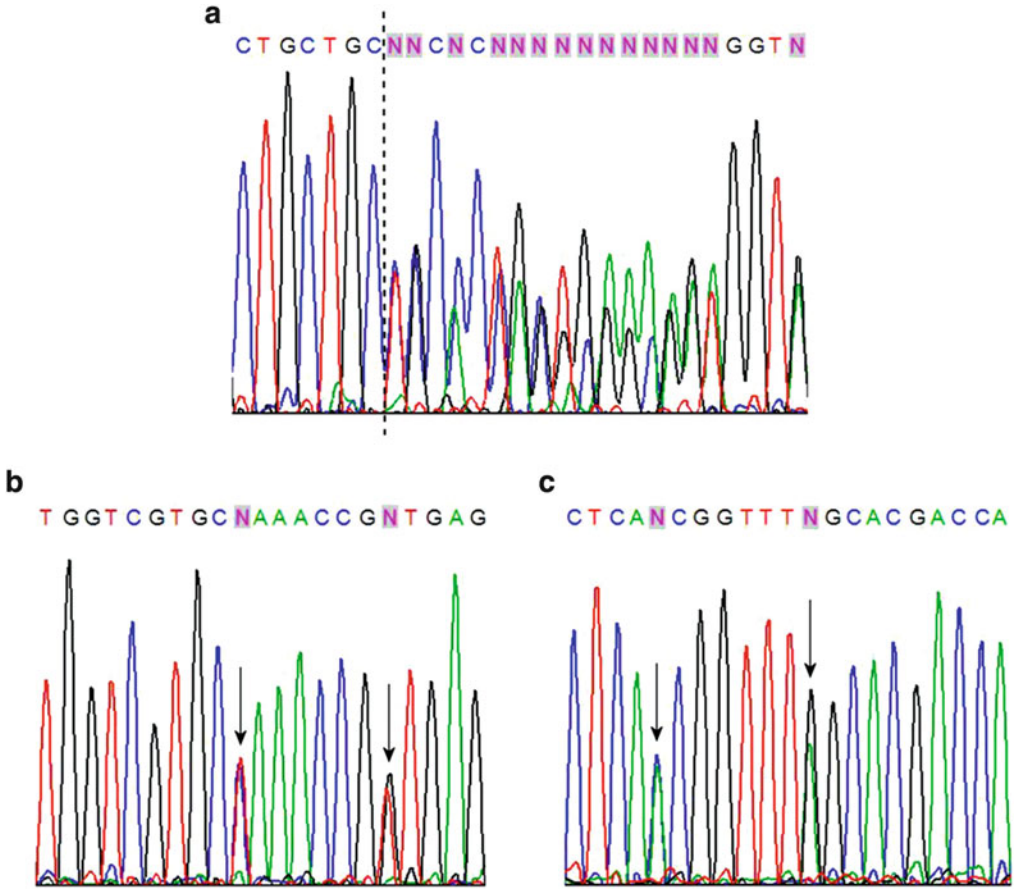

Fig. 1 Direct DNA sequencing of the *GBA* gene. (**a**) Direct sequencing of part of exon 7 from the forward primer. The *dashed line* shows where three nucleotides (TGC) have been inserted, creating the 982-983insTGC mutation. (**b**) Direct sequencing of part of exon 10 from the forward primer. The first arrow indicates the T to C substitution (c.1499T>C) in the L461P mutation, while the second arrow points to the G to T substitution in the IVS10+1G>T variant. (**c**) Direct sequencing of part of exon 10 from the reverse primer. The first arrow indicates the C to A substitution in IVS10+1G>T, while the second arrow points to the A to G substitution in L461P

The *GBA* of another type 1 GD patient has a T to G transversion at base 756 of the cDNA (exon 6 of the gene) predicting a phenylalanine to leucine substitution at residue 213 of the protein (F213L). The N370S mutation was also present in this compound heterozygous female type 1 GD patient. She is from the Southeast of Brazil and was diagnosed when she was 26 years old. Clinical features include hepatosplenomegaly and bone pain.

Two novel sequence variants are in exon 7. The first is a C to A change at position 850 of the cDNA predicting a proline to threonine substitution at amino acid 245 of protein (P245T). This was identified in a male type 3 GD patient from the southeast region of Brazil who is also heterozygous for Rec*Nci*I (c.1448T>C + c.1483G>C + c.1497G>C). This patient is more severely affected, with neurological involvement. The other alteration in exon 7 is an in-frame insertion of three nucleotides (TGC) that should insert an additional leucine residue between positions 327 and 328 of the mature protein (Fig. 1a). This mutation is referred to as 982-983insTGC and was found in a male type 1 patient from the southeast of Brazil diagnosed at 31 years of age. The other mutant allele in this patient is N370S, and his clinical features include hepatosplenomegaly.

Two novel G to C transversions are in exon 9. The first is at base 1251 of the *GBA* cDNA and causes a tryptophan to cysteine substitution at residue 378 of the mature protein (W378C). This alteration was found in unrelated alleles in six type 1 GD patients from the north and northeast regions of Brazil. One of these patients carries the G377S (c.1246G>A) mutation on the other chromosome, while the remaining five patients are compound heterozygotes [N370S] + [W378C]. All six patients have typical mild forms of the disease consistent with type 1 disease. The other G to C transversion is located at position 1312 and is responsible for an aspartate to histidine substitution at residue 399 of the protein (D399H). This mutation was found in a type 1 GD patient diagnosed at 45 years of age who is also heterozygous for the N370S mutation. The most characteristic finding in this patient was extreme hepatosplenomegaly.

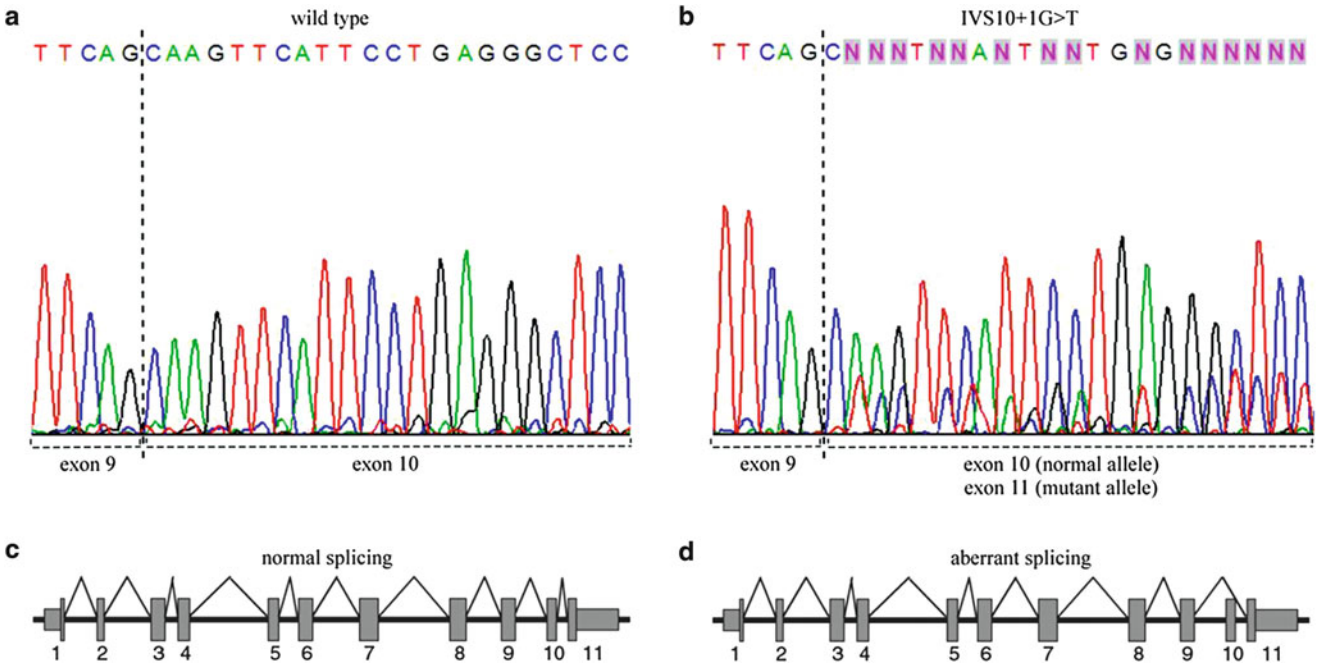

Fig. 2 Direct sequencing of *GBA* cDNA. (**a**) Wild-type alleles sequenced from the forward primer. The dashed line indicates the boundary between exons 9 and 10. (**b**) Normal and mutant alleles from a GD patient carrying IVS10+1G>T, sequenced from the forward primer. The dashed lines indicate the boundary between exons 9 and 10 in the normal allele and between exons 9 and 11 in the mutant allele, respectively. Schematic diagrams of (**c**) the normal splicing pattern and (**d**) the aberrant splicing pattern due to the IVS10+1G>T mutation

The remaining novel mutation involves a G to T transversion in the splice donor site at the first nucleotide of intron 10 (Fig. 1b and c). This mutation, named IVS10+1G>T, removes the conserved splice donor sequence and results in skipping of exon 10. RT-PCR amplification of the *GBA* cDNA from this patient revealed an 871 bp fragment in addition to the expected 988 bp fragment (from the other allele) (data not shown). This smaller product was sequenced and shown to lack exon 10, presumably due to loss of the splice donor site at the end of exon 10 (Fig. 2b). A control cDNA used at the same time yielded a normal splicing pattern and a fragment of the expected length (Fig. 2a). Schematic representations of the normal and aberrant splicing patterns are shown in Fig. 2c and d, respectively. This variant was found in a female type 1 patient from the south of Brazil diagnosed at 3 years of age. N370S and L461P (c.1499T>C) were also present in this patient. As DNA from her parents was also available, we were able to show that the N370S mutation was of paternal origin, while L461P and IVS10+1G>T were of maternal origin.

In silico evaluation of the five novel sequence variants using PolyPhen, SNPs3D, and SIFT predicted defective proteins for S125N, W378C, and D399H. However, the three bioinformatics tools differed in their predictions for the F213L and P245T variants. PolyPhen predicted that they would be deleterious whereas according to SNPs3D and SIFT, they were non-deleterious.

We also estimated the frequencies of the common previously reported mutations taking into account all 128 patients referred to our laboratory. As expected, the most frequent mutation was N370S, found in 113 alleles (44.1 %); the second most frequent mutation in our cohort, L444P, was found in 73 alleles (28.5 %); and G377S, the third most frequent mutation, accounted for 12 mutant alleles (11.1 %). This was followed by I489T (c.1583T>C) seven alleles (6.5 %), W378C and V398I (c.1309G>A) six alleles each (5.6 %), Rec*Nci*I three alleles (2.8 %), IVS2+1G>A two alleles (1.9 %), and [E326K + L444P] one allele (0.9 %).

The genotypes of the 48 GD patients examined in this study are shown in Table 4. Genotypes involving only combinations of the common mutations were excluded.

Discussion

The protocol described here allowed us to identify seven novel sequence variants of the *GBA* gene in patients with GD. These include five missense mutations (S125N, F213L, P245T, W378C, and D399H). We also found that the G377S mutation was common among the patients in this study, as previously reported by other Brazilian groups (Rozenberg et al. 2006; Sobreira et al. 2007). Therefore, G377S should be included in preliminary screening of Brazilian GD patients.

Table 4 Frequencies of the identified genotypes

Genotype	# of patients
[N370S] + [W378C]	5
[G377S] + [G377S]	4
[V398I] + [V398I]	3
[N370S] + [I489T]	3
[R120W] + [N370S]	3
[793delC] + [N370S]	2
[N370S] + [L461P + IVS10+1G>T]	1
[Y313H] + [L444P]	1
[G377S] + [I489T]	1
[R353W] + [Rec*Nci*I]	1
[F213L] + [N370S]	1
[W179X] + [M361I + N370S]	1
[N188S] + [Rec*Nci*I]	1
[M123T] + [E349K]	1
[G377S] + [W378C]	1
[S125N] + [N370S]	1
[P245T] + [Rec*Nci*I]	1
[N396T] + [N396T]	1
[N370S] + [D399H]	1
[413delC] + [N370S]	1
[I489T] + [I489T]	1
[N370S] + [G377S]	1
[N396T] + [I489T]	1
[N396T] + [L444P]	1
[G202R] + [N370S]	1
[R131C] + [N370S]	1
[84insG] + [R496H]	1
[H311R] + [N370S]	1
[982-983insTGC] + [N370S]	1
[G377S] + [E326K + L444P]	1
[N370S] + [?]	3
[R48Q] + [?]	1
Total	**48**

? denotes unidentified mutation

The S125N mutation involves the replacement of a polar uncharged amino acid with a hydroxyl group by an uncharged residue with an amide group containing a long side chain. Ser125 is conserved in several species from human to worms, arguing in favor of a role in enzyme function (Fig. 3). This mutation destroys a *Pvu*II restriction site. The S125N mutation was previously reported in patients with Parkinson disease (Lesage et al. 2011); however, this sequence alteration has not been identified in GD patients to date.

The F213L mutation replaces a bulky aromatic amino acid by a residue containing an open aliphatic chain, which possibly changes the protein conformation. Although in silico evaluation predicts a non-deleterious effect on GC, this phenylalanine residue is conserved in several species, such as chimpanzee, orangutan, rat, mouse, dog, pig, ox, and horse (Fig. 3).

The other mutation, P245T, involves the replacement of a cyclic aliphatic structure by an open polar uncharged chain and would be expected to affect protein structure and/or activity. In silico evaluation of P245T predicted a non-deleterious effect on the mature protein. However, the proline residue is conserved in mammals such as chimpanzee, orangutan, rat, mouse, dog, pig, ox, and horse (Fig. 3).

The mutation in exon 9, W378C, involves the replacement of an amino acid with an indole side chain by an amino acid with a sulfhydryl group, which may affect protein conformation. In silico analysis predicted a deleterious effect. The tryptophan residue is highly conserved, indicating a considerable degree of structural and functional relevance (Fig. 3). This mutation creates an *HpyCH4*V restriction site within *GBA*.

D399H involves the replacement of an acidic amino acid by a basic one, which is again likely to affect protein structure and/or function. Asp399 is highly conserved, and in silico analysis predicts a deleterious effect of the mutation (Fig. 3). This mutation destroys a *Hind*II restriction site.

Two other variants likely to have harmful effects are an insertion and a single base change at the first position of a splice donor site. Despite being an in-frame insertion, the insertion of an additional residue in the mature protein is likely to disturb normal folding and function. The point mutation in the first base of the splice donor site disrupts normal splicing, giving rise to exon 10 skipping, which yields a truncated protein of 458 amino acids (p.Lys464_Arg502del).

Most of the novel sequence alterations detected in this study were found in patients with the N370S mutation at the other locus. It appears that this allele when part of any genotype is able to protect patients from neurological involvement (Alfonso et al. 2001). Indeed, no neurological involvement was reported in any of our patients of this type. Moreover, neurological impairment is not seen in GD patients who carry N370S or 982-983insTGC, which further support a protective effect of the N370S mutation.

Interestingly, the G377S mutation was also found to be the third most frequent among GD patients in Portugal and Spain (Amaral et al. 1996). This alteration appears to be neuroprotective since homoallelic patients are essentially asymptomatic or have mild disease (type 1) (Amaral et al. 2000). However, other workers have detected type 3 patients with one copy of G377S (Beutler et al. 2005; Rozenberg et al. 2006). Thus we cannot rule out an allele-dose effect in which two copies of G377S generate sufficient residual enzymatic activity to prevent neurological involvement, whereas one copy combined with a severe second allele

Fig. 3 Alignment of the GC amino acid sequences of several organisms. (**a**) Human (Gene ID 2629); (**b**) chimpanzee (Gene ID 449571); (**c**) orangutan (Gene ID 100174563); (**d**) rat (Gene ID 684536); (**e**) mouse (Gene ID 14466); (**f**) dog (Gene ID 612206); (**g**) pig (Gene ID 449572); (**h**) ox (Gene ID 537087); (**i**) horse (Gene ID 100063514); (**j**) tick (Gene ID 8051161); (**k**) *C. elegans* (Gene ID 178535); (**l**) honeybee (Gene ID 409708). The boxes indicate conserved residues altered in novel missense mutations. The designation of each mutation is given above the appropriate box

leads to type 3 GD (Rozenberg et al. 2006). Such a model predicts that our patient with [G377S] + [E326K + L444P] is likely to develop neurological symptoms, although no such symptoms have been described to date.

There remain undefined mutant alleles in our collection that can be explained by alterations outside the *GBA* coding region. Such changes would not be detected by our approach, nor would sequence alterations in the promoter region of the gene, or in untranslated or in noncoding regions. We are currently testing further approaches to overcome these limitations and unmask the remaining mutant alleles.

The vast majority of mutations identified have not been found in homoallelic form, especially the new variants. Ultimately, it may be more accurate to envision the associated phenotypes as forming a continuum, with the major distinction being the presence and degree of neurological involvement (Sidransky 2004; Hruska et al. 2008).

The identification of mutant alleles is crucial for advancing knowledge of the worldwide *GBA* mutation spectrum, and should contribute to a better understanding of the molecular basis of the disease. Such information should also help in establishing genotype-phenotype correlations as well as in genetic counseling and/or in customized molecular analyses for families at risk.

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Synopsis

Novel and rare mutations in the *GBA* gene of patients with Gaucher disease.

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RESEARCH REPORT

Prevalence and Development of Orthopaedic Symptoms in the Dutch Hurler Patient Population after Haematopoietic Stem Cell Transplantation

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Abstract Hurler syndrome (MPS-IH) is a rare autosomal recessive lysosomal storage disease. Besides a variety of other features, Hurler syndrome is characterized by a range of skeletal abnormalities known as dysostosis multiplex. Despite the successful effect of haematopoietic stem cell transplantation on the other features, dysostosis remains a disabling symptom of the disease. This study analyzed the status and development of the orthopaedic manifestations of 14 Dutch Hurler patients after stem cell transplantation.

Data were obtained retrospectively by reviewing patients' charts, radiographs and MRIs. Existing methods to measure the deficiencies were modified to optimally address the dysostosis. These measurements were done by two of the authors independently. The odontoïd/body ratio, kyphotic angle, scoliotic angle and parameters for hip dysplasia and genu valgum were measured and plotted against age. The degree of progression was determined. The intraclass correlation coefficient (ICC) was calculated to determine the reliability of the measurements.

All patients showed hypoplasia of the odontoïd, which significantly improved during growth. Kyphosis in the thoracolumbar area was present in 13 patients and proved to be progressive. Scoliosis was observed in eight patients. Hip dysplasia was present in all patients and showed no tendency of improvement. In all but one patient, knee valgus remained more than two standard deviations above normal.

Dysostosis remains a major problem after haematopoietic stem cell transplantation in Hurler patients. Moreover, except for dens hypoplasia, it appears to be progressive and therefore surgical interventions may be necessary in the majority of these patients.

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Introduction

Hurler syndrome, also known as Mucopolysaccharidosis type IH (MPS-IH), is a rare genetic lysosomal storage disease with an autosomal recessive inheritance pattern and an incidence of 1 in 100,000 live births (Lowry and Renwick 1971). Because of a deficiency in the enzyme α-L-iduronidase (IUDA), the glycosaminoglycans (GAGs) dermatan and heparan sulphate accumulate in the lysosomes with a detrimental effect on cells and tissues (Neufeld and Muenzer 2001). Clinical features include cognitive delay, coarse facial features, hearing loss, corneal clouding, cardiorespiratory disease, inguinal and umbilical hernias, hepatosplenomegaly, carpal tunnel syndrome and skeletal abnormalities known as dysostosis multiplex (Cleary and Wraith 1995; Muenzer et al. 2009). Without therapy, most children die in the first decade of life (Muenzer and Fisher 2004).

In 1981 Hobbs et al. described the first successful haematopoietic stem cell transplantation (HSCT) in a Hurler patient (Hobbs et al. 1981). The donor derived blood cells, with normal enzyme function, appeared to effectively arrest the further accumulation of the incompletely degraded GAGs (Boelens 2006). Currently, HSCT

has become standard treatment and most patients live longer with less severe clinical features. However, despite the beneficial effect of HSCT on cognitive development and many physical functions, its effect on most orthopaedic problems remains limited (Hobbs et al. 1981; Braunlin et al. 2003; Peters et al. 1998; Souillet et al. 2003; Gatzoulis et al. 1995; Vellodi et al. 1997; Weisstein et al. 2004; Field et al. 1994; Guffon et al. 1998). 'Bone marrow transplantation does not appear to alter the natural history of the musculoskeletal disorders in Hurler syndrome…' stated Weisstein et al. (2004). Others, however, have the impression that some musculoskeletal problems are less severe in patients who underwent HSCT compared to untreated patients (Vellodi et al. 1997; Hite et al. 2000).

Dysostosis multiplex is a general term used for the large spectrum of skeletal abnormalities in Hurler syndrome. The general phenomenon is failure of ossification and aberrant bone remodelling of which the exact pathophysiological mechanism remains to be elucidated (Field et al. 1994; van der Linden et al. 2011). Recently it was shown that a reduced cathepsin K activity, due to the accumulation of GAGs, leads to impaired osteoclast activity and decreased subepiphyseal cartilage resorption (Wilson et al. 2009). Focal failure of ossification is seen in the processus odontoideus C1, the anterosuperior quarters of the vertebral bodies at the thoracolumbar junction, the lateral roof of the acetabulum and in the lateral margin of the metaphysis of the proximal tibia (Field et al. 1994). This may lead to, respectively, hypoplasia of the dens, vertebral beaking with thoracolumbar kyphosis, hip dysplasia and genu valgum.

The vast majority of the Dutch Hurler population is treated at our institution. Since April 2003, all patients receive bone marrow transplantation and are followed systematically in agreement with the recommendations of the worldwide MPS registry initiative (Muenzer et al. 2009). With increasing size and age of the population, many questions regarding the optimal treatment of the disabling musculoskeletal abnormalities have accumulated. To answer these questions, we recently performed a systematic review on musculoskeletal deformities in Hurler disease after HSCT (van der Linden et al. 2011). An important conclusion was that systematic patient follow-up is a first requirement to gain insight in the development of these skeletal abnormalities. In a first attempt to address the natural course of these abnormalities, the aim of the present study was to retrospectively analyze the status and development of the main orthopaedic manifestations in our Hurler population. We specifically investigated the rate of progression and whether there was an interrelation of the severity of deficiencies. Furthermore, we evaluated the accuracy of several radiological measurements to monitor these deficiencies.

Methods

Patients and Data Collection

All known patients with Hurler syndrome ($n = 14$), confirmed by enzyme or gene assay and treated with HSCT, were analyzed. Since April 2003, the patients were monitored systematically by means of history taking, physical examination and imaging by several medical disciplines. Data were obtained by reviewing the patient charts, radiographs and magnetic resonance images (MRI) made before September 1, 2011. For the patients that underwent surgical treatment ($n = 4$; see also Table 2 and 3), data were included until the moment of corrective surgery. Specifically designed radiological measurements for the amount of odontoïd dysplasia, degree of kyphosis and malformation of the pelvis were done by two authors (FS and MK or RS). Established measurements quantifying the degree of scoliosis and genu valgum were done by one author (FS). All images were analyzed using a Picture Archiving and Communications System (PACS) workstation (EasyVision DX/CL/RG/Home, Phillips Medical Systems, The Netherlands). All measurements were plotted against patient age in years. If applicable, right and left measurements were done separately. The study was approved by the local ethics board.

Odontoïd Dysplasia

Measurements were done on 3 or 4 mm T2-weighted MRI images. We used the method described by Cokluk et al. that calculates odontoïd process/body C2 ratio in the midsagittal plane (Fig. 1a) (Cokluk et al. 2006). In healthy children this ratio was found to be 2 (Cokluk et al. 2006). The length of the odontoïd process was defined as the distance between the tip of the odontoïd to the remnant of the dentocentral synchondrosis, the length of the body was the distance between the remnant of the dentocentral synchondrosis to the inferior border of C2. According to Cokluk et al. an odontoïd/body ratio ≥ 2 was considered normal, a ratio $\geq 1.5 < 2$ as mild hypoplasia, a ratio $\geq 1.0 < 1.5$ as moderate hypoplasia and a ratio < 1.0 as severe hypoplasia of the odontoïd. The presence of increased soft tissue around the tip of the odontoïd process was scored as yes or no.

Thoracolumbar Spine

To quantify the maximum kyphotic angle, the angle of kyphosis over the four thoracolumbar vertebrae with the largest kyphotic angle was measured on a lateral standing or sitting radiograph of the spine without brace (Fig. 1b). Beaking of the anterior vertebral body and disappearance of

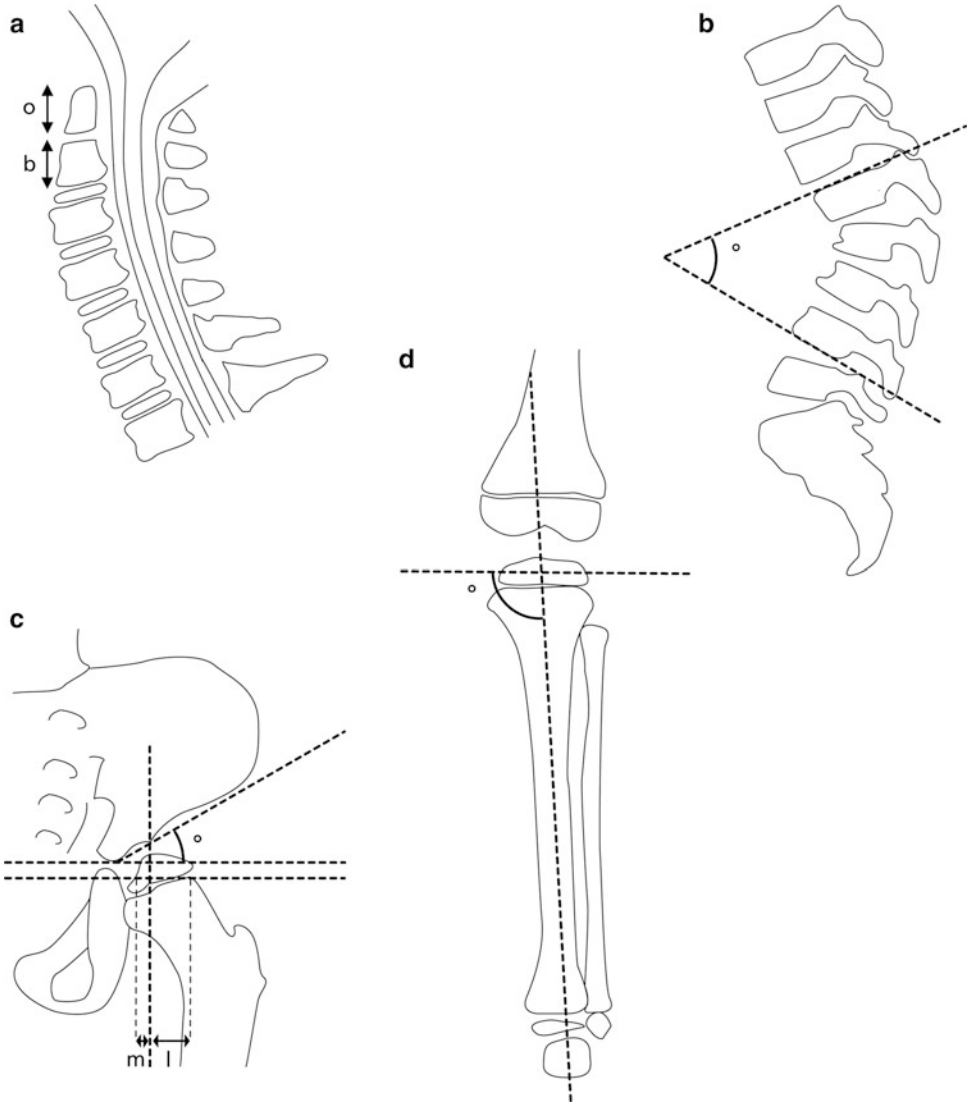

Fig. 1 Radiological measurements: (**a**) The *odontoïd/body ratio* was calculated by dividing the length of the odontoïd process (*o*) by the length of the body of the 2nd cervical vertebra (*b*). (**b**) The *kyphotic angle* was defined as the angle over the 4 thoracolumbar vertebrae with the largest angle of kyphosis. (**c**) The *acetabular index (AI)* was defined as the angle between the horizontal Hilgenreiner's line and the line connecting the superolateral edge of the acetabulum with the triradiate cartilage. The percentage of *bony coverage* was calculated by drawing the Hilgenreiner's line and perpendicular to this a line touching the lateral border of the bony acetabulum. A line parallel to the Hilgenreiner's line through the lateral subcapitalphyses was drawn next. By virtually connecting the metaphyseal and epiphyseal medial and lateral edges, the contour of the caput femoris was reconstructed and the intersection point with the transphyseal line was taken medially and laterally. The distance *m* represents the bony coverage. The percentage of coverage was calculated by dividing *m* by the distance *m* + *l*. (**d**) The *medial proximal tibial angle (MPTA)* was measured as the medial angle between a line drawn at the level of the tibial epiphysis, parallel to the growth plate of the proximal tibia and a line connecting the centre of the proximal tibial epiphysis to the midwidth of the talus at the ankle mortise

the disc were scored as yes or no and its corresponding level was noted. Antero- and retrolisthesis were scored using the Meyerding classification, where each grade represents 25 % of displacement with regard to the endplate of the vertebra below (Sharrard 1993). The degree of scoliosis was determined by measuring the Cobb angle on a sitting or standing anterior-posterior radiograph of the spine. Scoliosis was defined as a Cobb angle over 10°. Patient charts were reviewed to determine whether brace therapy was applied.

Hip Dysplasia

The acetabular index (AI) of both the right and left hip were measured on a standard X-ray of the pelvis. The angle was defined as the angle between the horizontal Hilgenreiner's

line and a line connecting the superolateral edge of the acetabulum with the triradiate cartilage (Fig. 1c) (Sharrard 1993). The average of both hips of both observers was plotted. The tables of Tonnis and Brunken (1968) were used for reference values. On the basis of the AI, the hips were classified as normal (AI within 1 SD of the mean), acetabular ossification delay (1 SD < AI < 2 SD), or dysplastic (AI > 2 SD), according to the classification system used by Tönnis and Brunken (Tonnis and Brunken 1968; Tonnis 1976). The development of AI in Hurler patients was calculated, as well as graphically compared to the control population (Tonnis and Brunken 1968). Since lateral acetabular ossification failure is a characteristic feature of Hurler patients, we strived to quantify this by measuring the percentage of bone coverage of the caput femoris. We used a modified version of Reimers lateral percentage of migration by drawing lines at the AP pelvis as described below (see also Fig. 1c) (Reimers 1980). First, Hilgenreiner's line through the triradiate cartilages with perpendicular to this, a line touching the lateral border of the bony acetabulum. Then parallel to Hilgenreiner's line, a line through the lateral subcapital physes. By virtually connecting the metaphyseal and epiphyseal medial and lateral edges, the contour of the caput femoris was reconstructed and the intersection point with the transphyseal line was taken medially and laterally. The distance m from the medial vertical line to the bold vertical line represents the bony coverage; the percentage of coverage with respect to the distance $m + l$ was calculated as well. The containment at the latest follow-up images (either AP radiograph or MRI) was scored as contained or dislocated.

Genu Valgum

To determine the lower limb alignment, the medial proximal tibial angle (MPTA) was measured according to Sabharwal et al. (2008). The MPTA was measured as the medial angle between a line drawn at the level of the tibial epiphysis, parallel to the growth plate of the proximal tibia and a line connecting the centre of the proximal tibial epiphysis to the midwidth of the talus at the ankle mortise (see also Fig. 1d). Since lower leg radiographs for follow-up were made of the left knee only, we analyzed the MPTAs just for the left knees. Reference values as described by Sabharwal et al. (2008) were used for comparison. Knees with an MPTA greater than the mean value plus two standard deviations were classified as having a valgus deformity.

Statistical Analysis

If applicable, the average of measurements obtained by two observers was used. To analyze the developmental changes in time, we made use of linear mixed models with fixed effects for age and random intercepts per child (Hedeker and Gibbons 2006). In the case of genu valgum, the skeletal changes are not linear, since the normal development of a knee valgus is parabolic. Therefore, a fixed quadratic term for age was included in order to better model the curve. To assess the reliability of the measurements we used, intra-class correlation coefficients (ICC) were calculated. For this purpose, we used a method described by Vangeneugden et al. (2004) which takes into account repeated measurements. To determine if the severity of one of the deformities was related to the severity of the other items, an interrelation coefficient was determined. In order to do so, we used calculated random intercepts to rank the children as per abnormality. Afterwards, an ICC of the rankings was calculated, based on the variance components from a one-way ANOVA with a random effect for patient.

Results

Patients

At the time of final data inclusion, the mean age of patients was 83 ± 27 months (range 45–126 months; see also Table 1). Successful bone marrow transplantation, defined as >10 % donor chimerism, was achieved at a mean age of 18.6 ± 8.3 months (range 8–37 months). Follow-up since transplantation ranged from 15 to 89 months (mean 52 ± 22 months). In ten cases, patients received their graft from an unrelated donor, three patients received their graft from a sibling and one patient received her graft from her mother. In five cases, successful transplantation was achieved by using bone marrow, in seven cases cord blood was used and in two cases peripheral blood. Enzyme replacement therapy (ERT) before HSCT was administered in nine patients. At the latest follow-up, there was full chimerism (≥ 95 % chimerism) in 13 patients. In one patient there was 18 % chimerism. Latest measurements of IUDA enzyme-activity showed a mean of 44 ± 21 nmol/h/mg (range 11–84 nmol/h/mg), with a normal reference range of 25–90 nmol/h/mg. GAG excretion in urine, which has age dependent reference values, ranged from 13–87 mg/mmol creatinine (mean 26.7 ± 18.9).

Odontoïd Hypoplasia

An MRI of the cervical spine was available for all patients. All patients had hypoplasia of the dens on their first MRI (odontoïd/body ratio below 2 (Cokluk et al. 2006); see also Table 2, Figs. 2a, 3). Aplasia was not seen. Severe hypoplasia (a ratio below 1) was seen in one patient. On the most recent MRI studies, two patients showed moderate

Table 1 Characteristics of 14 Dutch patients with Hurler Syndrome

Patient no.	Gender	Age at latest follow-up (months)	Age at HSCT (months)	Follow-up duration after HSCT (months)	Donor source (carrier status)	Donor material	ERT before HSCT	Chimerism (%)	IUDA-activity (nmol/h/mg)	GAG excretion (mg/mmol creat)
1	Male	126	42	84	Unrelated	Peripheral blood	No	100	24	26
2	Female	114	29	84	Mother (carrier)	Bone marrow	Yes	100	32	21
3	Male	112	29	83	Unrelated	Bone marrow	No	100	53	13
4	Female	109	8	101	Unrelated	Peripheral blood	No	18	11	26
5	Male	104	28	76	Unrelated	Cord blood	Yes	100	84	16
6	Male	101	20	81	Unrelated	Cord blood	Yes	100	74	18
7	Male	88	15	74	Unrelated	Cord blood	No	100	36	Unknown
8	Female	79	24	56	Unrelated	Cord blood	Yes	100	40	17
9	Male	67	13	54	Sibling (carrier)	Bone marrow	Yes	100	36	19
10	Male	60	12	48	Unrelated	Cord blood	Yes	100	41	23
11	Female	59	13	46	Sibling	Bone marrow	Yes	96	57	22
12	Female	59	12	47	Unrelated	Cord blood	Yes	100	66	25
13	Male	46	11	35	Sibling (carrier)	Bone marrow	No	98	26	34
14	Male	33	19	26	Unrelated	Cord blood	Yes	100	29	87

HSCT haematopoietic stem cell transplantation, *ERT* enzyme replacement therapy, *IUDA-activity* α-L-iduronidase activity, *GAG excretion* glycosaminoglycan excretion

odontoïd hypoplasia ($\geq 1.0 < 1.5$), ten patients showed mild hypoplasia ($\geq 1.5 < 2$) and one a normal odontoïd/body ratio (> 2). During growth, the odontoïd/body ratio increased in 11 patients, decreased in one patient and remained unchanged in two patients. In general, a slight but significant improvement in odointoïd/body ratio was seen (slope value 0.066 per year; 95 % C.I. 0.04–0.09). The ICC for this measurement technique was 0.74, which is generally interpreted as good or strong. Based on the radiological reports, increased soft tissue around the tip of the odontoïd process was observed in all 14 patients.

Thoracolumbar Kyphosis

Full weight bearing radiographs of the total spine were available for all patients. Kyphosis in the thoracolumbar area was present in 13 cases (see Table 2, Figs. 2b, 4a). A total of seven patients had had an attempt to control their kyphosis in a brace. However, in most cases, bracing was abandoned because of discomfort and absence of effect. The average angle of kyphosis at first presentation ranged from 23–60° (mean 42 ± 10.4°). At the latest follow-up visit, and in one case before corrective surgery, it ranged from 14 to 78° (mean 49 ± 19.1°). The kyphotic angle showed a slight but significant progression of 1.7° per year *(*95 % C.I. 0.82–2.5*)*. The ICC of our measurement technique was 0.84, which is considered excellent. There were six patients that showed a considerable increase of >10°, the remaining eight were quite stable. All 13 patients with a kyphotic angle >20° showed signs of anteriorinferior beaking. The anterior part of the vertebral body of L2 was most often affected (12/13), sometimes in combination with L1 (4/13) or L3 (1/13). Isolated beaking of L1 was observed in one patient. Disc disappearance at the level of Th12-L1 and/or L1-L2 was seen in eight patients. Spondylolisthesis at the thoracolumbar level, grade I-IV, was seen in 12 patients. A total of seven of these patients also showed signs of lumbar retrolisthesis, below the level of the kyphosis, ranging from grade I–III.

One of the patients (patient 5, see Table 2), with a progressive kyphotic angle > 80° was treated surgically. A short segment posterior fusion Th12 to L3 was done after

Table 2 Spinal deformities

Patient no.	Odontoïd/ body ratio at latest follow-up	Kyphotic angle at latest follow-up	Beaking	Disc disappearance	Anterolisthesis	Retrolisthesis	Scoliotic angle at latest follow-up	Myelum compression [level]
1	0.8[a]	56°	L1, L2	Th12-L1	–	–	56°	Yes [C0-1, C2-4]
2	1.4	14°	–	–	L4-L5 grade II L5-S1 grade IV	Th12-L1 grade II	–	No
3	1.7	63°	L2	L1-L2	L1-L2 grade II	–	12°	No
4	2.3	71°	L2, L3	–	L1-L2 grade IV	L3-L4 grade III	19°	No
5	1.7	78°[b]	L1, L2	Th12-L1	Th12-L1 grade IV	–	18°	No
6	1.3	38°	L1	Th12-L1	Th12-L1 grade III	–	–	Yes [Th12- L1]
7	1.9	66°	L2	Th12-L1 L1-L2	Th12-L1 grade II	L2-L3 grade II	15°	Yes [C3]
8	1.5	24°	L2	–	L1-L2 grade I	L2-L3 grade I	–	No
9	1.5	43°	L2	L1-L2	Th12-L1 grade I L1-L2 grade II	L2-L3 grade III	12°	No
10	1.5	71°	L2	Th12-L1 L1-L2	Th12-L1 grade III L1-L2 grade III	L2-L3 grade III	–	No
11	1.9	41°	L2	Th12-L1 L1-L2	L1-L2 grade II	–	12°	No
12	1.5	50°	L1, L2	–	Th12-L1 grade III	–	–	No
13	1.9	42°	L1, L2	–	Th11-Th12 grade I Th12-L1 grade II	–	20°	No
14	1.5	32°	L2	–	L1-L2 grade II	L2-L3 grade II	–	No

[a] Before cervical decompression

[b] Before thoracolumbar spondylodesis

pedicle subtraction osteotomy of L1. This resulted in a reduction from 80 to 12°. Unfortunately, the kyphosis of the segment superior to the spondylodesis progressed to 64° within 13 months. This was treated with a revision of the spondylodesis at the level of T11-L4 and superior extension with a growing rod system, which reduced the kyphosis to 14° (see also Fig. 4b). At the latest follow-up visit, 18 months after the revision, the kyphotic angle had again increased to 35°.

Scoliosis

Scoliosis (Cobb angle >10°) was observed in eight patients (57 %; see Table 2 for characteristics). Mostly, scoliosis became manifest around 4 years of age. At the latest follow-up visit, the scoliotic angle ranged from 12–56° (mean 20 ± 14.8°). Statistical analysis indicated a nonsignificant slight progression of 2.5° per year (95 % C.I. –0.03–5.1).

Spinal Cord Involvement

A full spine MRI was available for all patients. Asymptomatic narrowing of the spinal canal with signs of mild spinal cord compression on the T2-weighted images was found in two patients (pt. no. 6 at level Th12-L1 and pt. no. 7 at level C3: see also Table 2). Another patient developed a symptomatic cervical myelopathy at 85 months of age (pt. no.1, level C0-1 and C2-4). This patient was treated with a cervical decompression without fusion elsewhere, but developed a progressive kyphosis with neurological symptoms at and above the level of the decompression. Subsequently,

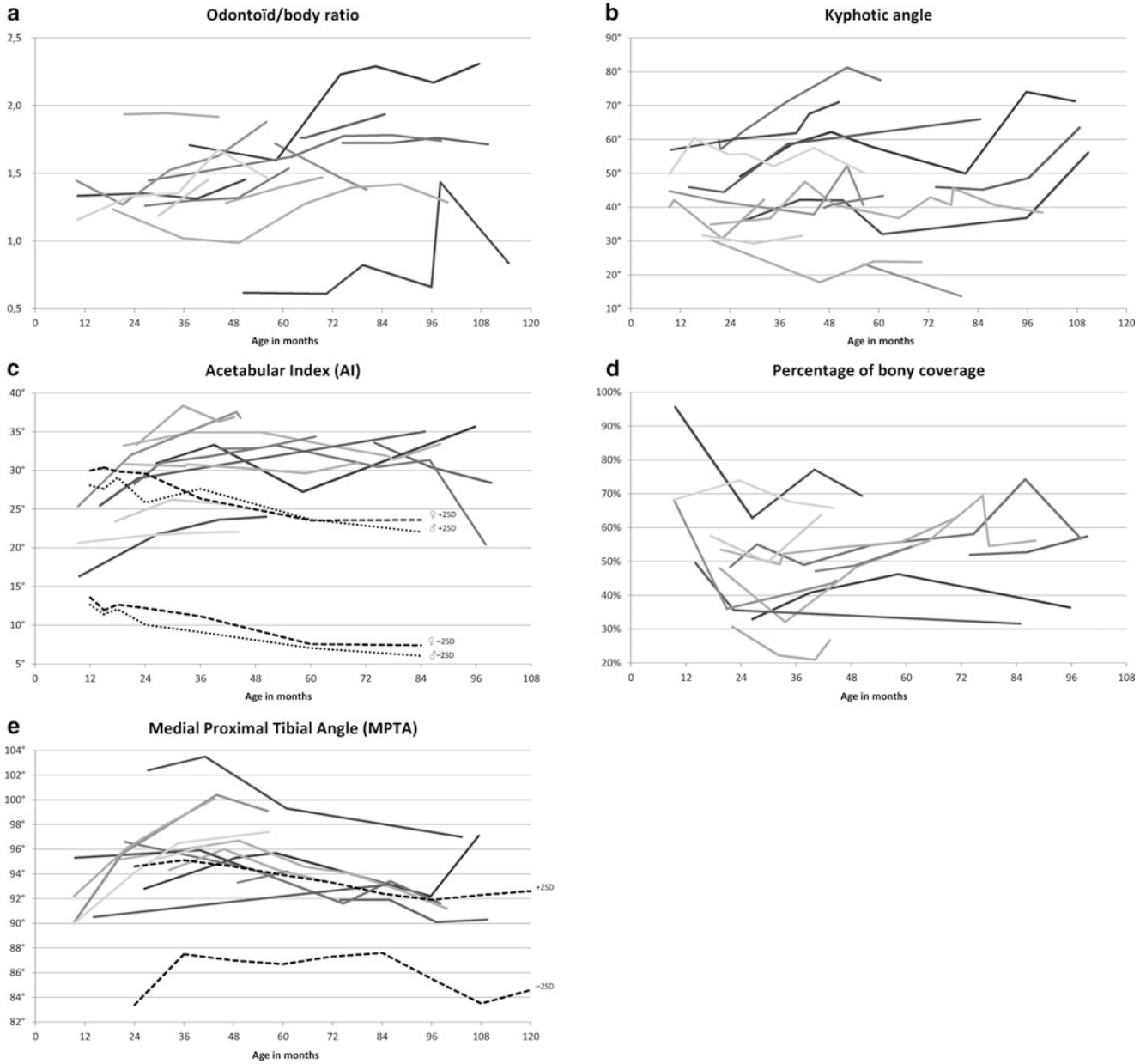

Fig. 2 Progression of the skeletal abnormalities of the Hurler patient population over time: (**a**) odontoid/body ratio, (**b**) maximum kyphotic angle, (**c**) acetabular index, (**d**) percentage of bony coverage of the caput femoris, and (**e**) medial proximal tibial angle (MPTA). All measurements are plotted against patient age. Each line represents an individual patient. Reference lines of ± 2 standard deviations (SD) of the mean, are based on the data of Tonnis and Brunken (1968) for the acetabular index (**c**) and Sabharwal et al. (2008) for the MPTA (**e**)

a posterior spondylodesis from the occiput to C4 was done in combination with decompression of the foramen magnum. The fusion was still stable 18 months after surgery, and the patient regained some function. However, the strength of his right arm remained decreased (MRC 4/5).

Hip Dysplasia

Multiple radiographs of the pelvis were available for all but one patient. Another patient showed such a deformed acetabulum that adequate measurements were impossible. In the remaining patients ($n = 12$), the average AI at presentation was 28 ± 6.0° (range 15–35°) for the left and 28 ± 6.4° (range 18–37°) for the right hip (see Fig. 2c and Table 3). At the latest follow-up visit, the mean AI was 30 ± 7.1° (range 16–38°) at the left and 31 ± 6.1° (range 21–38°) at the right side. Although it seems that the right and left hip were almost similarly affected, the similarity within patients was rather poor with an ICC of only 0.53. In eight patients, an increase in the average AI of both hips

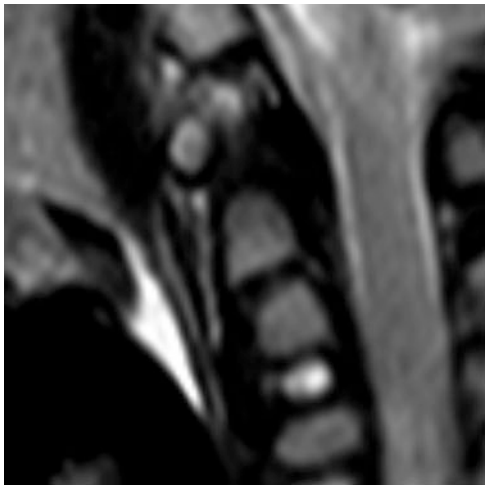

Fig. 3 Example of odontoïd hypoplasia. Illustrated is a T2 weighted MRI of the midsagittal cervical spine of a 5 year old female patient. The body of C2 and odontoid process can clearly be observed with in-between the dentocentral synchondrosis. This patient had an odontoid/body ratio of 1.4, which was classified as moderate hypoplasia

was seen, indicating worsening of the hip dysplasia (see Fig. 5 for an example). Only two patients showed a decrease in the average AI of both hips over time, as seen in a healthy population. Another two patients showed neither improvement nor worsening of the AI. Overall, the AI increased minimally with an average angle of 0.37° per year (95 % C.I. −0.086–0.82, not significant). The interobserver ICC of the AI angle was 0.66 per hip, which is considered fair to moderate.

The percentage of bony coverage appeared to remain quite stable with an initial value of 54 ± 19.9 % (range 26–100 %) for the left and 54 ± 16.0 % (range 35–91 %) for the right hip, and average values of, respectively, 53 ± 15.9 % (range 18–78 %) for the left and 52 ± 14.4 % (range 22–69 %) for the right hip at latest follow-up (0.66 % per year; 95 % C.I. −0.47–1.8, not significant). Again, the similarity between the left and right side was moderate with an ICC of 0.66. There was an increase in seven and a decrease in five patients. The interobserver ICC of this measurement was a fair 0.59. All patients showed some degree of failure of ossification at the lateral acetabulum, delayed ossification of the femoral head and coxa valga. Therefore, we did not attempt to score subluxation separately. Only the oldest patient (patient no. 1), who received successful HSCT relatively late, at 37 months of age, developed bilateral hip dislocations at the age of 40 months. This situation has been accepted. In two patients we treated the bilateral severe hip dysplasia at the age of, respectively, 4 years and 1 month (pt. no. 11) and 7 years and 11 months (pt. no. 3; see Table 3) with a combination of a Pemberton osteotomy and proximal femoral osteotomy.

Genu Valgum

Radiographs were available for all but one patient. Compared to the values described by Sabharwal et al., all patients in our study appeared to have an MPTA far above the mean of healthy children of similar age (see Fig. 2e and Table 3) (Sabharwal et al. 2008). In all but one patient the increase was more than two standard deviations, at least once in their life, indicating a valgus deformity (see Fig. 6 for an example). At initial presentation, the average MPTA for the left knee was 94 ± 3.3° (range 90–102°). At latest follow-up, a mean MPTA of 95 ± 3.1° (range 90–100°) was found. Like healthy children, the included patients showed a parabolic knee development with a maximum MPTA between 3 and 5 years of age. This appears to be delayed as compared to the healthy population, where this maximum is reached around the age of 2 years (Sabharwal et al. 2008). None of the patients were surgically treated for their valgus deformity during the course of this study.

Correlation Between Abnormalities

We could not demonstrate an interrelation between the severity of the different dysostosis multiplex manifestations within the subjects. However, as could be expected, a significant correlation between the acetabular index and the percentage of bony coverage of the caput femoris existed.

Discussion

In the current study, we collected data to evaluate the prevalence, progression and interrelation of the orthopaedic manifestations in the Dutch Hurler patient population. Although the general Hurler manifestations after stem cell transplantation were less severe compared to untreated patients, all patients showed worrisome dysostosis multiplex without improvement. More specifically, odontoïd dysplasia, kyphosis, spondylolisthesis, scoliosis, hip dysplasia and genu valgum were frequently observed.

Thoracolumbar kyphosis was present in nearly all patients regardless of the use of brace treatment. It usually occurred in combination with anterior beaking of L1 or L2, and disappearance of the intervertebral disc at the apex of the deformity. The radiological method we used, measuring the maximal angle between the four vertebrae at the apex of the kyphosis, is not clearly described in literature but appeared to work well with a very acceptable interobserver variability. During follow-up, the patients had a significant progression in their kyphotic angle. A progression of more than 10° was observed in nearly half of the patients. This could not be related to the presence of vertebral beaking or disc disappearance, but seemed related instead to larger

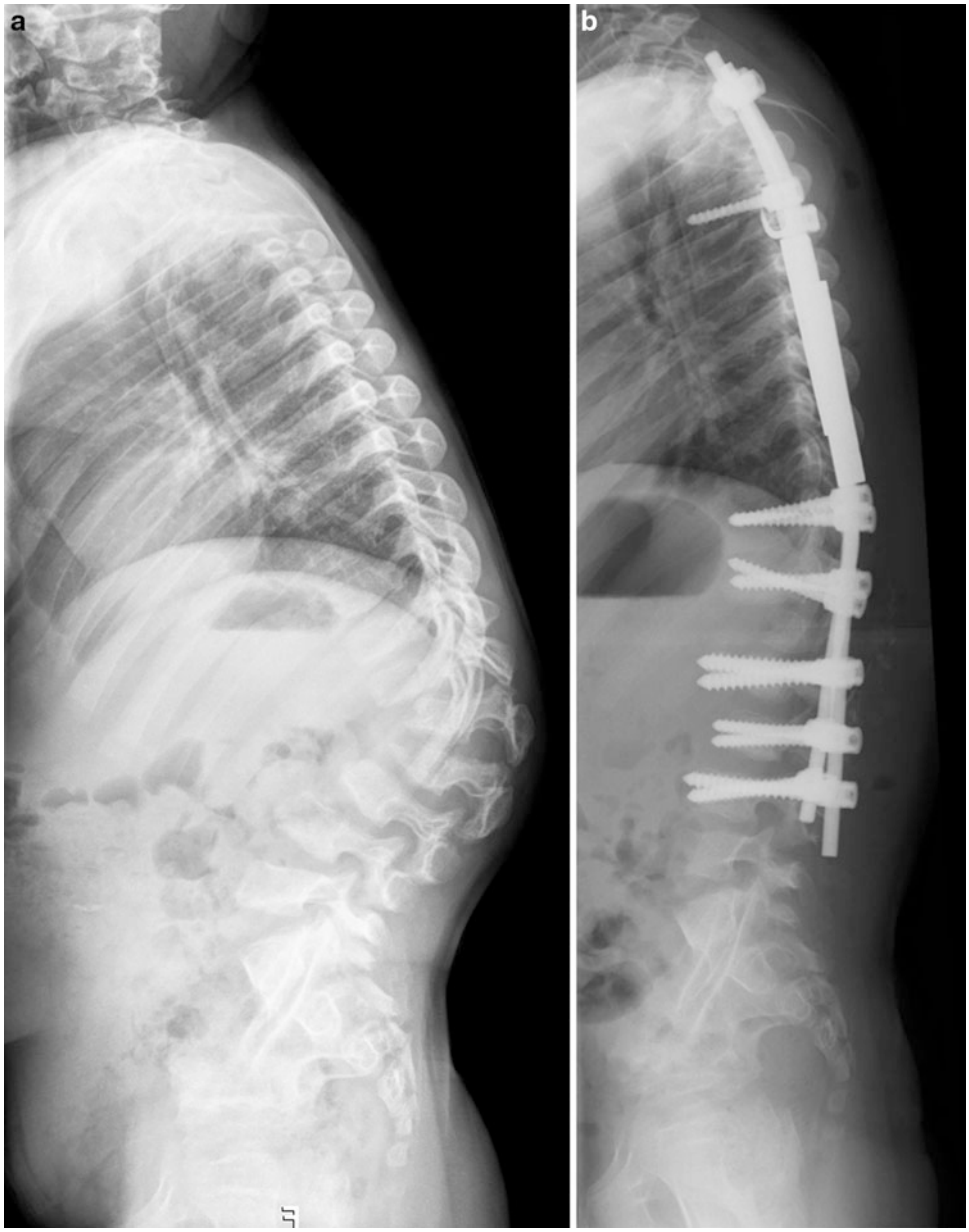

Fig. 4 Example of a thoracolumbar kyphosis. (**a**) Lateral radiograph of the thoracolumbar spine of a 5 year old sitting male patient. There is an obvious kyphosis of 78°, measured between T11 and L2, in addition to anterior beaking of T12 and L1 and severe spondylolisthesis of T11. (**b**) Radiograph after revision of an initial short segment posterior spinal fusion (Th11-L3) in the patient shown in Fig. 4a. To maintain correction, the segment above was fixated with a growing rod system

kyphotic angles at initial presentation (see also Fig. 2b). How to treat these patients remains an important question. Short segment posterior fusions seem insufficient, possibly because the posterior ligamentous tether is deficient in this disorder as well. We used a growing rod extension over a short posterior fusion in one revision case, also with limited success because of the inherent kyphogenic tendency. The optimal solution for this problem has not been described yet. As suggested in our recent literature review, short segment anterior fusions combined with posterior stabilization may be required (van der Linden et al. 2011; Dalvie et al. 2001). Another potential way to avoid failure might be stabilization over a longer trajectory or earlier intervention. The patient that was operated on in the current study had a kyphotic angle of approximately 80°. However, we now consider a kyphosis angle $> 60°$ an indication for surgery, since it is very likely that the angular deformity will progress from this point, resulting in unacceptable deformities in adult life (van der Linden et al. 2011). In addition to the kyphosis, the other frequently observed spinal deformities like antero-, retrolisthesis and scoliosis may require surgical treatment at some point in time as well.

Table 3 Measurements of the hips and knee at latest follow-up

	Left hip		Right hip		Left knee
Patient no.	AI	Percentage of bony coverage	AI	Percentage of bony coverage	MPTA
1	55°	0 %[b]	53°	0 %[b]	97°
2	37°[a]	31 %[a]	31°[a]	35 %[a]	–
3	26°[c]	58 %[c]	31°[c]	57 %[c]	90°
4	35°	36 %	37°	37 %	97°
5	16°	63 %	25°	51 %	92°
6	30°	56 %	37°	57 %	91°
7	34°	41 %	36°	22 %	93°
8	36°	57 %	26°	69 %	93°
9	38°	49 %	31°	60 %	94°
10	23°	78 %	25°	61 %	95°
11	36°[c]	46 %[c]	38°[c]	43 %[c]	99°
12	24°	67 %	21°	65 %	97°
13	38°	18 %	36°	35 %	100°
14	26°	63 %	25°	64 %	96°

AI acetabular index, *MPTA* medial proximal tibial angle

[a] Only one follow-up visit available

[b] Patient with bilateral hip luxation

[c] Before corrective surgery (Pemberton with femoral osteotomy)

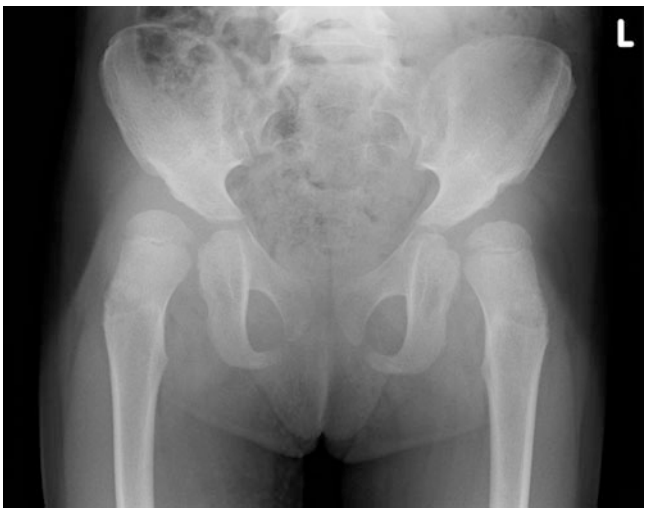

Fig. 5 Example of hip dysplasia. Illustrated is a pelvic radiograph of a 3.5 year old female patient with bilateral hip dysplasia. Note the lateral acetabular failure of ossification. The AI in this patient was 38° on the right and 36° on the left side. The percentage of bony coverage was, respectively, 43 % and 46 %

The majority of patients showed mild to moderate odontoïd hypoplasia, which improved during follow-up. When critically reviewing the existing literature on Hurler´s disease, we found that dens hypoplasia was recently

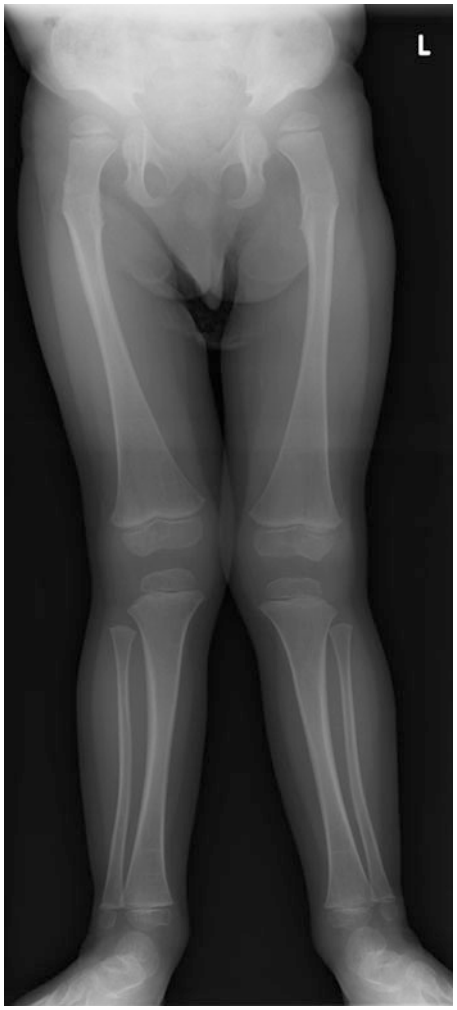

Fig. 6 Example of genu valgum. Illustrated is a long leg radiograph of a 3.5 year old male patient. The left valgus angle was 12° with an MPTA of 100°

described in a similar manner in 30 Hurler patients (Miebach et al. 2011). Unfortunately, this paper reported absolute dens lengths instead of the ratio between the vertebral body and dens as described by Cokluk et al. (2006). Other papers on dens hypoplasia in Hurler´s disease failed to describe a clear measurement method (Vellodi et al. 1997; Hite et al. 2000; Tandon et al. 1996; Belani et al. 1993). We chose the current quantification method because it is easy to use and healthy control values are available. The interobserver reliability appeared to be good. In agreement with previous findings, dens hypoplasia improved in the large majority of patients (Hite et al. 2000; Miebach et al. 2011). The one patient that showed worsening was a patient that was not fully engrafted. When this trend continues, we believe that dens hypoplasia per se is not a major problem with respect to cervical stability or mobility in Hurler patients.

In addition to the odontoïd dysplasia, soft tissue deposition around the dense was observed in all patients as well.

This is in line with the high percentage reported in our previous literature review (85.7 %, van der Linden et al. 2011). Although soft tissue deposition around the dense has often been described in Hurler's disease (Weisstein et al. 2004; Kachur and Del Maestro 2000; Vellodi et al. 1997; Hite et al. 2000; Tandon et al. 1996), it is difficult to reliably quantify it, and reference values are lacking (Hite et al. 2000; Tandon et al. 1996). A potential serious consequence of both odontoïd hypoplasia with atlanto-axial subluxation and (extra)dural soft tissue deposition is cervical spinal cord compression, requiring surgical intervention. Cervical spinal cord compression was present in two of our patients, which is roughly in accordance with the 3.4 % reported in the literature (van der Linden et al. 2011). It is therefore, presumably, a less frequent complication in Hurler's disease as compared to, for example, Hunter's and Morquio's disease (MPS II and IV), where cervical cord compression has been reported in 64 % to 100 % of the cases (Stevens et al. 1991; Parsons et al. 1996; Hughes et al. 1997).

Hip dysplasia appears to be a concern as well. Only one of our patients showed some improvement of the Acetabular Index over time. Hip dysplasia resulted in bilateral hip dislocation in one of our patients. Inspired by the good results of others (van der Linden et al. 2011; Taylor et al. 2008), we now started to surgically treat this problem with combinations of pelvic and femoral osteotomies. The clinical and technical results of the first two patients are positive. A pitfall of measuring the AI in Hurler patients was the impression of a false acetabulum on the X-ray, due to the lateral failure of ossification. Despite this knowledge, we encountered difficulties in measuring the AI reproducibly, resulting in an ICC in the fair to moderate range. The modified Reimers assessment of bony coverage of the caput femoris was even less reproducible with a fair ICC. Both measurements indicated a relatively weak correlation between the right and left hip, which means that both hips have to be addressed and followed separately in the clinic. In contrast to the healthy control population, there was no improvement of both the acetabular index and the percentage of bony coverage in the Hurler population. This can thus be considered a gradual worsening of the status of these hips. Therefore, it is likely that the vast majority of these patients will need bilateral surgical treatment to preserve hip containment in the future. As previously reported, surgical procedures to correct hip dysplasia in Hurler´s disease should always include corrections of the acetabulum (Weisstein et al. 2004; Field et al. 1994; Taylor et al. 2008; Masterson et al. 1996).

Knee valgus was obvious as well, and did not show a trend for significant improvement over time. A predictable correction of the mechanical axis of the leg in Hurler's disease can be achieved with growth modulation techniques (Odunusi et al. 1999). This is a simple and effective method, which improves function and can be combined with other surgical interventions. Although none of our patients were treated with this technique yet, we are planning to perform this surgery in the near future. To analyze the degree of genu valgum we used the MPTA. Sabharwal et al. found an average of 89° in children from 1 to 2 years old. Between the age of 2 and 3 years, healthy children reach their peak valgus with an average angle of 91°. Thereafter the MPTA declines to an average angle of 88° in children between 8 and 9 years of age (Sabharwal et al. 2008). The tibial valgus in the Hurler population appeared to follow a similar trend; however, the peak was around 3.5–4 years of age and the valgus angle was generally more than 2SDs above normal.

Besides a correlation between the acetabular index and the percentage of bony coverage of the caput femoris, we were unable to detect a correlation between the severity of the different orthopaedic abnormalities within the individual patients. The absence of a clear interrelation between the severity of the different forms of dysostosis indicates that dysostosis cannot be generally categorized as mild or severe, but should be classified per patient per item.

An obvious limitation of this study is the retrospective design, with a relatively large variation in diagnostic delay and exact type of treatment. A subgroup of patients was treated with enzyme replacement therapy before transplantation. In addition, varying transplantation regimes were applied. Also, the spine problems of a subgroup of patients were initially treated with various bracing regimes. Furthermore, different X-ray regimes were used at first, for example sitting versus standing spine radiographs. Finally, sizes are small and preclude statistical comparisons. Since 2 years we have standardized the frequency and type of follow-up measurements according to the suggestions of the MPS registry (Muenzer et al. 2009). Hopefully this strategy will help our understanding of the disease and aid in developing new treatment algorithms.

In conclusion, dysostosis multiplex was found in all Dutch Hurler patients, despite successful bone marrow transplantation. In nearly all patients, progressive bilateral hip dysplasia was observed, which seems to warrant surgical intervention at some point. In addition, progressive thoracolumbar kyphosis was frequently observed, which did not respond well to brace treatment and short posterior segment fusion. Knee valgus was frequently observed as well, and appeared stable in most patients. If surgery for genu valgum is indicated, guided growth seems an appropriate technique. Only dens hypoplasia showed a tendency to improve over time, of which the clinical relevance remains speculative. Longer follow-up periods and a combination of our data with that of other institutes are necessary to determine more precisely how and when to optimally treat these patients.

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Take-Home Message

Dysostosis multiplex remains a major problem in Hurler patients after haematopoietic stem cell transplantation. Except for dens hypoplasia, it appears progressive and often requires surgical intervention.

Contribution of Individual Authors

The authors FS, MK and RS measured the degree of odontoïd dysplasia, kyphosis and malformation of the pelvis. The degree of scoliosis and genu valgum was measured by FS. Authors FS, MK and MV pooled and analyzed the data, and constructed the tables, figures and the first draft of the article. Authors FS, MK, MV, RS, PV and RC were involved in the set-up of the study, and in critically reviewing and editing the various drafts of the manuscript.

Guarantor of the Article

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None of the authors have competing interests, based on the 'Competing Interests Questions'.

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RESEARCH REPORT

Nutritional Changes and Micronutrient Supply in Patients with Phenylketonuria Under Therapy with Tetrahydrobiopterin (BH_4)

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Abstract *Background:* Since 2008 patients with BH_4-sensitive phenylketonuria can be treated with sapropterin dihydrochloride (Kuvan®) in addition to the classic phenylalanine (Phe) restricted diet. The aim of this study was to evaluate the nutritional changes and micronutrient supply in patients with phenylketonuria (PKU) under therapy with tetrahydrobiopterin (BH_4).

Subjects and Methods: 19 children with PKU (4–18 years) and potential BH_4-sensitivity were included, 14 completed the study protocol. Dried blood Phe concentrations as well as detailed dietary records were obtained throughout the study at preassigned study days.

Results: Eight patients could increase their Phe tolerance from 629 ± 476 mg to 2131 ± 1084 mg ($P = 0.006$) under BH_4 while maintaining good metabolic control (Phe concentration in dried blood 283 ± 145 μM vs. 304 ± 136 μM, $P = 1.0$), therefore proving to be BH_4-sensitive. They decreased their consumption of special low protein products and fruit while increasing their consumption of high protein foods such as processed meat, milk and dairy products. Intake of vitamin D ($P = 0.016$), iron ($P = 0.002$), calcium ($P = 0.017$), iodine ($P = 0.005$) and zinc ($P = 0.046$) significantly declined during BH_4 treatment while no differences in energy and macronutrient supply occurred.

Conclusion: BH_4-sensitive patients showed good metabolic control under markedly increased Phe consumption. However, the insufficient supply of some micronutrients needs consideration. Long-term multicenter settings with higher sample sizes are necessary to investigate the changes of nutrient intake under BH_4 therapy to further evaluate potential risks of malnutrition. Supplementation may become necessary.

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Introduction

Phenylketonuria (PKU, OMIM 261600), one of the most common inborn errors of metabolism, is caused by mutations in the gene encoding phenylalanine hydroxylase (PAH, EC 1.14.16.1), leading to deficient enzyme activity. More than 500 mutations in the PAH gene have been identified (http://www.PAHdb.mcgill.ca). PAH is primarily expressed in the liver, its activity depending on tetrahydrobiopterin (BH_4) as a cofactor. The enzyme catalyses the irreversible hydroxylation of phenylalanine (Phe) to tyrosine (Tyr). In PAH deficient patients, Phe accumulates in blood and tissues while a deficiency of Tyr develops. In untreated patients, this results in severe, irreversible psychomotor retardation. Newborn screening and initiation of dietary treatment within the first weeks of life nowadays allow for a normal neurocognitive development (Scriver et al. 1998). The treatment of PKU consists in dietary

restriction of Phe and therefore natural protein and a supplementation with Phe free amino acid mixtures (AAM). Next to essential amino acids except for Phe the AAM contain energy, carbohydrates and fat as well as vitamins, minerals and trace elements as currently recommended by the national councils (for the German speaking countries, DACH 2000). The extent of Phe restriction depends on the patients` individual Phe tolerance, which depends on residual PAH activity as well as the patients` age and weight (Scriver et al. 1998). Phe tolerance is determined by regularly monitored dried blood Phe concentration. Protein rich foods such as meat or dairy products, which are also important sources for other nutrients such as iron, vitamin B_{12} or calcium, must be strictly avoided. Most natural food has to be replaced by special low protein products.

Phe restriction and AAM supply are well established as an efficient and safe treatment for PKU patients. However, this therapy remains a great challenge and burden for the patients and their families. In the classical treatment of PKU it is necessary to plan meals exactly and to calculate the Phe content of all foods. Only recently, some specialized centres allow the unrestricted consumption of fruits and vegetables under certain conditions (Macdonald et al. 2003; Rohde et al. 2012; Zimmermann et al. 2012). However, the individual Phe tolerance has to be respected. Moreover, the AAM have an unpleasant taste. As a consequence patients often show a low diet adherence, particularly older children and adolescents (Macdonald 2000; Macdonald et al. 2010). Especially during adolescence many patients drop out of specialized care (Mütze et al. 2011). Discontinuation of dietary treatment bears a high risk of nutrient deficiencies, cognitive and emotional dysfunction as well as behavioral problems. Therefore, lifelong diet is strongly recommended (Stemerdink et al. 2000; Burgard et al. 1997; Weglage et al. 1992).

In recent years, BH_4 supplementation has been investigated as a new therapeutic tool in PKU treatment. It was shown that in some PKU patients BH_4 enhanced the residual PAH activity and partially restored Phe oxidation (Muntau et al. 2002). As a result, blood Phe concentrations decrease while Phe tolerance can increase up to 2- to 3-fold (Hennermann et al. 2005; Lambruschini et al. 2005; Burlina and Blau 2009; Trefz et al. 2009). This now allows some patients to consume protein rich foods. The dosage of Phe free AAM can be reduced. Due to the higher Phe tolerance, quality of life as well as therapy adherence may be enhanced (Ziesch et al. 2012). However, BH_4 monotherapy seems to be sufficient in only few cases (Lambruschini et al. 2005).

Available reports on BH_4 supplementation have focused on the identification of patients who may benefit from BH_4, and the quality of metabolic control (Bélanger-Quintana et al. 2005; Fiori et al. 2005; Burton et al. 2007, 2010; Burlina and Blau 2009; Trefz et al. 2009). The small number of studies investigating the nutrition of PKU patients under BH_4 treatment revealed heterogeneous results (Lambruschini et al. 2005; Singh et al. 2010).

The aim of this study was to investigate eating habits of BH_4-sensitive patients under BH_4 therapy compared to the classical Phe restricted diet. We evaluated food and nutrient intake in order to examine whether BH_4 treated PKU patients are adequately supplied with critical nutrients under relaxed dietary conditions.

Study Design

This open, monocentric, prospective intervention trial follows the principles of the Declaration of Helsinki and ICH/GCP. It was approved by the University of Leipzig's ethics committee (registration-number 087-2009-20042009). All included patients and/or their guardians gave written informed consent.

Inclusion was restricted to patients aged 4–18 years with PKU and potential BH_4 sensitivity. Patients with a BH_4 deficiency as well as additional diseases or abnormal signs in the general or neurological examination, a need for concomitant medication except the Phe free AAM, an implementation of other diets except the Phe restricted diet and an existing or planned pregnancy were excluded.

The study design is shown in Fig. 1. In each of the four study periods the patients were asked to send dried blood samples and dietary records to the hospital on preassigned study days.

Study period 1 consisted in the reevaluation of the current dietary treatment by review of Phe concentrations in dried blood one year prior to enrolment and determination of current Phe tolerance using a 3 day dietary record (days −3 to 0). In study period 2 (days 0 to 14), the patients were instructed to double their daily Phe intake from natural protein (from any food) to achieve Phe concentrations $\geq$ 600 µmol/l. No standardized meals were given throughout the study. The intake of the Phe free AAM was continued. Dietary records were performed three times a week and dried blood samples for analyses of Phe concentrations were obtained. In study period 3 (day 14 to 42), the patients received sapropterin dihydrochloride (Kuvan®, Merck Serono) at 20 mg per kg body weight daily, administered as single oral dose in the morning, while continuing the doubled Phe intake. On day 42, patients were defined as BH_4-sensitive if they showed a reduction in Phe concentration of $\geq$ 30 % and/or an increase of Phe consumption by $\geq$ 100 % while Phe concentrations remained within the therapeutic range. All other patients were classified as BH_4-resistant. During study period

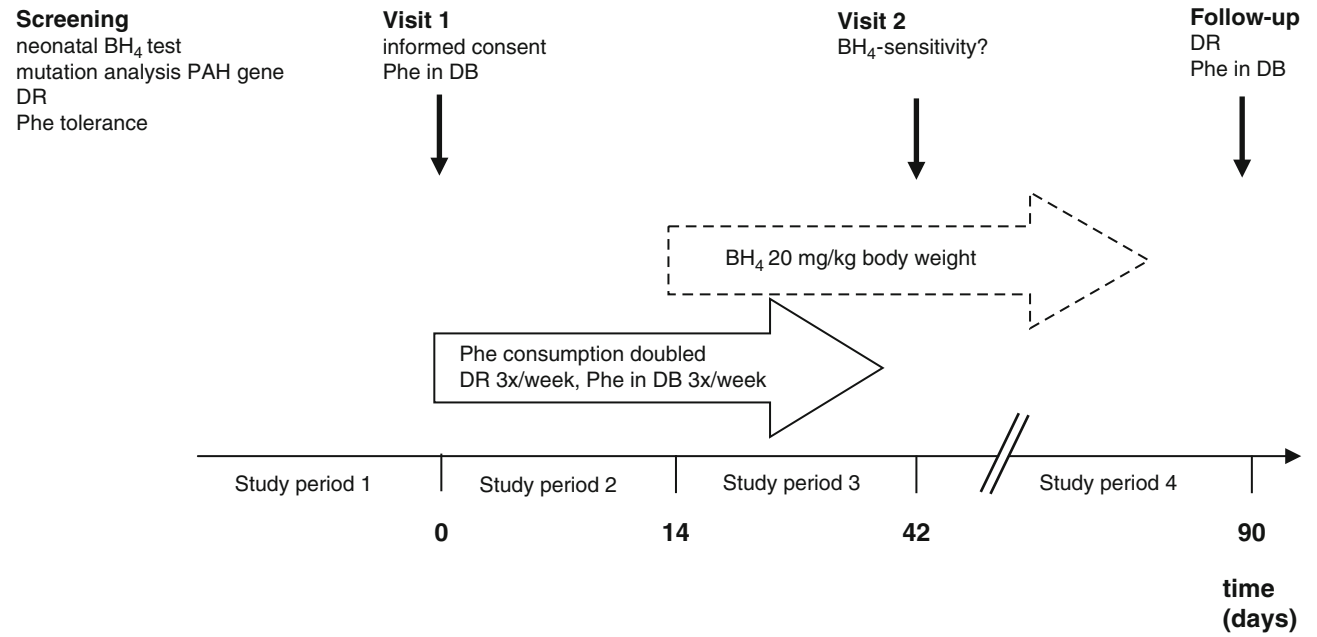

Fig. 1 Study design of BH_4-intervention trial in PKU patients. *BH4* tetrahydrobiopterin, *PAH* phenylalanine hydroxylase, *Phe* phenylalanine, *DB* dried blood, *DR* dietary record

4 (days 43 through 90 = follow-up), BH_4-sensitive patients continued BH_4 treatment. During follow up, every actual Phe concentration was evaluated and followed by personal contact with the patient or the parents to advise on further increase of Phe consumption. Once the patients had reached an adequate protein consumption from natural foods (DACH 2000) AAM dosage was reduced.

Subjects and Methods

Subjects

A total of 41 patients (4–18 years), treated at the outpatient clinic for inborn metabolic diseases at the University Hospital in Leipzig, Germany, were screened for participation. They were tested for potential sensitivity to BH_4 by determination of their PAH mutation and reevaluation of the neonatal BH_4-test. In 20 of the patients BH_4-sensitivity seemed to be implausible. The other ($n = 21$), including those whose results were incomplete, were offered to test BH_4 (as sapropterin dihydrochloride, Kuvan®) as a potential additional treatment. Two of the patients declined participation. Nineteen patients were included into the study.

Of these, 18 completed the protocol. One patient refused to take the medication and terminated study participation. Four other patients ignored essential parts of the study protocol and had to be excluded from the analysis. All participants had been diagnosed with PKU by newborn screening and dietary treatment had been initiated within the first 2 weeks of life. All but four underwent a test for BH_4-sensitivity when first diagnosed. None of them showed a deficiency in tetrahydrobiopterin (Kaufman et al. 1978; Blau et al. 2011). All patients were regularly followed in the outpatient clinic for inborn metabolic diseases and their reliable adherence to a Phe restricted diet had been recorded. The dosage of substitution with synthetic amino acids followed the current recommendations for protein intake in children (DACH 2000) with an added surplus of 20 % to account for possible differences in biological value relative to natural protein.

Methods

Assessment of Plasma Phe Concentrations

Patients took samples of capillary whole blood on filter paper at preassigned study days. All parents were familiar with the proper technique, having been trained during the patients` newborn period. Phe and Tyr concentrations in dried blood were determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as previously described (Ceglarek et al. 2002).

Assessment of Food and Nutrient Intake

The patients and/or their guardians performed dietary records throughout the study on preassigned study days. All foods and beverages, including special low protein foods as well as the AAM, were weighed and documented.

All ingested food was allocated to the following food groups: bread and cereal products, potatoes/ rice and pasta, vegetables, fruit, milk and dairy products, other food of animal origin (meat and processed meat, fish, egg), edible fat (butter, margarine, oil), sweets and snacks, special low-protein products. This classification was chosen according to the recommendations for age-based food consumption issued by the German Research Institute of Child Nutrition (Kersting and Alexy 2005).

Nutritional analysis was performed using the Food and Control Management System "Diät 2000" based on the updated version of the Bundeslebensmittelschlüssel (Hartmann 2009). Additional information on AAM, low protein foods as well as other processed foods provided by the manufacturer was added to the database. Besides Phe intake (mg/day) the following nutrients were calculated as percentage of regular daily allowance, recommended for the German speaking countries (DACH 2000): energy, protein, carbohydrate, fat, vitamin C, vitamin D, vitamin B_{12}, iron, calcium, iodine and zinc.

Mean nutrient supply and food consumption of BH_4-sensitive patients under the new therapeutic regime was compared to the classical dietary treatment and to the current recommendations for nutrient intake (DACH 2000). In addition, mean nutrient supply was compared to data from a cohort of age-matched healthy German children (Mensink et al. 2007).

Statistical Analysis

All procedures were performed using SPSS for Windows 17 (SPSS Inc., Chicago, Illinois).

To reduce the number of variables, laboratory data from each study period were averaged and used for analyses. If normality of distribution could be assured, longitudinal changes in nutrient supply over the four study periods were analysed by Wilk's multivariate analysis of variance (MANOVA) with "time" as the within-subject factor with four levels (study periods 1 through 4). If Wilk's analysis yielded a significant effect of "time", this was followed by repeated (sequential) contrasts to locate pairs with significant changes over time. In analogy, data that were not normally distributed were analysed by Friedman test and Wilcoxon test.

Significance was accepted for $P < 0.05$. Data are given as mean $\pm$ standard deviation (SD) unless otherwise stated.

Results

Patient Characteristics

A total of 14 patients (6 females, 8 males) could be considered in the analysis of metabolic control and Phe intake. All of them had a normal body weight with a mean BMI-SDS of 0.21 $\pm$ 1.0. In one of these patients no data of nutrient supply under classical therapy except for Phe consumption were available. Thus, this patient had to be excluded from the nutrition analyses. No adverse events were observed throughout the study.

Metabolic Control and Phe Consumption

The data of Phe concetration in dried blood, Phe and natural protein consumption are presented in Table 1. Over the last year prior to study entry all included patients showed good metabolic control. Eight patients proved to be BH_4-sensitive. In the other six patients, BH_4 therapy was not effective. Only BH_4-sensitive patients stayed on BH_4 therapy during follow-up. They continued to show good metabolic control with stable dried blood Phe concentrations within the therapeutic range that did not differ from study entry (see Table 1, $P = 1.0$).

In BH_4-resistant patients we found a slight increase of Phe intake and a significantly higher Phe concentration in dried blood during follow-up compared to study entry (621 µmol/l $\pm$ 117 vs 474 µmol/l $\pm$ 141 at study entry; $P = 0.003$). It took them another three months to regain the original metabolic control (data not shown).

Food Consumption of BH_4-Sensitive Patients

Figure 2 shows the food consumption of BH_4-sensitive patients under classical dietary treatment compared to BH_4 therapy. The serving sizes of consumed food remained unchanged throughout the study. In contrast, remarkable changes with regard to food choice could be observed. Before study entry all BH_4-sensitive patients followed a Phe restricted diet, having some characteristics of a vegan diet. Due to the partially very low Phe tolerance they mainly consumed fruit and vegetables. A further important share of total food consumption was spent on special low protein products, mainly low protein sausages, bread, pasta, rice, potato products. In addition, the patients consumed a relatively large amount of sweets (in particularly jelly beans), jelly and sweet powdered instant drinks. No differences compared to BH_4-resistant patients could be revealed.

From study period 2 onwards all BH_4-sensitive patients increased their consumption of foods with a higher protein content such as bread, pasta, rice and dairy products as well as meat, egg and fish. The mean fruit and vegetable consumption under BH_4 administration dropped compared to classical treatment, but no significance was found ($P = 0.274$ and $P = 0.139$, respectively). In contrast, the intake of edible fats as well as sweets and snacks remained stable. The consumption of special low protein products declined significantly.

Table 1 Phe concentration in dried blood, Phe and natural protein consumption of the patients throughout the study

				Phe concentration (µmol/l) in dried blood (mean ± SD)				Phe decrease on day 42 (%)	Phe (mg/day) and *natural protein (g/day)* consumption (mean ± SD)				Increase of Phe intake on day 42 (%)	Increase of Phe intake on day 90 (%)	DACH RDA (g/day)
			Mean-Age *(range)*	**SP1**	**SP2**	**SP3**	**SP4**	**SP3 vs SP2**	**SP1**	**SP2**	**SP3**	**SP4**	**SP3 vs SP1**	**SP4 vs SP1**	
All patients ($n = 14$)			10 *(4–16)*	364 (169)	516 (241)	420 (245)	440 (204)	**25.8**	550 (378) –	1098 (611) –	1099 (611) –	1442 (1153) –	112.4	164.1	
	ID	**Sex**	**Age (years)**												
BH_4-sensitive patients ($n = 8$)	2	m	6	100 (88)	273(75)	225 (109)	215 (44)	17.6	351 (4) *10.5 (1.2)*	1062 (179) *24.7 (4.6)*	1103 (143) *24.3 (2.8)*	1039 (87) *23.7 (2.2)*	214.1	195.8	18
	5	m	16	215 (42)	233(39)	131 (46)	198 (49)	43.7	733 (21) *18.9 (2.4)*	1739 (318) *37.5 (11.6)*	1526 (244) *35.8 (5.2)*	3048 (641) *69.6 (16.5)*	108.1	315.7	60
	8	m	8	201 (58)	231(55)	158 (37)	186 (36)	31.6	657 (64) *16.0 (2.9)*	1360 (39) *32.0 (2.1)*	1521(206) *34.9 (5.6)*	3162 (355) *69.0 (7.3)*	131.5	381.0	24
	9	f	16	520 (133)	618(61)	386 (103)	487 (183)	37.5	432 (23) *11.4 (0.5)*	1557 (643) *35.8 (12.5)*	1556 (607) *34.4 (13.4)*	2851 (1245) *65.3 (29.4)*	260.0	559.6	46
	14	f	8	203(48)	269(88)	163 (23)	164 (47)	39.4	385 (2) *9.6 (0.5)*	787 (9) *17.9 (1.1)*	790 (48) *17.9 (1.5)*	1566 (132) *34.8 (2.7)*	105.3	306.8	24
	24	f	11	278 (122)	375 (195)	259 (68)	377 (126)	39.9	382 (10) *9.3 (0.1)*	968 (85) *22.3 (2.3)*	1019 (44) *23.4 (1.1)*	1298 (18) *29.1 (2.2)*	167.1	240.1	35
	25	m	5	277(98)	427 (165)	199 (74)	308 (59)	53.4	342 (47) *8.6 (0.9)*	728 (60) *16.8 (1.2)*	759 (61) *17.6 (1.8)*	704 (33) *16.0 (1.1)*	121.8	105.8	18
	26	f	15	475(54)	589(52)	381 (88)	498 (99)	35,3	1750 (0) *38.8 (0)*	2777 (477) *88.9 (9.9)*	2810 (826) *88.0 (20.5)*	3380 (249) *76.2 (5.3)*	60.8	93.2	46
Total mean (SD)				**283(145)[a]**	**376(157)[a]**	**238(99)[a]**	**304(136)**	**37,3**	**629(476)[b]**	**1372(679)[b]**	**1386(661)[b]**	**2131(1084)[b]**	**146.1**	**274.8**	**n.a.**
MANOVA (P)					**0.018***					**0.006***					

Abbreviations: *SP* study period, *SP 1* Evaluation of patients (neonatal BH_4 test, Phe tolerance), *SP 2* Phe challenge, *SP 3* Phe challenge + BH_4 intake, *SP 4* Follow-up, *DACH-RDA* regular daily allowance, recommended for the German speaking countries (DACH 2000), *n.a.* not applicable, *f* female, *m* male

[a] Significant difference between period 1 and 2 ($P = 0.016$) and period 2 and 3 ($P = 0.007$)

[b] Significant difference between period 1 and 2 ($P = 0.001$); period 1 and 3 ($P = 0.001$) and period 1 and 4 ($P = 0.009$)

* Significant effect of "time" yielded by Wilk´s multivariate analysis of variance (MANOVA)

Macronutrient Intake of BH_4-Sensitive Patients

Under classical treatment (study period 1) as well as in study periods 2 and 3 all BH_4-sensitive patients regularly took an AAM. During follow-up six of the eight BH_4-sensitive patients could end any AAM supply. In the other two BH_4-sensitive patients the dosage could be reduced.

Under classical treatment (study period 1) total protein intake (sum of synthetic protein from AAM and intact protein from natural food) was markedly above the recommended range (165 ± 38 %). Total protein intake remained stable throughout the study, but the proportions of synthetic and natural protein changed. In study period 1 the intake of synthetic protein from AAM was 120 ± 28 % compared to 45 ± 13 % from intact natural protein of the current recommendation (DACH 2000). By doubling the Phe intake in study periods 2 and 3 the patients ingested twice as much natural protein and further increased this amount during follow-up. Under BH_4 therapy during follow-up, the total protein intake was similar to that of classic treatment ($P = 0.292$). However, the patients now consumed 142 % of the recommended protein as intact protein from natural foods and only 12 % as synthetic protein from AAM.

Under classical treatment BH_4-sensitive patients showed a carbohydrate intake below the current recommendations (93 ± 22 %; DACH 2000), which even further decreased during the other study periods (during follow-up 70 ± 18 %, $P = 0.032$). The mean fat intake was stable over the course of the study, but lower than the recommendations (81 ± 25 % throughout the study).

Micronutrient Intake of BH_4-Sensitive Patients

Figure 3 shows the supply of selected micronutrients of BH_4-sensitive patients under BH_4 therapy (study period 4) as well as under classical treatment. In addition the data are compared to data from aged-matched healthy German children (Mensink et al. 2007).

Under the Phe restricted diet (study period 1) overall micronutrient intake except for vitamin C was adequate. The highest percentage of vitamins and minerals derived from AAM and only a small amount from natural food. During study periods 2 and 3, mean total supply of vitamin D, vitamin B_{12}, iron, calcium, iodine and zinc remained stable and no significant differences occurred compared to study period 1 as the patients still consumed the original dose of AAM.

In patients who reduced or stopped AAM intake during follow-up (study period 4), the micronutrient supply dropped markedly below the current recommendations (DACH 2000) and deteriorated compared to the classic treatment. The differences were significant for vitamin D, iron, calcium, iodine and zinc. Considering the vitamin C content of Kuvan® (5 mg vitamin C per 100 mg sapropterin dihydrochloride), total vitamin C supply did not differ between study periods 1 and 4 ($P = 1.0$). However, due to a reduced fruit and vegetable intake, BH_4-sensitive patients consumed a lower amount of vitamin C from natural foods compared to classical dietary treatment.

Comparing the results to data from age-matched healthy German children (Mensink et al. 2007) several differences could be noted (Fig. 3). In general, healthy German children aged 6 to 17 years almost met the recommendations for all investigated micronutrients except for vitamin D and iodine. Under classical dietary treatment, the BH_4-sensitive PKU patients showed a higher mean intake of vitamin D, iron, calcium and iodine, but a lower mean intake of vitamin C and vitamin B_{12}. Under BH_4 treatment the supply of almost all micronutrients proved to be markedly lower compared to the healthy German children.

Discussion

The study presented here investigated metabolic control and eating habits, including food and nutrient supply, of BH_4-sensitive PKU patients. BH_4-sensitive patients could increase their Phe tolerance by 100 % or more while dried blood Phe concentrations remained within the therapeutic range. In comparison to the classical dietary treatment the patients consumed more protein rich natural foods. In six of the BH_4-sensitive patients the AAM administration could be stopped. Two of the BH_4-sensitive patients further needed some AAM. While mean total protein intake did not change compared to the classical dietary treatment, the mean intake of carbohydrate, energy and also of some micronutrients declined and dropped below the current recommendations (DACH 2000).

Under the new therapeutic regime, the BH_4-sensitive patients could increase their Phe tolerance and consumed more protein rich foods. In contrast, the markedly declined fruit and also the slightly reduced vegetable intake must be considered as critical, since they are important sources of vitamins, minerals, trace elements, phytochemicals and dietary fibre. Several factors may explain this phenomenon. Due to the increased consumption of protein rich food, patients may feel satisfied earlier, reducing their desire for more food. Furthermore, the classical Phe restricted diet quite likely does not reflect the true food preferences of our patients.

In general, factors influencing food choice are very complex, including socio-economic components as well as psychological aspects (Zabinski et al. 2006; Elfhag et al. 2008). Moreover, parents´ eating habits are an important factor influencing their children's choice of healthy or less healthy foods (Kremers et al. 2003; De Bourdeaudhuij et al. 2008).

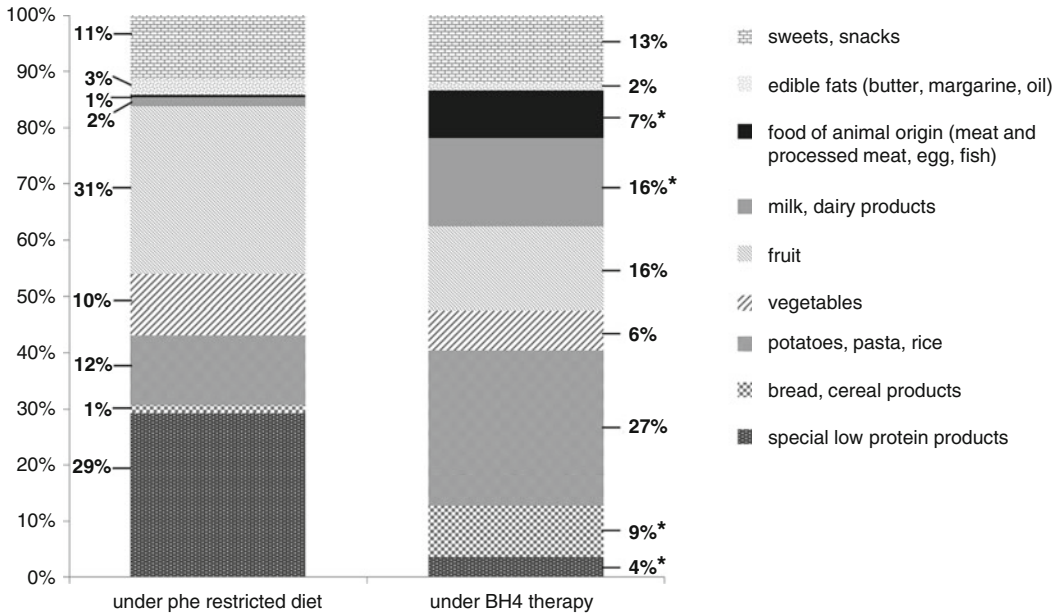

Fig. 2 Food consumption of BH_4-sensitive patients on classical dietary treatment (*left bar*) compared to BH_4 therapy during follow-up (*right bar*). Shown are the shares of food groups (%) of total food consumption (*significant difference between period 1 and 4: bread ($P = 0.022$), dairy products ($P = 0.002$); potatoes, pasta and rice ($P = 0.021$), food of animal origin (meat, processed meat, fish, egg, $P = 0.016$); special low protein products ($P = 0.009$))

Interestingly, our patients did not realise their markedly decreased fruit consumption. They were only aware of and enjoyed the possibility to consume protein rich foods like meat or dairy products. Over a longer term these patients are at risk to adopt some of the bad eating habits of healthy German children (Mensink et al. 2007). The study period has certainly been too short to completely develop a new dietary regime, especially with regard to seasonal differences in food choice. Extending the study would have permitted the patients to slowly accustom to a liberalized nutrition. However, this requires a high adherence to the study protocol by the patients and it is associated with higher costs. Reevaluating the nutrient intake at least 6 months after introduction of BH_4 treatment and identifying the new correct Phe tolerance would be advisable.

The changed food choice of BH_4-sensitive patients resulted in changes of macro- and micronutrient supply. Under classical dietary treatment the patients` mean nutrient intake was still adequate except for fat, total energy and vitamin C. Under BH_4 therapy their supply of carbohydrates and most of the investigated micronutrients dropped below the recommended range (DACH 2000). The mean total protein intake, however, was sufficient and comparable to classical dietary treatment. This observation is in accordance with data from a recent study (Singh et al. 2010). Likewise, the fat supply did not change compared to classical dietary treatment, but stayed below the recommendations (DACH 2000). A low fat intake in PKU patients under Phe restricted diet has already been described (Schulz and Bremer 1995, Rohde et al. 2012). Many of the protein rich foods, which must be avoided in the PKU diet, are simultaneously sources rich in fat. This is especially true for meat, processed meat and dairy products. Although the BH_4-sensitive patients increased their consumption of these foods they did not meet the recommendation for fat supply.

Under BH_4 therapy the carbohydrate intake declined and dropped below the current recommendations (DACH 2000). Taken together, the BH_4-sensitive patients did not meet the recommendations for the energy supply in healthy children (DACH 2000). Although none of the patients was underweight or growth retarded, a regular examination of energy – and macronutrient supply is strongly recommended, in particular when switching from classical dietary treatment to BH_4 therapy with a liberalized diet.

During the Phe restricted diet (study period 1) some of the patients showed a vitamin C supply slightly below the recommended range (DACH 2000). None of them took an AAM containing vitamin C. As a consequence of reduced fruit and vegetable consumption under the BH_4 treatment, the vitamin C supply of our patients further declined. Only taking into account the vitamin C content of Kuvan®, there were no differences compared to study period 1. However, a special supplementation of vitamin C seems not to be necessary for PKU patients, as the most fruit and vegetables, the main sources for vitamin C, are low in protein and could be consumed in adequate amounts. Recent studies even showed that free consumption of fruits

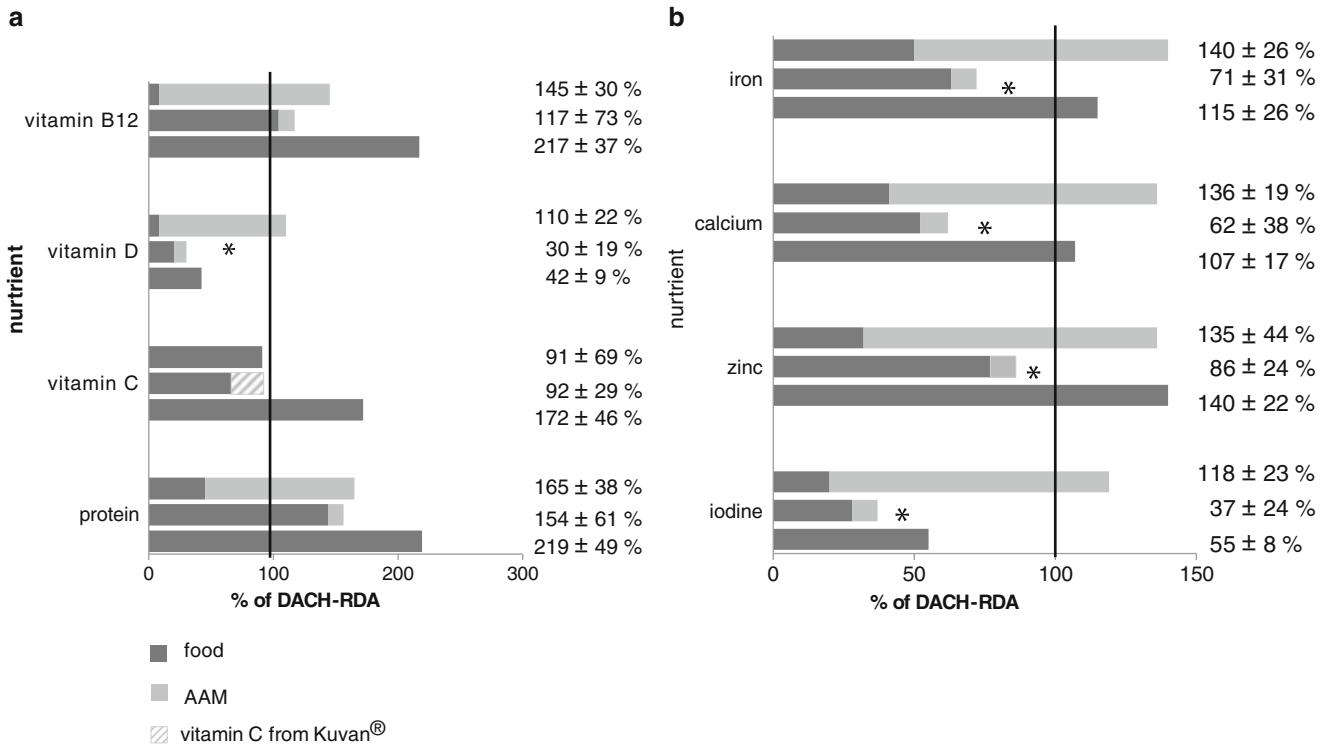

Fig. 3 (**a**–**b**) Protein and micronutrient supply of BH_4-sensitive patients ($n = 7$) under Phe restricted diet (*upper bar*) and under BH_4 therapy during follow-up (*middle bar*) compared to age-matched healthy German children (*lower bar*) as percent of DACH-RDA. Shown are the shares of food and amino acid mixture (AAM) of the total nutrient supply. The vitamin C content of Kuvan® was included into the calculation. Under the Phe restricted diet all these patients took an amino acid mixture while under BH_4 therapy only two patients took some (* *significant differences in total micronutrient supply in BH_4-sensitive patients under Phe restricted diet compared to BH_4 therapy: $P_{vitamin\ D} = 0.016$; $P_{iron} = 0.002$; $P_{calcium} = 0.017$; $P_{iodine} = 0.005$, $P_{zinc} = 0.046$)*

and vegetables does not impair the metabolic control in PKU patients (MacDonald et al. 2003; Rohde et al. 2012; Zimmermann et al. 2012). Hence, independent from the therapeutic regimes (Phe restricted diet + AAM alone or in combination with BH_4) it should be the objective to encourage the patients to eat more fruits as well as vegetables, rather than supplementing vitamin C.

Following the stopped or reduced AAM administration, the patients also showed an insufficient supply of some other micronutrients, in particular of vitamin D, iron, calcium, iodine and zinc. With respect to the long-term outcome of this group of patients, an insufficient micronutrient supply should be avoided. This might be the reason why some specialized centres still supply BH_4-sensitive patients with a relatively high amount of AAM despite the fact that their pure protein supply from natural food is adequate (Singh et al. 2010). It should be remarked that the micronutrient content of all AAM is calculated according to a very limited Phe tolerance. A reduction of AAM supply under BH_4 treatment without adapting eating habits therefore bears the risk of an insufficient micronutrient supply. On the other hand overdosing AAM in order to supply adequate amounts of micronutrients may lead to obesity. One could argue that even healthy German children do not reach the currently recommended intake of several micronutrients, especially vitamin D. However, their micronutrient intake is still above that of the investigated children with PKU. In addition vitamin D deficiency continuous to be a common problem among otherwise healthy children. Endogenous production, cleavage by sunlight and further enzymatic modification into active vitamin D_3 are not satisfactory to prevent rickets. This even led to compulsory vitamin D_3 supplementation of dairy products in some countries (Unuvar and Buyukgebiz 2010).

As a consequence a micronutrient supplement not containing any protein, carbohydrate or fat should be developed for these patients. Unfortunately specifically developed supplements are only available in some countries. Alternatively, the intake of an existing vitamin and mineral supplement for healthy children is recommended at least for the period until eating habits change completely towards an adequate supply from natural food.

In conclusion, the results of the study confirm that some patients benefit from a BH_4 therapy. Their Phe tolerance increases markedly, allowing a relaxed diet containing more protein rich food. Furthermore, BH_4-sensitive patients do no longer need special low protein products. Nevertheless, BH_4 supplementation with a relaxed diet currently bears the risk of an imbalanced nutrition. Especially, with respect to nutrition related diseases the potential micronutrient defi-

ciency needs consideration. Supplementation may become necessary. Nutritional education seems to be important for PKU patients under a BH_4 therapy as the new dietetic regime contradicts almost everything they were taught before. To prevent deterioration of eating habits a close follow-up of these patients by dieticians is recommended. Long-term multicenter settings with a higher sample size are necessary to further investigate the nutrient supply under BH_4 therapy and to satisfy the dietary requirements of this special group of patients in the future.

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Take Home Message

Although BH_4 therapy is an effective and helpful additional treatment in some patients with PKU, changes in eating habits and consequently insufficient supply of micronutrients has to be judged critically.

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RESEARCH REPORT

Effects of Switching from Agalsidase Beta to Agalsidase Alfa in 10 Patients with Anderson-Fabry Disease

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Abstract Anderson-Fabry disease (AFD) is a multiorgan X-linked lysosomal storage disease that particularly affects the heart, kidneys, and cerebrovascular system. Current treatment is enzyme replacement therapy (ERT) with agalsidase beta (Fabrazyme®, Genzyme Corporation, Cambridge, MA, USA) or agalsidase alfa (Replagal®, Shire Human Genetic Therapies AB, Lund, Sweden). It was recommended that patients switch to agalsidase alfa due to a manufacturing shortage of agalsidase beta beginning in June 2009. This study assessed the effect of switching to agalsidase alfa on clinical outcomes in patients with AFD previously treated with agalsidase beta. Ten patients (seven male, three female) with genetically confirmed AFD and at least 48 months' continuous data collected during treatment with agalsidase beta 1 mg/kg every other week were switched to agalsidase alfa 0.2 mg/kg every other week for at least 20 months, with prospective clinical evaluations every 6 months. Pre-switch data was collected retrospectively from patient charts. Cardiac functional parameters were assessed using magnetic resonance imaging. Results showed that renal function was normal (estimated glomerular filtration rate $\geq$90 mL/min/1.73 m^2) in 8 of 10 patients prior to agalsidase alfa and generally remained stable after the switch. Cardiac mass decreased significantly ($p < 0.05$ vs pre-ERT) after agalsidase beta and remained unchanged after switching to agalsidase alfa. Symptoms of pain and health status scores did not deteriorate during agalsidase alfa therapy. Adverse events were mostly mild and infusion related. In conclusion, switching to agalsidase alfa was relatively well tolerated and associated with stable clinical status and preserved renal and cardiac function.

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Introduction

Anderson-Fabry disease is an X-linked lysosomal storage disorder caused by a deficiency of the hydrolytic enzyme α-galactosidase A (α-Gal A). Consequently, globotriaosylceramide (Gb3) accumulates in cells and tissues of the body resulting in a multisystem pathology (Brady et al. 1967; Desnick et al. 2001), affecting the skin, nervous and cerebrovascular systems, kidneys, and heart, and is associated with a reduced life expectancy (Zarate and Hopkin 2008). Renal involvement is progressive, eventually leading to end-stage renal disease (ESRD) by the fourth and fifth decades of life if untreated; prior to the advent of dialysis and renal transplantation, ESRD was the leading cause of death (Branton et al. 2002). Cardiac manifestations include left ventricular (LV) hypertrophy, valvular disease, conduction abnormalities leading to arrhythmias, congestive heart failure, and coronary artery disease (Weidemann et al. 2005; Linhart and Elliott 2007; Kampmann et al. 2008; Linhart 2008; Morrissey et al. 2011).

Two recombinant enzyme formulations for enzyme replacement therapy (ERT) of Anderson-Fabry disease have been commercially available in Europe for almost

10 years: agalsidase alfa (Replagal®, Shire Human Genetic Therapies AB, Danderyd, Sweden) and agalsidase beta (Fabrazyme®, Genzyme Corporation, Cambridge, MA, USA) (Smid et al. 2011). Numerous clinical trials, observational studies, and registry data have provided some evidence for the safety and efficacy of ERT in improving disease symptoms, cardiac mass, renal function, and quality of life (Eng et al. 2001; Schiffmann et al. 2001; Weidemann et al. 2003; Wilcox et al. 2004; Banikazemi et al. 2007; Hughes et al. 2008; Koskenvuo et al. 2008; Imbriaco et al. 2009; Mehta et al. 2009; Feriozzi et al. 2012). To date, there have been limited comparisons of the two agents, from which no firm conclusion can be drawn regarding their relative efficacy and safety (Lidove et al. 2010).

As of June 2009, viral contamination in the agalsidase beta production facility resulted in a worldwide supply shortage of agalsidase beta (European Medicines Agency 2009). It was initially recommended by the European Medicines Agency (EMA) that priority patients continue on a reduced dosage of agalsidase beta (European Medicines Agency 2009) but this appeared to be associated with an increase in the adverse event rate, prompting the EMA to revise their recommendation about a year later to treatment with either the full dose of agalsidase beta (1 mg/kg every 2 weeks) or agalsidase alfa (0.2 mg/kg every 2 weeks) (European Medicines Agency 2010a, b). An expert consensus guidelines published in 2011 recommended a similar approach (Linthorst et al. 2011). Data on the effect of switching between the two therapies are limited to one observational study in 11 Japanese patients (Tsuboi and Yamamoto 2012) and a subgroup of 20 patients in a retrospective cohort Dutch study (Smid et al. 2011). Both reported clinical outcomes, including effects on cardiac abnormalities, but neither assessed cardiac effects via cardiac magnetic resonance imaging (cMRI).

We aimed to assess the effect of switching from agalsidase beta to agalsidase alfa on renal function, cardiac functional parameters (assessed via cMRI), health status, pain, and adverse events in patients with Anderson-Fabry disease by comparing retrospective data during treatment with agalsidase beta with prospective data during 20 months' treatment with agalsidase alfa.

Materials and Methods

Study Design

This was a single-center observational study conducted at the University Hospital Federico II, Italy, of patients with Anderson-Fabry disease who switched from treatment with agalsidase beta to agalsidase alfa. Data for the period during which patients received agalsidase beta was gathered retrospectively. Data collected at the time patients switched to agalsidase alfa and during agalsidase alfa treatment was gathered prospectively.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee. All patients switched to agalsidase alfa and included in the final study population provided written informed consent.

Patients

Patients eligible for study inclusion were those with a genetically confirmed diagnosis of Anderson-Fabry disease, at least 48 months of continuous data during treatment with agalsidase beta 1 mg/kg (i.e., prior to switching), those switched to agalsidase alfa after the start of the agalsidase beta shortage, and patients who provided written informed consent. Patients excluded from the study included those who had received agalsidase beta at other than the recommended dose for >12 months prior to the study or who had used any investigational drug within 30 days prior to study entry.

Treatments

Patients receiving agalsidase beta at a dosage of 1 mg/kg every other week for a minimum of 48 months were switched without any crossover period or interval to agalsidase alfa 0.2 mg/kg every other week, given according to the manufacturer's prescribing information. Patients received agalsidase alfa for a minimum of 20 months. Pretreatment with an antihistamine and/or ibuprofen was allowed to minimize infusion-associated reactions (IAR). Concomitant treatments such as antihypertensive agents were allowed.

Data Collection and Study End Points

Study endpoints included renal function, selected cardiac parameters, pain symptoms, and patient health status. The following clinical evaluations were performed at baseline, after 48 months of ERT with agalsidase beta, and again after 20 months ERT with agalsidase alfa: (1) physical examination, routine blood chemistries, hematology, and urinalysis; (2) serum creatinine levels and the mean estimated glomerular filtration rate (GFR), calculated by the Modification of Diet in Renal Disease (MDRD) study equation (Levey et al. 1999) on the basis of patients' age, race, sex, and serum-creatinine values; (3) proteinuria (estimated via the urine protein to creatinine ratio); (4) 12-lead electrocardiography (ECG); (5) cMRI was used to determine LV mass, LV wall thickness, and LV ejection fraction (see separate section for cMRI Techniques and

Analysis); (6) health status, evaluated with the Short Form-36 (SF-36) Health Status Survey (Ware 1997); and (7) pain score, evaluated with the short form McGill pain questionnaire (Melzack 1987). Use of concomitant medications, including pain medication and antihypertensive agents was recorded. In addition, α-Gal A enzyme activity was assessed at diagnosis (Desnick et al. 1973).

Data collected during treatment with agalsidase beta was obtained retrospectively from patient medical charts for the 48 months prior to the treatment switch. Retrospective data included all endpoints as described above for the prospective part of the study and, in addition, the results of a physical examination and cMRI prior to initiation of agalsidase beta.

cMRI Technique and Analysis

cMRI assessments were performed pre-ERT, after 48 months' agalsidase beta (before switching), and after 20 months' agalsidase alfa, using a 1.5 Tesla MRI system (Gyroscan Intera, Philips Medical System, Best, the Netherlands) equipped with high-performance gradients (maximum gradient amplitude 30 mT/m, maximum slew rate 150 mT/m/ms). Images were acquired with a 5-element cardiac phased-array coil using a vector cardiographic method for ECG gating and respiratory gating. After performing a survey scan, LV long axis and 4-chamber horizontal long axis images were acquired using a breath-holding 2D balanced turbo field echo multiphase-multislice sequence (TR/effective TE, 2.8/1.4; matrix, 160X256; slice thickness, 10-mm; flip angle, 50°); subsequently, biventricular short-axis images were obtained using 9–10 slices covering the left ventricle from the apex to the base for evaluation of LV mass. The total acquisition time ranged between 25 and 30 min for each assessment.

Post-processing was performed on a dedicated workstation (Viewforum, Philips Medical System, Best, the Netherlands). LV wall thickness was measured at the level of the mid-septum. Analysis of LV mass was performed choosing the slice with the greatest cardiac diameter of the 2D-balanced turbo field echo multiphase-multislice acquisition in the biventricular short axis; subsequently, the endocardial and the epicardial borders were manually traced, carefully including the papillary muscles, on each end-diastolic and end-systolic frame for each of the 9–10 slices.

The evaluation of late gadolinium enhancement (LGE), looking for areas of myocardial fibrosis, although not a goal of this study, was assessed and only in two cases were these additional MR sequences observed. In particular, a commercially available gadolinium-based contrast agent, gadodiamide (gadopentetate dimeglumine – Magnevist, Schering AG, Berlin, Germany; 0.15 mmol/kg), was injected intravenously at a dose of 0.1 mmol/kg of body weight, and 10–15 min after the injection, a segmented inversion-recovery fast-gradient echo sequence was acquired. LGE images were acquired in multiple short-axis views identical to those obtained for cine cardiac MRI. Finally, a visual estimation of LGE extent in LV horizontal, vertical long-axis, and short axis views was subsequently performed.

Safety

The safety of agalsidase alfa was assessed by recording all adverse events (AEs), which were assessed for their severity and relationship to the study drug.

Statistical Analysis

Data are mean ± standard deviation (SD) unless specified otherwise. Differences in LV parameters between pre-ERT and agalsidase beta treatment, and between agalsidase beta and agalsidase alfa treatment, were analyzed using a Student t-test for paired observations. Value of $p < 0.05$ was considered statistically significant.

Results

Patients

Of the 14 patients with Anderson-Fabry disease receiving agalsidase beta at our institution, 10 agreed to switch to agalsidase alfa, 2 patients opted for the reduced agalsidase beta dosage (0.3 mg/kg every other week) and 1 discontinued ERT. Ten patients (mean ± SD age 44 ± 5 years) with genetically confirmed Anderson-Fabry disease met study inclusion criteria and were enrolled (seven male, three female) into the prospective part of the study. A causal mutation/deletion was identified in all patients included in this study.

Renal Function

Renal function, assessed by serum creatinine levels and eGFR, remained stable in both male and female patients 24 months after switching treatment (Table 1). Individual eGFR values confirmed this finding, with no patient demonstrating clinically significant changes in eGFR after the ERT switch (Table 2). At entry to the evaluation, eGFR was normal (eGFR ≥90 ml/min/1.73 m^2) in 8 of the 10 patients, and remained normal during the 20-month follow-up. Two patients had elevated serum creatinine levels at the start of the switch but these levels did not worsen with agalsidase alfa treatment (data not shown). The median urinary protein to creatinine ratio was 0.96 (in mg protein/

Table 1 Selected study endpoints at pre-ERT, after 48 months' treatment with agalsidase beta and 20 months' agalsidase alfa. Data are mean ± SD values unless stated otherwise

	Pre-ERT	After 48 months' agalsidase beta	After 20 months' agalsidase alfa
Renal function			
eGFR (L/min/1.73 m^2)	92.4 ± 13.1	91.3 ± 14.9	90.3 ± 17.5
Median (range) eGFR (L/min/1.73 m^2)	98 (67–100)	97.5 (60–100)	96 (60–100)
Proteinuria			
Urinary protein to creatinine ratio	0.96 ± 0.29	0.36 ± 0.14	0.38 ± 0.07
Cardiac parameters			
LV mass (g/m^2)	106 ± 32	73 ± 24[a,b]	70 ± 24
LV wall thickness (mm)	16 ± 4	13 ± 4[a,b]	13 ± 3
LVEF[c] (%)	63 ± 4	65 ± 6	64 ± 6

eGFR estimated glomerular filtration rate; *LV* left ventricular; *LVEF*[c] LV ejection fraction, corrected; *SD* standard deviation

[a] $p < 0.05$ vs baseline

[b] $p > 0.05$ vs agalsidase alfa

[c] $p > 0.05$ baseline vs agalsidase beta vs agalsidase alfa

Table 2 Individual patient data for estimated glomerular filtration rate (GFR; L/min/1.73 m^2) at baseline and after 48 months' treatment with agalsidase beta and after 20 months' agalsidase alfa treatment

	GFR, L/min/1.73 m^2		
Patient	Pre-ERT	After 48 months' agalsidase beta	After 20 months' agalsidase alfa
1	100	100	98
2	68	68	65
3	67	60	60
4	100	100	100
5	98	98	96
6	98	96	95
7	97	97	97
8	98	96	96
9	98	98	96
10	100	100	100

mMol creatinine) pre-ERT, 0.36 after 48 months' agalsidase beta therapy (before switching to agalsidase alfa), and 0.38 after 20 months' agalsidase alfa therapy, indicating a stabilization in urine protein excretion. Individual patient data showed that those patients who experienced an increase in urine protein excretion had relatively high proteinuria at baseline (data not shown).

Cardiac Functional Parameters

Cardiac function (assessed by LV ejection fragment [LVEF]) remained unchanged after a switch from agalsidase beta to agalsidase alfa (Table 1); pre-ERT LVEF values did not change significantly after agalsidase beta or agalsidase alfa therapy. LV mass index and LV wall thickness decreased significantly ($p < 0.05$) from pre-ERT values after 48 months' treatment with agalsidase beta as shown in Table 1. These reductions were maintained when patients were switched to agalsidase alfa, as there were no significant differences in these parameters after 20 months' treatment with agalsidase alfa compared with values after 48 months' agalsidase beta treatment. Individual changes in left ventricular functional parameters measured at baseline after 48 months of therapy with agalsidase beta and after 20 ± 3 months of agalsidase alfa therapy are shown in Table 3.

No significant correlation was observed between the age of the patients and the variation in LV functional parameters ($p > 0.05$) with agalsidase beta or with agalsidase alfa treatment.

Five male patients showed signs of LV hypertrophy (LVH) before starting ERT with agalsidase beta. LVH improved after 48 months' therapy with agalsidase beta, and this remained at a stable level after 20 months' agalsidase alfa ERT. Figure 1 shows no changes in LV wall thickness and LV mass, between study 2 and 3 in a 50-year-old male patient with Anderson-Fabry Disease.

Quality of Life

For most components of the SF-36 questionnaire, there were no differences in mean scores after agalsidase alfa treatment compared with the pre-switch period (data not shown). No variations from pre-treatment through month 20 were seen for the components of Physical Functioning, Role Emotional, Body Pain, and Standardized Physical

Table 3 Left ventricular functional parameters measured at baseline (1), after 48 months of therapy with agalsidase beta (2) and after 20 ± 3 months of therapy with agalsidase alfa (3)

Pts ($n = 10$)	Age/Gender	LVMI (g/m^2)	LVMI (g/m^2)	LVMI (g/m^2)	LVWT (mm)	LVWT (mm)	LVWT (mm)
Time point		1	2	3	1	2	3
1	56/M	167	113	92	21	15	15
2	43/M	82	70	64	15	15	13
3	44/M	77	55	53	15	10	12
4	43/F	147	111	116	22	22	20
5	38/F	93	62	64	15	11	13
6	40/M	66	71	54	13	9	10
7	41/M	98	75	64	12	10	10
8	50/M	121	50	52	15	14	13
9	40/F	85	41	42	14	12	11
10	48/M	124	86	96	14	12	15

LVMI left ventricular mass index, *LVWT* left ventricular wall thickness

Fig. 1 Left ventricular short axis view in a 50-year-old male with Anderson-Fabry disease showing no changes in LV mass index and LV wall thickness between 48 months' ERT with agalsidase beta (50 g/m^2, 14 mm) and after 20 months' ERT with agalsidase alfa (52 g/m^2, 14 mm)

Component Scale; only small improvements were observed for the other components, although none reached statistical significance (data not included).

Pain Symptoms

Mean pain scores improved during agalsidase beta ERT and then remained stable (i.e., no worsening) during treatment with agalsidase alfa (data not inclued). Of the 10 patients, 8 had no pain at switching and remained pain free during treatment with agalsidase alfa; 2 patients were experiencing pain at the time of switching, but then improved slightly during the agalsidase alfa ERT period.

Tolerability

There were no reports of treatment-related atrial fibrillation, ventricular premature beats, tachyarrhythmia, cardiac failure, or other cardiac adverse events within 24 h of administration of ERT for every infusion given during the 20 months' agalsidase alfa ERT period. Six patients experienced at least one AE during treatment with agalsidase alfa. Most AEs were mild in nature and unrelated to treatment. The most common treatment-related AEs were IAR, consisting of rigors, temperature change sensations, fever, nausea, headache, vomiting, flushing, rhinitis, pruritus, and somnolence. Most IARs were assessed as mild, and the total number of patients who experienced an IAR markedly decreased over time (data not shown). There were no abnormal laboratory test values. No major AE were observed during treatment with agalsidase beta.

Discussion

Renal and cardiac function, pain symptoms and health status were largely unchanged when 10 patients with Anderson-Fabry disease switched from ERT with agalsidase beta to

agalsidase alfa for 20 months, suggesting patients maintained disease stability. The switch in treatment was generally well tolerated.

These results largely confirm those reported by Tsuboi et al. in a similarly designed study in 11 Japanese patients with Anderson-Fabry disease (Tsuboi and Yamamoto 2012). In their study, eGFR also remained stable when patients switched to agalsidase alfa. Unlike the current study, their echocardiographic findings suggested improvements in LV mass index and wall thickness after 12 months agalsidase alfa treatment compared with agalsidase beta treatment (Tsuboi and Yamamoto 2012). They reported a similar lack of increase in pain symptoms or deterioration in quality of life with the switch in treatment to the current study. In addition, the results of the current study also support those of a retrospective cohort study of the effects of the agalsidase beta shortage on patients with Anderson-Fabry disease, where 20 patients switched to agalsidase alfa, 18 of whom did so only after taking reduced-dose agalsidase beta, and 15 stayed on agalsidase beta but at a reduced dose (Smid et al. 2011). There was no increase in the clinical event incidence rate during the shortage (clinical events included neurological, renal, and cardiac events).

Most, but not all (Koskenvuo et al. 2008; Kovacevic-Preradovic et al. 2008), prior studies that investigated the effects of ERT (agalsidase beta or agalsidase alfa) on cardiac functional parameters reported improvements in cardiac parameters after initiation of ERT (Weidemann et al. 2003; Beck et al. 2004; Hughes et al. 2008; Vedder et al. 2008; Imbriaco et al. 2009). Results of the current study were in agreement with the majority of these studies, i.e., LV mass and wall thickness decreased significantly from pre-ERT values after initiation of ERT, which in this case was with agalsidase beta (retrospective data; see Table 1). In particular, Eng et al. (2001) demonstrated in a placebo-controlled, double-blind study that agalsidase beta (1 mg/kg) resulted in a histological clearance of Gb3 deposits in myocardial endothelial cells; this effect was sustained over 54 months of therapy (Germain et al. 2007). Furthermore, Weidemann et al. suggested that clearance of Gb3 by agalsidase beta leads to a regression of LV hypertrophy, which was documented by echocardiography and confirmed by MRI (Weidemann et al. 2003). Sustained treatment with adequate doses of enzyme is of particular importance as an in vitro study has shown that a relatively limited percentage of administered enzyme reaches the cardiac compartment (Ioannou et al. 2001). The current study did not measure plasma or myocardial Gb3 levels; thus, whether myocardial Gb3 deposits were reduced after sustained ERT for 48 months with agalsidase beta and then 20 months with agalsidase alfa in this study is not known. The Tsuboi et al. switch study did not measure myocardial Gb3 levels either, but their patients experienced an increase in plasma Gb3 levels after the switch from agalsidase beta to alfa treatment (Tsuboi and Yamamoto 2012). Nevertheless, patients still experienced reductions in LV mass after the switch. The authors queried the relevance of Gb3 levels as biomarkers of Fabry disease.

It has been recommended that patients receiving ERT are also prescribed angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB) to reduce the risk of proteinuria (Ortiz et al. 2008; Oqvist et al. 2009; Mehta et al. 2010; Warnock et al. 2010). In our study, seven patients were receiving ACEI and/or ARB therapy. Most of these patients started ACEI/ARB therapy at the outset of ERT. Some patients received both ACEI and ARB agents; the dosages of ACEI/ARB therapy and other agents that could affect kidney function and proteinuria were adjusted empirically, depending on individual baseline proteinuria levels, response to antiproteinuric therapy, and tolerance of the antihypertensive effects. Antiproteinuric therapy was continued throughout the study period. We ascribed the significant drop in urinary protein observed during the ERT period to concomitant use of ACEI/ARB therapy and ERT.

The present study has some potential limitations that should be considered, including the relatively small number of evaluated patients and the relatively short time of switching therapy. Further study in larger patient populations is warranted, and according to ClinicalTrials.gov, a switching study with a planned enrolment of 200 patients is under way in the USA (NCT01268241) (ClinicalTrials.gov. 2012). It is the nature of many studies of rare diseases that they are underpowered due to the difficulty in enrolling sufficient eligible patients within a reasonable time frame. However, underpowered studies for rare diseases are justified and ethical in that they allow clinical data to be amassed over time and these data can then be consolidated in meta-analyses, thereby increasing the power and strength of the findings and adding to the clinical evidence base.

Development of neutralizing IgG or IgE antibodies to agalsidase alfa or agalsidase beta were not determined in this study, although switching treatments is not expected to prevent IgG antibody formation or related adverse effects because of complete cross reactivity (Linthorst et al. 2004). Nevertheless, our study is the first MRI study reporting positive long-term effects of switching therapy ERT on cardiac performance in patients with Anderson-Fabry disease.

Conclusions

The results of the present study demonstrate that switching therapy from agalsidase beta to agalsidase alfa, the two products currently available for treatment of patients with

Anderson-Fabry disease, did not result in significant changes in renal function or cardiac functional parameters, pain symptoms or health status, and was generally well tolerated. Patients were able to maintain a clinically stable disease state after the switch to long-term agalsidase alfa treatment.

Appendix

Details of the Contributions of Individual Authors

AP was primarily involved in patient management, literature search, and preparation of manuscript. BV, IC, MS, and ER assisted in patient management and were involved in manuscript preparation. LS was involved in the literature search. GM performed the cMRI analysis. MI was involved in the literature search and manuscript preparation. All authors read and approved the final manuscript. The results presented in this chapter have not been published previously in whole or part, except in abstract form.

Guarantor

Dr. Antonio Pisani serves as guarantor for the chapter, accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

Competing Interest Statement

No authors at any time received payment or services from a third party for any aspect of the submitted work and they have nothing to declare.

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Details of Ethics Approval and Patient Consent

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee. All patients switched to agalsidase alfa and included in the final study population provided written informed consent.

Synopsis

Patients with Anderson-Fabry disease can be safely switched from long-term treatment with agalsidase beta to agalsidase alpha and maintain their health status, without any worsening of renal function, cardiac mass, or pain symptoms.

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RESEARCH REPORT

Molecular Genetics and Genotype-Based Estimation of BH4-Responsiveness in Serbian PKU Patients: Spotlight on Phenotypic Implications of p.L48S

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Abstract Phenylketonuria (PKU) is caused by mutations in the gene encoding phenylalanine hydroxylase (PAH) enzyme. Here, we report the updated spectrum of *PAH* mutations in 61 Serbian PKU patients. By using both DGGE/DNA sequencing and PCR-RFLP, we identified 26 disease-causing mutations (detection rate 99%). The most frequent ones were p.L48S (31%), p.R408W (16.4%), p.P281L (6%), p.E390G (5.2%), and p.I306V (5.2%). Homozygosity value indicated high heterogeneity of Serbian population.

To overcome possible pitfalls of patients' phenotypic classification, we used two parameters: pretreatment/maximal phenylalanine blood concentration and Phe tolerance. The two phenotypes did not match only for patients with p.L48S. Therefore, we used Mann-Whitney statistical test to compare pretreatment/maximal blood Phe concentration and Phe tolerance detected in patients with p.[L48S];[null] and p.[missense];[null] genotypes. For patients with p.L48S, our results implied that Phe tolerance is a better parameter for phenotypic classification. Also, Fisher's exact test was used to compare p.L48S effect on phenotype of homozygous and functionally hemizygous patients. Our findings showed that effect of p.L48S was altered in functional hemizygotes. Moreover, phenotypic inconsistency found in homozygotes suggested that interallelic complementation and/or additional factors play a role in genotype-phenotype correlation.

Since BH4-supplementation therapy is not available in Serbia, we made the first estimation of its potential benefit based on patients' genotypes. In the analyzed cohort, the total frequency of BH4-responsive mutations was 52.6%. Furthermore, we found a significant number of genotypes (26.2% BH4-responsive and 51% probably BH4-responsive) that may respond to BH4 therapy. This led us to a conclusion that BH4-supplementation therapy could bring benefit to Serbian PKU patients.

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Introduction

Phenylketonuria (PKU, MIM#261600) is the most severe form of hyperphenylalaninemia (HPA). It is a metabolic pathology that represents the paradigm of hereditary disease that can be treated. With the average incidence of 1 per 10,000 newborns, PKU is considered to be the most common inborn error of amino acid metabolism in Caucasians and one of the most studied rare diseases (Scriver et al. 2008).

Hyperphenylalaninemias are a result of deficient hepatic enzyme, phenylalanine hydroxylase (PAH, EC 1.14.16.1). PAH is responsible for the conversion of phenylalanine (Phe) into tyrosine, in the presence of the molecular oxygen and cofactor tetrahydrobiopterin (BH4). If this metabolic pathway is impaired, tyrosine becomes an essential amino acid

while the concentration of phenylalanine rises above normal. Deficiency of the PAH enzyme is caused by mutations in the phenylalanine hydroxylase gene (*PAH*, GenBank accession no. AF404777). *PAH* gene is mapped to human chromosome 12q24.1, it spans approximately 90 kb and contains 13 exons and 12 large introns (Scriver 2007).

PKU is a monogenic disease which is transmitted in an autosomal recessive pattern and the two mutations found in the patient's genotype stand as the main determinant of PKU phenotype (Kayaalp et al. 1997; Guldberg et al. 1998). The knowledge about mutations in the *PAH* gene and their effect on the phenotype is the best example of nutrigenetics – a new scientific discipline with a goal to develop an optimal diet based on genotype analysis (Guttler and Guldberg 2000; Fenech et al. 2011). Nevertheless, the PKU phenotype is not simple and there are numerous factors (genetic and nongenetic) that contribute to its complexity (Scriver and Waters 1999; Dipple and McCabe 2000).

More than 600 different mutations have been identified in *PAH* gene and recorded in the literature and the *PAH* locus knowledgebase (Scriver et al. 2003, http://www.pahdb.mcgill.ca). Depending on mutation type (missense, nonsense, splice site, small or large insertions and deletions) and position (regulatory, catalytic, or tetramerization domain), the effect of a mutation on the structure and activity of the PAH varies greatly. As a consequence, the activity of mutant protein ranges from 0% to almost 100% compared to normal PAH enzyme (Waters et al. 1998). Correspondingly, hyperphenylalaninemic phenotypes range from mild hyperphenylalaninemia (MHP) that does not require treatment to a classic PKU characterized by severe mental retardation and epilepsy in the absence of treatment. Furthermore, the effect of some mutations on PAH activity and PKU phenotype is steady and consistent through numerous in vitro and in vivo studies performed in different populations, while for others its effect is inconsistent and unpredictable. While mutations with a consistent effect enable accurate prediction of PKU phenotype based on genotype, PKU phenotype becomes more complex in case of mutations with an inconsistent effect.

For more than two decades, efforts had been made to fully understand the effect of mutations on PKU phenotype. However, there is ongoing need to further analyze mutations in genotype-phenotype correlation studies, particularly in the group of patients of identical genotype and genetic background.

Recently, a new therapeutic approach is becoming a valid option for the dietary treatment of PKU patients. It relies on the observation that pharmacological doses of the tetrahydrobiopterin (BH4), a natural cofactor of PAH enzyme, can lower blood phenylalanine concentration (Kure et al. 1999). Molecular mechanism of BH4 action is multifactorial, but mainly based on its chaperon-like activity which results in the stabilization of altered conformations of PAH enzyme. By binding to an unstable enzyme, BH4 protects it from degradation, prolongs its half-life and enables it to perform conversion of phenylalanine to tyrosine. (Erlandsen et al. 2004; Pey et al. 2004; Pérez et al. 2005). The BH4-supplementation therapy (Kuvan) can be used to loosen or even replace burdensome dietary treatment of PKU patients (Levy et al. 2007a; Trefz et al. 2009b). However, not all PKU patients are BH4 responsive, meaning that at the molecular level, not all mutated proteins benefit from the increased concentration of BH4. In order to understand which mutations are good candidates, many studies analyzed the enzyme activity of mutated enzymes in vitro in the presence of BH4 precursors (Kim et al. 2006; Aguado et al. 2007). Also, it was shown that PAH function and response to BH4 administration result from interplay between genotype, metabolic state, and cofactor concentration (Staudigl et al. 2011). Several studies analyzed the correlation of genotype and patient's responsiveness to BH4 and made an attempt to predict the BH4-responsiveness on the basis of genotype (Zurflüh et al. 2008; Karacic et al. 2009; Rivera et al. 2011; Sterl et al. 2012). In the Kuvan era, genotypization of patients became important, not only because of the definitive diagnosis and prediction of the optimal diet, but also to point out those patients that could benefit from new therapeutic approach.

BH4 loading test is a definitive diagnostic test for cofactor sensibility. However, estimation based on genotyping answers an important question in countries where BH4 is not an approved drug: Is the frequency of BH4 responsive mutations in a population high enough to consider giving opportunity to patients for a new therapeutic method?

The first study on Serbian PKU population was performed 6 years ago (Stojiljkovic et al. 2006). Here, we presented molecular and phenotypic characteristics of enlarged cohort of Serbian PKU patients with focus on phenotypic effect of the most frequent mutation, p.L48S (c.143T>C). We discussed possible pitfalls of phenotypic classification of PKU patients and genotype clustering that could influence genotype-phenotype correlation studies. Also, we assessed the potential benefit from BH4-supplementation therapy in Serbia.

Subjects and Methods

Patients and Phenotypic Classification

In this study, 61 patients with hyperphenylalaninemia from Serbia (comprising three pairs of siblings) were included. Also, 10 parent samples were analyzed in order to facilitate

and clarify mutation detection of a patient or to explain phenotypic status of a mother. Neonatal screening was established in Central Serbia in 1982, and in 2003 it was expanded to Vojvodina (Serbian Northern province). According to regional agreement, it was recommended to start with dietary regiment if the pretreatment phenylalanine level exceeded 360 μmol/l. Although most patients included in this study were detected by the newborn screening program, 16 children were diagnosed later in life during evaluation of psychomotor retardation or by routine measurement of blood Phe level at the Mother and Child Healthcare Institute "Dr Vukan Cupic" in Belgrade.

Clinical data collected include: pretreatment and maximal phenylalanine blood concentration, phenylalanine tolerance, IQ, age at the diagnosis, the application of low Phe diet, and other relevant data. The phenylalnine tolerance is the highest amount of Phe from food that a patient can tolerate to keep blood Phe concentrations in the safe range (120–360 μmol/l) (Guldberg et al. 1998). It is possible to reliably assess Phe tolerance already at the age of 2 years (van Spronsen et al. 2009).

In this study, patients were classified into three categories according to pretreatment/maximal phenylalanine blood level and according to phenylalanine tolerance: classical PKU (pretreatment Phe >1200 μmol/l; Phe tolerance <20 mg/kg/day – equivalent 250–350 mg/day), mild PKU (pretreatment Phe 600–1200 μmol/l; Phe tolerance 20–25 mg/kg/day – equivalent 350–600 mg/day), and MHP (pretreatment Phe <600 μmol/l; Phe tolerance >25 mg/kg/day – equivalent >600 mg/day) (Trefz et al. 1985; Guldberg et al. 1998). The pretreatment level is a quantitative blood/serum Phe measurement under standardized condition. Phe tolerance was determined in cooperation with the patient/parents or during hospitalization. Maximal Phe level is the highest pretreatment blood Phe concentration (μmol/L) or the highest Phe in the loading Phe test (180 mg/kg/day) if performed.

This study was approved by the Ethics Committee at the Mother and Child Healthcare Institute "Dr Vukan Cupic" and all parents and/or patients gave informed consent. They were further referred to the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, for molecular genetic analysis.

Molecular Genetic Analysis

Genomic DNA was extracted from peripheral blood by QIAamp DNA Blood Mini Kit. Mutations were identified by multiplex "broad range" denaturing-gradient gel electrophoresis (DGGE) (Guldberg and Guttler 1994) followed by DNA sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). When necessary, PCR-RFLP or PCR-ACRS analyses for several common point mutations (p.L48S (c.143T>C), p.R408W (c.1222C>T), p.R261Q (c.782G>A), p.R261X (c.781C>T), IVS10-11G>A (c.1066-11G>A), and p.R158Q (c.473G>A)) were also performed (Eiken et al. 1993).

We used referent sequence (GenBank accession no. AF404777) to identify changes in *PAH* gene and HGVS guidelines for nomenclature of genetic variants. Also, we used the *PAH* locus knowledgebase (PAHdb, http://www.pahdb.mcgill.ca) and literature found on PubMed to determine if a mutation has already been reported.

Results

Genotyping

This study included molecular characterization of 61 Serbian patients with hyperphenylalaninemia. Given that the cohort included three pairs of siblings, we analyzed the total number of 116 unrelated mutant alleles. On 113 mutant alleles we identified one disease-causing mutation, and, interestingly, on two unrelated mutant alleles we detected two disease-causing mutations. Since 115 out of 116 *PAH* alleles were characterized, the mutation detection rate in this study was 99%. High diagnostic efficiency was achieved by combining PCR-RFLP and DGGE/DNA sequencing analysis of *PAH* gene. Additionally, our genetic analysis established PKU diagnosis for two patients' mothers that have not been diagnosed before.

In this cohort of Serbian patients, we identified 26 different disease-causing mutations. Seven of them were not reported in the previous study on Serbian population. The frequency of the most typical Serbian mutation, p.L48S (c.143T>C), is 31%. Other significant mutations had the following frequencies: 16.4% of p.R408W (c.1222C>T), 6% of p.P281L (c.842C>T), 5.2% of p.E390G (c.1169A>G), and 5.2% of p.I306V (c.916A>G). These five mutations accounted for almost two thirds of all mutant alleles in the Serbian population. The remaining mutations are listed in Table 1. Calculated homozygosity value was expectedly low (0.14), confirming the mutational heterogeneity of *PAH* locus for Serbian population.

Genotypic homozygosity was observed in 12 unrelated patients for the following mutations: p.L48S, p.R111X, p.R243X, p.P281L, and p.R408W. Among them, there were 8 [p.L48S];[p.L48S] genotypes. Interestingly, homozygosity for p.I306V mutation was detected in two additional cases. However, presence of the third disease-causing mutation was also confirmed in these patients' genotypes: p.[R261Q; I306V]; [I306V] and p.[R408W; I306V]; [I306V].

Table 1 Spectrum and frequency of mutations causing PKU in Serbia

Mutations					
Trivial name	Systematic name	Exon	Number of chromosomes	Relative frequency (%)	Type of mutation
p.S16fs	c.47_48delCT	1	1	0.9	Frameshift
p.Q20X	c.58C > T	1	2	1.7	Nonsense
p.L48S	c.143T > C	2	36	31.0	Missense
p.R111X	c.331C > T	3	3	2.6	Nonsense
p.R158Q	c.473G > A	5	4	3.4	Missense
p.V177L	c.529G > C	6	3	2.6	Missense
p.V177M	c.529G > A	6	1	0.9	Missense
p.L213P	c.638T > C	6	1	0.9	Missense
p.P225T	c.673C > A	6	3	2.6	Missense
p.S231F	c.692C > T	6	1	0.9	Missense
p.R243X	c.727C > T	7	2	1.7	Nonsense
p.R252Q	c.755G > A	7	1	0.9	Missense
p.R261Q	c.782G > A	7	4	3.4	Missense
p.R261X	c.781C > T	7	2	1.7	Nonsense
p.P281L	c.842C > T	7	7	6.0	Missense
p.R297H	c.890G > A	8	1	0.9	Missense
p.I306V	c.916A > G	9	6	5.2	Missense
IVS10 + 3A > G	c.1065 + 3A > G	*	1	0.9	Splice site
IVS10-11G > A	c.1066-11G > A	*	2	1.7	Splice site
p.E390G	c.1169A > G	11	6	5.2	Missense
p.A403V	c.1208C > T	12	2	1.7	Missense
p.R408W	c.1222C > T	12	19	16.4	Missense
p.R413P	c.1238G > C	12	1	0.9	Missense
p.Y414C	c.1241A > G	12	2	1.7	Missense
p.P416Q	c.1247C > A	12	1	0.9	Missense
IVS12 + 1G > A	c.1315 + 1G > A	*	3	2.6	Splice site
Unspecified			1	0.9	
Total			116		

* Mutations affecting splice junction, located in flanking intron regions

PKU mutations were variously combined into 39 different genotypes, among which 33 genotypes were not present in more than one family. The relative frequency of unrelated genotypes that included p.L48S mutation was 48% (28/58).

Phenotypic Characterization

All PKU patients were assigned to phenotypic category according to pretreatment/maximal blood Phe concentration level. The analyzed cohort included 34 classical PKU (58.6%), 14 mild PKU (24.2%), and 10 MHP (17.2%) patients.

In the majority of cases, phenylalanine tolerance was used as an additional parameter for classification of PKU patients. We compared phenotype categories based on two different parameters and found that patients without p.L48S in the genotype had matching categories according to both parameters. However, for almost 20% of patients carrying p.L48S, phenotype category based on pretreatment/maximal blood Phe concentration was more severe than the phenotype obtained by Phe tolerance. In order to further analyze observed discrepancies, we listed detailed phenotype characteristics and other relevant data (e.g., start and compliance to the low Phe diet) for homozygous and heterozygous p.L48S patients (Table 2).

Furthermore, we used Mann-Whitney test to compare pretreatment/maximal blood Phe concentration and Phe tolerance for p.[L48S];[null] and p.[missense];[null] genotypes. We have found that patients with p.[L48S];[null] genotype had significantly higher pretreatment Phe concentrations ($p = 0.001$).

Table 2 Phenotypic characteristics of homozygous and heterozygous p.L48S patients

Patient number	Genotype	Max Phe levels (μmol/l)	Phe tolerance (mg/day)	Phenotype		IQ	Other data
				Max Phe levels	Phe tolerance		
17[1]	p.[L48S];[S231F]	>1200	230	cPKU	cPKU	20	LD
18[1]	p.[L48S];[S231F]	>1200	240	cPKU	cPKU	24	LD
10	p.[L48S];[P225T]	>1200	280	cPKU	cPKU	100	DBNS
5	p.[L48S];[I306V]	>1200	280	cPKU	cPKU	110	DBNS
9	p.[L48S];[R158Q]	>1200	280	cPKU	cPKU	85	DBNS
6	p.[L48S];[R158Q]	>1200	300	cPKU	cPKU	87,5	DBNS, BDC
21	p.[L48S];[R158Q]	>1200	300	cPKU	cPKU	90	DBNS
22	p.[L48S];[Q20X]	>1200	300	cPKU	cPKU	45	LD, LPD at the age of 3 years
2	p.[L48S];[R261Q]	>1200	300	cPKU	cPKU	50	LD, LPD at the age of 4 months, seizures
15	p.[L48S];[R408W]	>1200	300	cPKU	cPKU	84	DBNS, PCD
24	p.[L48S];[R408W]	>1200	300	cPKU	cPKU	97,5	DBNS
25	p.[L48S];[R408W]	>1200	340	cPKU	cPKU	100	DBNS
4	p.[L48S];[R408W]	>1200	350	cPKU	cPKU	102,5	DBNS
11	p.[L48S];[R408W]	>1200	350	cPKU	cPKU	105	DBNS
12	p.[L48S];[R408W]	>1200	500	**cPKU**	**mPKU**	110	LD, LPD, from 22 months to 5 years
7	p.[L48S];[P281L]	>1200	350	cPKU	cPKU	60	LD, LPD at the age of 20 months
1	p.[L48S];[P281L]	>1200	400	**cPKU**	**mPKU**	59	LD, LPD at the age of 6 years
23	p.[L48S];[L48S]	>1200	310	cPKU	cPKU	77,5	LD, LPD at the age of 1 year
8	p.[L48S];[L48S]	>1200	330	cPKU	cPKU	80	DBNS, BDC
19	p.[L48S];[L48S]	>1200	>600	**cPKU**	**MHP**	84	LD, never on LPD
20	p.[L48S];[L48S]	>1200	>600	**cPKU**	**MHP**	82,5	LD, never on LPD
13[2]	p.[L48S];[L48S]	1200	450	mPKU	mPKU	115	DBNS, LPD at the age of 11 months
14[2]	p.[L48S];[L48S]	1200	450	mPKU	mPKU	110	DBNS, LPD at the age of 11 months
3	p.[L48S];[L48S]	1200	450	mPKU	mPKU	85	DBNS, refused LPD
26	p.[L48S];[L48S]	900	550	mPKU	mPKU	100	DBNS never on LPD
16	p.[L48S];[V177L]	600	500	**MHP**	**mPKU**	95	DBNS
27	p.[L48S];[V177M]	300	/	MHP	/	100	DBNS

Maximal Phe level is the highest Phe pretreatment blood concentration (μmol/L) or the highest Phe in the loading Phe test (180 mg/kg/day) if performed; cPKU – classical PKU; mPKU – moderate PKU; MHP – mild hyperphenylalaninemia; DBNS – diagnosis by neonatal screening; LD – late diagnosis; LPD – low Phe diet; PCD – poorly control diet; where it was not stated differently, LPD was introduced at the age of 1 month; patients 24–27 are under 2 years of age and Phe tolerance value could be revised later; two pairs of siblings are indicated by superscripts 1 and 2; differences between two phenotype categories based on different parameters are in bold

Genotype-Phenotype Correlation Study

Genotype-phenotype correlation study was performed for patients homozygous for the mutation or functionally hemizygous patients (the mutant allele is combined with null mutation and therefore acts on its own). Nonsense, frameshift, and splice site mutations, as well as missense mutations which resulted in zero enzyme activity in vitro, were considered as null mutations (Kayaalp et al. 1997; Guldberg et al. 1998). Patients with three mutations in the genotype were not taken into account.

The most frequent mutation, p.L48S, is known to have an inconsistent phenotypic outcome (Guldberg et al. 1998). To avoid the effect attributable to interallelic interaction, we separately analyzed genotype-phenotype correlation for 8 p.[L48S];[L48S] and 11 p.[L48S];[null] patients. Also, we analyzed genotype-phenotype correlation when phenotypes were classified according to pretreatment blood Phe concentration and according to Phe tolerance. We used Fisher's exact test to compare the effect of p.L48S on phenotype in homozygous versus functionally hemizygous patients. We found that distribution of phenotypes significantly varied between two genotype groups ($p = 0.018$ and $p = 0.039$, respectively). Interestingly, when functionally hemizygous p.L48S genotypes were correlated with phenotypes determined according to pretreatment blood Phe concentration, these genotypes were associated with classical PKU in 100% cases. On the other hand, p.L48S homozygotes were associated with all three phenotype categories determined according to Phe tolerance.

We also analyzed the effect of several other missense mutations found in functional hemizygotes. We found that p.P225T, p.R261Q, and p.R413P mutations were associated with classical PKU, p.V177L and p.R297H with mild PKU, while p.I306V and p.A403V were associated with MHP. The inconsistency was noticed for p.E390G mutation which was found in patients with one classical PKU, three mild PKU, and one MHP phenotype.

BH4 Responsiveness

The p.L48S mutation as well as seven others (p.R158Q, p.R261Q, p.I306V, p.E390G, p.A403V, p.R413P, and p.Y414C) were characterized as BH4-responsive ones in previous European studies (Zurflüh et al. 2008; Trefz et al. 2009a; Karacic et al. 2009). Accordingly, the sum of relative frequencies of BH4-responsive mutations for Serbian population is 52.6%.

However, some mutations have the status of consistently BH4-responsive while others are considered as inconsistently responsive. According to the classification given in Sterl et al. (2012), we classified all 39 Serbian *PAH* genotypes into BH4 responsive (26.2%), probably BH4 responsive (51%), and non-BH4 responsive (22.8%) (Table 3). Furthermore, we made distinction between genotypes with one and two BH4-responsive alleles.

Discussion

This is an update study on molecular genetics of Serbian hyperphenylalaninemic patients and the first study that estimates potential of BH4-supplementation therapy based on patients' genotypes. Furthermore, we conducted genotype-phenotype correlations to analyze phenotypic effect of mutations.

Unlike recent studies conducted in European PKU patients, all mutations identified in this cohort of patients have already been reported in the literature (Sterl et al. 2012; Groselj et al. 2012). However, the mutational spectrum was enlarged with seven mutations that were not reported in the previous study on Serbian population. Furthermore, five mutations accounted for almost two thirds of all mutant alleles and the homozygosity value was low. All these findings indicated rather high heterogeneity of Serbian population. As it was stated previously, genetics of *PAH* locus is concordant with historically documented migrations across the Western Balkans (Stojiljkovic et al. 2006).

Mutation p.L48S reassured its status as the most frequent in Serbia. Interestingly, its relative frequency (31%) is the highest ever reported in any population. This mutation was initially detected in a Turkish patient with phenylketonuria (Konecki et al. 1991). Since then it has been identified in many European populations usually with a frequency of 2–5% (Zschocke 2003). Higher frequencies have been found in Croatian (10%), Italian (9.7%), and Turkish (7%) populations which are geographically and historically interconnected with the Serbian population (Giannattasio et al. 2001; Karacic et al. 2009; Dobrowolski et al. 2011). In the preliminary haplotype study of patients with p.L48S, it was shown that the mutation was imported to Serbia from populations with different genetic backgrounds (Stojiljkovic et al. 2007).

Mutation p.R408W remains the second frequent Serbian mutation. It is the most prevalent mutation worldwide, which is associated with two different haplotypes and in Slavic populations it follows the cline distribution from Baltic to Mediterranean countries (Zschocke 2003). Since its frequency is 84% in Estonia, 46% in Slovakia, and 36% in Croatia, detected frequency in Serbia (16.4%) fits into the established gradient distribution. Mutations p.P281L and p.E390G are relatively common in Balkans, but rare in other European populations. Thus, p.P281L was found on 10% alleles in Greece, 8.4% in Turkey, 8% in Croatia, and 6% in Serbia, while p.E390G accounted for 7% of alleles in Croatia, 5.2% in Serbia, and 4.1% in Turkey (Zschocke

Table 3 Genotype-based estimation of BH4-responsiveness in Serbian PKU patients

BH4 responsive		Probably BH4 responsive		Non-BH4 responsive
R + IR	R + N	IR + IR	IR + N	N + N
26.2%		51%		22.8%
7%	19.2%	21%	30%	22.8%
p.[I306V];[L48S] (2)	p.[I306V];[R261X] (1)	p.[L48S];[L48S] (8)	p.[L48S];[Q20X] (1)	p.[R408W];[V177L] (1)
p.[I306V];[R261Q] (1)	p.[I306V];[R408W] (1)	p.[L48S];[R158Q] (3)	p.[L48S];[V177L] (1)	p.[R408W];[P225T] (1)
p.[E390G];[R158Q] (1)	p.[I306V];[P416Q] (1)	p.[L48S];[R261Q] (1)	p.[L48S];[V177M] (1)	p.[R408W];[R252Q] (1)
	p.[E390G];[R261X] (1)		p.[L48S];[P225T] (1)	p.[R408W]; [IVS10 + 3A > G] (1)
	p.[E390G];[P281L] (1)		p.[L48S];[S231F] (1)	p.[R408W];[R408W] (1)
	p.[E390G];[R408W] (3)		p.[L48S];[P281L] (2)	p.[P281L];[P225T] (1)
	p.[A403V];[R408W] (1)		p.[L48S];[R408W] (7)	p.[P281L];[P281L] (1)
	p.[Y414C];[IVS10- 11G > A] (1)		p.[R261Q];[L15fs] (1)	p.[P281L]; [IVS12 + 1G > A] (1)
	p.[Y414C];[L213P] (1)		p.[R261Q]; [IVS12 + 1G > A] (1)	p.[R111X];[R111X] (1)
			p.[R413P];[R408W] (1)	p.[R111X];[IVS10- 11G > A] (1)
				p.[V177L];[Q20X] (1)
				p.[R243X];[R243X] (1)
				p.[R297H]; [IVS12 + 1G > A] (1)

PAH mutations are designated as: R – responsive, IR – inconsistently responsive, and N – nonresponsive/unknown to be responsive according to BH4 responsiveness reported in previous studies (Muntau et al. 2002; Zurflüh et al. 2008; Trefz et al. 2009a; Karacic et al. 2009; Sterl et al. 2012) and according to BIOPKU db. Between parentheses is the number of the same genotypes detected in Serbian PKU patients. Genotype with one unidentified mutation was excluded from the table

2003; Karacic et al. 2009; Dobrowolski et al. 2011). Although mentioned frequencies are quite similar, we could observe gradient from southeast to northwest for p.P281L and the inverse for p.E390G.

Interestingly, we found two unrelated patients with three PKU mutations. Although it is rarely found that a PKU patient carries three mutations, our finding highlights the need to screen the complete *PAH* gene in order to accurately define PKU genotype. It is possible to imagine that incomplete genotyping could also lead to inaccurate conclusions regarding genotype-phenotype correlation.

Genotype-Phenotype Inconsistencies

Analysis of mutation's effect, based on genotype-phenotype correlation, could encounter a problem of inadequate phenotype classification. Up to date, two distinct parameters have been widely used for phenotype classification. The first parameter, pretreatment blood Phe level was quite helpful in the past and was convenient because it was known for every diagnosed patient. However, this parameter corresponded mostly to the screening Phe level. The second one, phenylalanine tolerance, depicts more realistically the ability of a patient to metabolize Phe from the food. Unfortunately, there are no universal guidelines which parameter should be used for classification of HPA patients. In order to overcome possible pitfalls of patients' phenotypic classification, we used both parameters.

Among the most frequent mutations in this study, we found two null mutations (p.R408W and p.P281L) and two missense mutations (p.L48S and p.E390G) known to have inconsistent phenotypic effect. Therefore, Serbian cohort was suitable and interesting for investigation of genotype-phenotype correlation in homozygous and functionally hemizygous patients. Given that the inconsistency noticed for p.E390G mutation was found in a small number of patients, we further focused on p.L48S mutation. It is worth noting that other missense mutations, analyzed in genotype-phenotype correlation, showed consistent effects which were already known in the literature.

Our results from Mann-Whitney statistical test for patients carrying p.L48S implied that categorization based on Phe tolerance instead of pretreatment blood Phe concentration is particularly important for patients carrying

p.L48S mutation. Furthermore, Fishers's exact test suggested that the effect of p.L48S mutation is more reliably determined in the correlation of homozygous genotypes and phenotypes based on Phe tolerance. In this study, the majority of p.L48S functionally hemizygous patients had classical PKU phenotype. However, when p.L48S mutation was found in the homozygous form, its inconsistent nature susceptible to different influences became obvious. Therefore, we could conclude that the real effect of p.L48S was masked if the mutation was combined with a null mutation.

For some mutations, such as p.I65T, inconsistency could be explained by interallelic complementation between different subunits of heterotetrameric PAH (Leandro et al. 2006; Leandro et al. 2011). This mutation is frequent in Western European populations (Spanish, Portuguese, French, British, and Norwegian) while it is not frequent in Slavic populations (Zschocke 2003). It is worth mentioning that both mutations (p.L48S and p.I65T) in a homozygous state tend to produce a milder degree of PKU than when associated with a null allele, demonstrating that each of the two mutations encode residual PAH activity (39% and 26.5%, respectively). Although p.L48S has not been studied for interallelic complementation effect, comparison between homozygous vs. functionally hemizygous Serbian patients showed that p.L48S residual activity (39%) was decreased in interaction with null residual activity PAH subunit. Thus, our finding implies existence of negative interallelic complementation in functionally hemizygous p.L48S patients.

Interallelic complementation is only one of the possible explanations for mutation's inconsistency. Phenylalanine concentration in the peripheral circulation is controlled by various genetic loci and modifying factors at least at two different levels (Scriver and Waters 1999; Dipple and McCabe 2000). At the cellular level, a variant PAH enzyme with disturbed stability is susceptible to enhanced degradation. However, if there is a flaw in proteolytic degradation system or a defect in chaperones, both of which could be genetically determined, it would to some extent have influence on the blood Phe concentration. At the level of the whole organism, blood Phe concentration could be influenced by alterations in Phe absorption in the gut or variations in the transport of Phe through the hematoencephalitic barrier. The identification and characterization of modifier genes involved in the determination of phenylketonuria phenotype is the field that needs to be explored.

Furthermore, it has been shown that the genetic variant in the intron of *PAH* gene has the ability to regulate gene expression (Stojiljkovic et al. 2010). Numerous variants are embedded in the large noncoding regions of *PAH* gene, and their effect remains unknown. Therefore, variants found in introns as well as those found upstream and downstream of the gene coding region should be further investigated.

Since we noticed inconsistency in identical genotypes of patients with the same population background, it could be expected that variations in modifier genes would provide an answer to interindividual rather than interpopulation inconsistencies.

BH4 Responsiveness in Serbia

The first study which suggested that BH4-responsiveness could be deduced from *PAH* genotype stated that at least one BH4-responsive mutation would be enough to lead to the physiological BH4 responsiveness (Zurflüh et al. 2008). In this study on Serbian PKU patients, total frequency of BH4-responsive mutations was 52.6%.

Later on, it became clear that BH4-responsive genotype has a greater BH4-responsive predictive value (Trefz et al. 2009a; Karacic et al. 2009). In order to make the most accurate estimation of the potential of BH4-supplementation therapy, we classified Serbian *PAH* genotypes into BH4 responsive, probably BH4 responsive, and non-BH4 responsive (Table 3) (Sterl et al. 2012). Furthermore, we made distinction between genotypes with one and two BH4-responsive alleles. Previously, Zurfluh and colleagues postulated that BH4 responsiveness ought to be in the 17–79% range in European populations with a north to south gradient (Zurflüh et al. 2008). Accordingly, we found that only 22.8% of patients could not benefit from BH4 therapy and that as much as 77.2% of PKU population should be subjected to a standardized BH4 loading test.

Genotype p.[L48S];[L48S] is among the most frequent genotypes reported in the BIOPKU database (www.biopku.org). It was reported that when patients carrying this genotype were subjected to BH4 loading test, they were found to be responders, slow responders, or partial responders. Patients with p.[L48S];[R408W] genotype, which could be regarded as probably responsive according to the genotype, were frequently reported as nonresponders in reality. Interestingly, similar to our findings on phenotypic effect of p.L48S, Karacic et al. observed regularity in p.L48S BH4 responsiveness. They found that p.L48S tends to be a nonresponder in functional hemyzigotes, while it usually acts as a responder in homozygotes (Karacic et al. 2009).

Without a doubt, BH4 loading test is the only way to determine BH4 responsiveness (Blau et al. 2009; Levy et al. 2007b). However, the genotype-based estimation is valuable for countries where BH4 is not yet included in the drug lists. This study, which represents the first estimation on BH4 responsiveness for Serbian population, led us to a conclusion that BH4 supplementation therapy could bring benefit to Serbian PKU patients.

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Synopsis

Analysis of Serbian PKU patients showed that Phe tolerance was a better parameter for phenotypic classification of patients carrying p.L48S mutation and the genotype study identified significant number of individuals that may respond to BH4 supplementation therapy.

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RESEARCH REPORT

Subjective and Objective Assessment of Hand Function in Mucopolysaccharidosis IVa Patients

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Abstract

Introduction: Morquio A is classically described as a skeletal dysplasia, but the impact of the abnormalities on the upper limb has not been described.

Aim: The aim of our study is to assess the level of subjective and objective functions of the hand and upper limb in Morquio A patients in order to advance understanding as this may change future management for this complex group of patients.

Method: We have evaluated a series of ten patients with Morquio syndrome who are currently part of the enzyme replacement therapy trials in our unit. To establish functional levels, we developed a questionnaire based on the principles of the World Health Organization International Classification of Functioning, Disability and Health (WHO ICF). Furthermore, we measured patients' grip function and range of motion.

Results: All patients showed hypermobility and limited control of the wrist. Their grip strength was reduced and they showed higher difficulty levels for activities involving strength. It is feasible that the wrist abnormalities may be the major cause of functional loss for Morquio patients.

Conclusion: Our findings suggest the wrist itself represents an area that we should focus on to be able to improve the patients' functional abilities and hence their level of independence on day-to-day basis.

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Introduction

Mucopolysaccharidosis type IV is an autosomal recessive lysosomal storage disease that can be subdivided into types A and B (A OMIM # 253000, B OMIM # 253010) depending on the type of enzyme defect. They are genetically distinct with some overlapping clinical features. Patients with the type A phenotype are thought not to survive beyond their third decade, whereas in type B phenotype patients can survive into their seventh decade (Montano et al. 2007). Type A, which is the focus of this study, is caused by a deficiency in the enzyme acetylgalactosamine-6-sulfatase (GALNS, EC 3.1.6.4; encoded by GALNS gene at 16q24.3).

Morquio syndrome was first described in 1929 by Morquio and Brailsford as a description of a series of patients (Morquio 1929). The reported incidence varies from 1 in 76,000 in Ireland (Nelson 1997) to 1 in 450,000 in Portugal (Pinto et al. 2004). The classical description is of a skeletal dysplasia associated with short stature, but it has become apparent that it shares many features with the other MPS disorders like cardiac valve abnormalities, limited endurance, abnormal dentition, corneal clouding and cervical instability (Northover et al. 1996).

Growth failure is one of the main features of many of the MPS disorders and may contribute to many of the limitations of these disorders. A recent study looked at the growth patterns of a large number of Morquio type A

patients and found that the growth pattern was characterised by impaired growth starting shortly after the first year of age (Montano et al. 2008).

With advances in enzyme replacement therapy (ERT), there are increased survival rates amongst MPS1 patients (Kakkis et al. 2001). ERT for Morquio patients is still undergoing clinical trials but in vitro studies and animal models are providing promising insights (Dvorak-Ewell et al. 2010).

One of the characteristic radiological findings for Morquio patients is seen in their hand and wrist x-rays. Conically shaped bases of the second to fifth metacarpals with shortening of the metacarpals and distal tufts, but with relatively soared proximal and middle phalanges, have been described (Lachman 1979). Eggli and Dorst (1986) go on to describe the delayed ossification of the carpal bones and the relative shortness of the radius and ulna, much more so in the ulna leading clinically to an ulnar deviation of the hand. Despite these well-known radiological characteristics, which are mirrored in clinical findings, the impact of this on hand function has not been described.

The aim of this study is to assess the level of subjective functioning as well as objective functioning of the hand and upper limb in MPS IVa patients.

Patients and Methods

Questionnaires

Hand surgeons and hand therapists developed a questionnaire to evaluate the patients' perspective on their hand and upper limb functioning. To establish functional levels, the principles of the World Health Organization International Classification of Functioning, Disability and Health (WHO ICF) were applied (www.who.int/classifications/icf). The WHO ICF framework was developed and endorsed in May 2001 and provides an international standard to describe and measure health and disability. It provides a holistic approach to patient health assessment and gives equal importance to all of its four sections: Body Structures, Body Functions, Participation and Activities and Environment. Three questionnaires were developed and age differentiated for 2 to 4 years, 5 to 9 years and above 10 years. The questions were designed in conjunction with senior hand therapists and reflect age-appropriate activities in each of the respective age groups. Parents completed the questionnaires for children under 10 years and the above 10 years questionnaire is completed by the patients themselves. Each question is answered on an ordinal scale of 1 to 5, with 1 reflecting no difficulty or disability and 5 representing the highest level of dysfunction in that parameter. The questionnaires represent all areas of the ICF and looks specifically at daily hand and upper limb function. The section activity limitation asks to indicate the level of difficulty encountered in performing certain day-to day tasks. Body function was reviewed by frequency of symptoms of pain, stiffness, weakness as well as paraesthesia. Participation was also assessed by reviewing the school and classroom activities and the ability to join in with peers. The questions for support and environment requested a response of either agreeing or disagreeing with statements. Support received from family members, school environment, and understandings of the wider health-care team were key issues in this section.

Measurements

The objective part of the assessment takes the form of range of movement (ROM) and grip strength measurements. The ROM measurements were taken from each of the small joints of the hand and the wrist, actively and passively. Measurements for each patient were averaged for extension and flexion at each joint level, i.e. all distal interphalangeal joints (DIPJ) readings for each digit were averaged to give one overall average of extension and flexion for the DIPJ, etc. Patients were given standardised instructions for positioning when taking measurements to reflect unsupported individual joint movements hence reducing bias. Grip strength measurements were taken for gross grip strength with standardised and calibrated dynamometer (Jamar®, Bolingbrook, IL) in position 1 and lateral key grip with a pinch gauge (Jamar®, Bolingbrook, IL). All assessments were performed by two experienced assessors (RA, AJ).

Study Population

We have clinically studied a series of ten patients with a diagnosis of Morquio A syndrome currently involved in the ERT trials within our unit. There are five girls and five boys with an average age of 9.4 years (range of 5 to 18 years). The baseline characteristics are shown in Table 1.

Results

Questionnaires

The individual numerical results from the questionnaires have been graphically demonstrated for each patient to show trends.

In terms of physical health, 40 % (4/10) patients answered excellent to the question on how they regarded their physical health with 60 % (6/10) answering very good. When asked how they regarded their emotional health, 20 % (2/10) answered very good, 30 % felt it was good,

Table 1 Table showing the demographics for the patient series including level of mobility at the time of assessment

Patient	Age (years)	Morquio type	Gender	Time on ERT	Mobility
1	5	A	F	6 months	Independent
2	5	A	M	6 months	Independent
3	6	A	F	1 .5 years	Independent
4	8	A	F	2 years	Independent
5	10	A	F	2 years	Assistance for transfers
6	10	A	M	2 years	Assistance for transfers
7	10	A	F	2 years	Independent
8	11	A	M	2 years	Assistance for transfers
9	11	A	M	2 years	Independent
10	18	A	M	2 years	Mobile in wheel chair

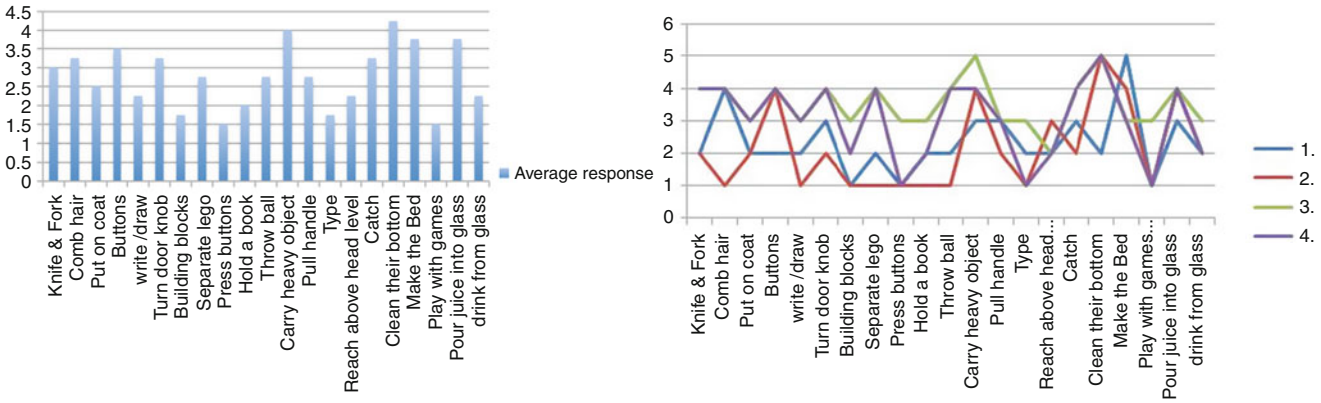

Fig. 1 Bar chart showing the average response and line graph depicting trend in answers to questions on activities of daily living for patients aged 5 to 9 years

40 % responded with fair and 1 patient felt their emotional health was poor.

Within activity limitation for the 5 to 9 years age group (Fig. 1), the highest average response, indicating most difficulty, was generated by questions on carrying a heavy object (mean 4, range 3–5), cleaning their bottom (mean 4.5, range 2–5), making their bed (mean 3.75, range 3–5) and pouring juice from a bottle into a glass (mean 3.75, range 2–5). The lowest average response, indicating least difficulty, was seen with playing with games consoles (mean 1.5, range1–3), pressing buttons (mean 1.5, range 1–3), typing (mean 1.75, range 1–3) and building blocks (mean 1.75, range 1–3). When analysing the overall responses, 17 % (15/84) of responses were scored no activity limitation and 6 % (5/84) of responses were unable to complete task.

In the greater than 10 years age group (Fig. 2), the most difficulty was caused by carrying a heavy object (mean 4.75, range 4–5), washing themselves (mean 4.25, range 4–5), pouring juice from a bottle into a glass (mean 4.25, range 2–5) and making their bed (mean 4, range 3–5). The least difficulty was seen with playing with games consoles (mean 1.25, range1–3), typing (mean 1.25, range 1–3), text messaging (mean 1.5, range 1–2) and writing (mean 2.5, range 2–3). Nine percent (11/120) of responses within this section were representing no activity limitation and 10 % (12/120) were unable to complete the task.

All patients responded that they had weakness in their hand or upper limb from some to all of the time, whereas pain, paraesthesia and stiffness were variable (Fig. 4).

Nine children were attending school on a regular basis; one child was a school leaver at the age of 18 years. Patient's response in terms of limitation at school and participation was that they felt moderately limited (7/10) regarding normal classroom activities, which progressed to very limited (10/10) when physical education or sports were reviewed. Eight out of ten patients answered only slightly or not limited on regular school attendance. All patients answered strongly agree or agree on support from family and health-care team. Eight out of ten patients

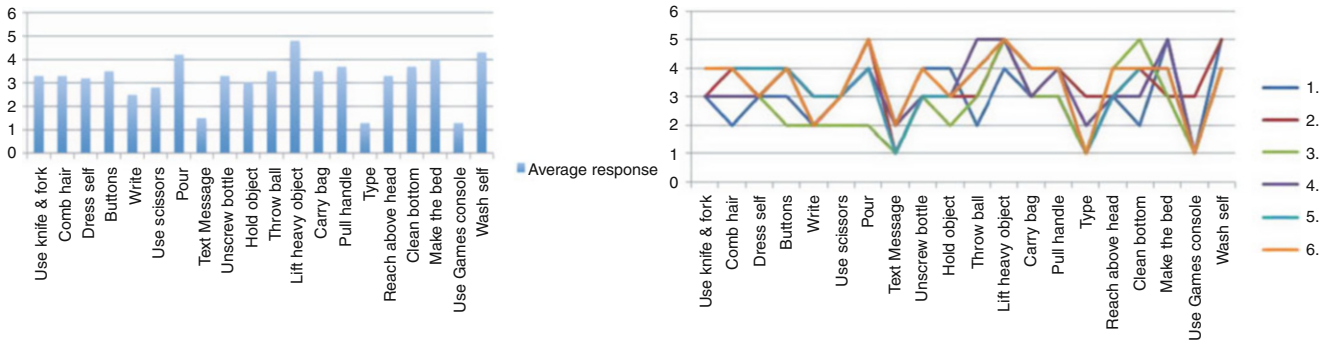

Fig. 2 Bar chart showing the average response and line graph depicting trend in answers to questions on activities of daily living for patients aged 10 years and above

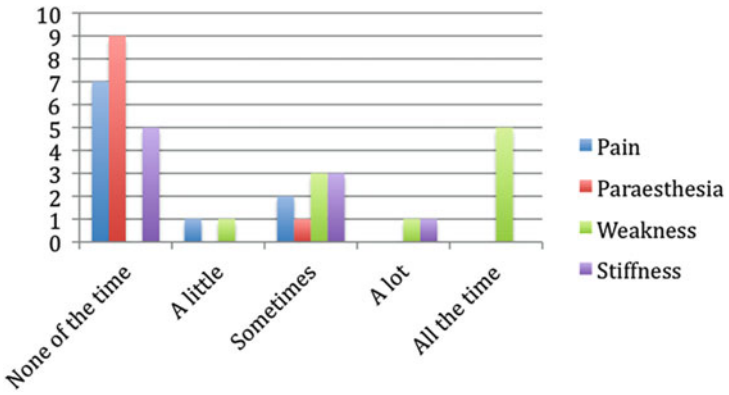

Fig. 3 Bar chart representing the distribution of responses to questions within the body functions category

agreed they had support from their school, whereas 2/10 patients neither agreed nor disagreed (Fig. 3).

Clinometry

The results for the measurements of range of joint movements for the DIPJ and proximal interphalangeal joints (PIPJ) of the digits did not reveal a clinically significant difference between active and passive movements for the patients beyond what is expected with normal ranges. The metacarpophalangeal joints (MCPJ) were found to have a slightly decreased ROM in flexion with the average flexion of PIPJ limited to 76.5° actively (range 50–95) and 83° passively (range 65–90) (Table 2).

The wrist showed significant differences in active and passive measurements (Table 3). The greatest difference in extension/flexion of 195° was seen in the eldest Morquio patient who was male and aged 18 years (Case 10). The average difference between active and passive movements was 93° in extension/flexion (range 60–195). The average ROM was decreased for radial deviation with 6° active and 12.5° passive. The average ROM for ulnar deviation showed a greater difference of 30° active and 61° passive.

The grip strength measurements (Table 4) show the strength in the gross handgrip and lateral key for the dominant hand. Gross grip strength results were an average of 19.1 kg below the normative values. The pinch gauge results showed an average reduction in strength of 2.4 kg in lateral key grip.

Discussion

Being able to achieve optimal hand function in children is the main goal in paediatric hand surgery. This is widely recognised and much research has gone into achieving this goal. When presented with the challenging group of MPS patients, we are forced to start from the very beginning, as there is very little documentation of dysfunction let alone surgical or non-surgical intervention.

Even though Morquio A patients have relatively normal levels of intelligence and a survival usually into the third decade of life, there has been no attempt to assess a level of functioning in these patients. Interest in MPS from hand

Table 2 Mean active and passive range of movement for the small joints of the hands through extension and flexion

Hand	Patient	1	2	3	4	5	6	7	8	9	10
DIPJ	Active	0-5-65	0-0-70	0-5-40	0-0-55	0-0-70	0-0-65	0-5-60	0-0-65	0-0-55	0-0-75
	Passive	0-0-85	5-0-95	5-0-60	5-0-80	40-0-85	15-0-90	15-0-85	5-0-90	0-0-85	5-0-90
PIPJ	Active	0-0-95	0-0-85	0-0-90	0-0-90	0-0-85	0-0- 90	0-0-95	0-0-90	0-0-85	0-0-90
	Passive	0-0-105	0-0-105	0-0-95	5-0-100	0-0-100	0-0-105	5-0-110	0-0-105	0-0-90	0-0-100
MCPJ	Active	10-0-85	0-0-75	0-0-80	5-0-70	0-0-70	0-0-85	0-0-85	5-0-90	5-0-75	0-0-50
	Passive	60-0-90	40-0-80	60-0-90	45-0-75	40-0-85	50-0-95	45-0-80	45-0-90	40-0-85	60-0-60
Thumb IP	Active	0-0-55	0-0-80	0-0-40	0-5-60	0-0-85	0-0-75	0-0-80	0-0-80	0-0-75	0-0-80
	Passive	0-0-60	5-0-95	10-0-40	0-0-60	5-0-90	10-0-90	0-0-90	25-0-90	20-0-85	50-0-100

Table 3 Active and passive range of joint movement at wrist level with the difference in degrees for each patient

Wrist	Patient	1	2	3	4	5	6	7	8	9	10
Extension/ flexion	Active	30-0-30	50-0-40	10-0-90	45-0-65	30-0-40	35-0-30	30-0-60	90-0-45	40-0-80	45-0-10
	Passive	90-0-70	90-0-70	70-0-90	95-0-100	90-0-90	120-0-30	85-0-90	120-0-95	90-0-90	150-0-100
Difference		100	70	60	85	110	85	85	80	60	195
Ulnar/radial deviation	Active	20-0-10	30-0-0	30-0-0	20-0-0	40-0-20	40-0-0	30-0-20	35-0-0	35-0-0	20-0-10
	Passive	60-0-20	85-0-10	70-0-0	40-0-10	65-0-20	60-0-0	60-0-30	90-0-10	40-0-5	80-0-20
Difference		50	65	40	30	25	20	40	65	10	70

Table 4 Mean strength measurements of the dominant hand for each patient for lateral key grip and gross grip. (Normative data: Mathiowetz et al. 1986)

Patient		1	2	3	4	5	6	7	8	9	10
Gender		F	M	F	F	F	M	F	M	M	M
Age		5	5	6	8	10	10	10	11	11	18
Lateral key grip	Kg force	1	2.5	2.5	2	2	2	2.5	1.5	1.5	1
Normative		3.03	3.2	3.03	3.44	4.4	4.5	4.4	4.5	4.5	7.7
Gross grip	Kg force	0.5	2	2	1	2	0.5	2	1	2	2
Normative		12.9	14.7	12.9	19	22.5	24.4	22.5	24.4	24.4	48.9

surgeons has almost exclusively involved patients that have developed carpal tunnel syndrome (CTS) requiring surgical release. Haddad et al. (1997) studied a group of 48 MPS patients for signs of CTS. They commented that none of the Morquio's patients had signs or symptoms suggestive of CTS, but no mention was made to the hand dysfunction that they did possess.

Patients with MPS IVa in particular undergo multiple surgical operations to not only prolong survival (e.g. c-spine fusion) but also to improve function (knee and hip). With the advent of ERT, the expectation is that survival will further improve and that disease severity will decline.

The level of hand functioning therefore takes on a greater role in these patients who have normal intelligence and are keen to lead as independent a life as is possible for them.

The first step in establishing a management plan for these patients is to determine the level of their disability contributed by their hands. We have conducted twofold assessments of hand function involving an activity of daily living questionnaire as well as ROM and strength.

The questionnaires show that in certain areas such as activity limitation and participation, there are trends that reflect the reduced strength seen in the hands of Morquio patients. For example in questions such as "carry objects" or "pouring from a bottle", a majority of patients were very limited and sometimes unable to carry out the task. In the 5 to 9 year age group, bimanual activities such as "using a knife and fork" and "doing up buttons" represented particular areas of difficulty. In contrast, tasks that involved "pressing buttons" or "typing" were areas that were well

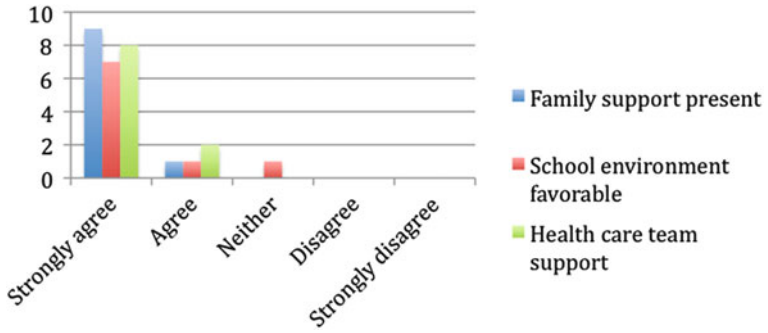

Fig. 4 Bar chart representing the distribution of responses to questions on environment and support

adapted to and hence posed little difficulty on a day-to-day basis (Fig. 4).

In the above 10 years age group, once again, activities involving use of strength such as pouring from a bottle or carton, or lifting heavy objects gave the responses of most difficulty. Bimanual activities such as doing up buttons, using scissors, using a knife and fork also were areas of difficult with responses averaging 3 or above out of 5. Text messaging and typing were again areas that were found to be areas of very low or no difficulty. It is highly likely that the lack of strength leads to these activity limitations.

Interestingly, the participation area of the questionnaire showed that patients are less limited in normal classroom activities but are still very limited in terms of sports and physical education. Even though these children go to schools that provide special attention and adaptations they still have significant areas of limitation compared with their healthy peers.

In terms of attitudes and support from health-care teams and family, all patients felt well supported, with their needs and requirements well understood. This is recognised by the WHO ICF as an important part of a patient's progress and hence prognosis.

The hypermobility of the wrist joints is also a unique point seen in the Morquio group of patients. The average difference of 93° between active and passive ROM at the wrist joint reflects the loss of stability at this joint and that although they have a wider range, they are unable to utilise this actively. This was seen universally in all Morquio patients but with more striking results in the older age groups. Patient 10 (18 years old) had the widest range of passive movement in extension and flexion at the wrist joint, yet active movements were much reduced. This particular patient's ROM in extension/flexion was 250° passively, yet he had only 55° of active range.

The results of the grip strength tests were also significant. When compared with normative data (Mathiowetz et al. 1986), the strength for gross grip was reduced on average by 19.1 kg and 2.4 kg for pinch strength. When comparing ROM measurements, the finger joints seem to have an almost normal range, whereas the wrist displays hypermobility on extension and flexion, as well as ulnar deviation, but markedly reduced radial deviation. We were unable to determine if the hypermobility is purely an effect of the skeletal abnormalities or if a combination of ligamentous laxity and tendon displacement either primary or secondary lead to these findings. Progressive muscle weakness may contribute furthermore to the loss of strength.

Our study has several limitations. First, the questionnaires we used are not yet validated. However, we found no existing questionnaires that are designed to reflect this aspect of functioning in this specific group of patients. We developed these questionnaires based on the principles of the WHO ICF. All domains are included to provide a clear overall view of the patients' abilities. Second, the active ROM of patients might not be representative of their real ability as patients could become quickly distracted. Also, all patients had been commenced on the ERT trial for varying lengths of time prior to their evaluations. Since ERT is still in clinical trial phase, it is unclear the influence this has on hand function.

Although the study has those limitations, this article provides a clear view of the disabilities of the hand and its influence on daily living.

Conclusion

Morquio patients show hypermobility of the wrist with limited control with the vastly reduced grip strength. This, coupled with the higher difficulty levels for activities involving strength, makes it feasible that the wrist may be the cause of major areas of functional and functioning deficit for Morquio patients.

In our experience, the older children often had limited control of the movements at the wrist level, almost "dragging" their hands to positions or areas required. It is a possibility that the hand function progressively deteriorates as the patients get older. The wrist itself represents an area that we should focus on to be able to improve the patients' functional abilities and hence their level of

independence on a day-to-day basis. More research is needed in this area to be able to measure if ERT will have any effect on these parameters.

Take-Home Message

Patients with Morquio syndrome show difficulties in daily living and the upper limb abnormalities contribute significantly to their disability.

The authors have nothing to declare or any competing interests.

The authors confirm independence from any sponsors.

Ethical approval was obtained prior to the study from NRES Committee West Midlands – Staffordshire. REC reference: 11/H1203/5

Contributors

Riffat Aslam is the first author and was involved in the conception and design of the research, the seeking of ethics approval, assessment of patients and collection of data, interpretation of data and write up of the research. She is the corresponding author.

Annelotte van Bommel was involved in data collation and interpretation as well as in revising the article in various draft formats.

Chris Hendriksz is a leading academic on Morquio patients and is a senior author to the article. He was involved in drafting the article and revising it critically for intellectual content.

Andrea Jester is the senior author and guarantor of the article. She has been involved in conception, methodology, assessment of patients, data analysis, drafting and revising the article critically for important intellectual content.

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RESEARCH REPORT

Ceftriaxone for Alexander's Disease: A Four-Year Follow-Up

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Abstract In 2010, we reported the successful clinical outcome related to a 20-month course of intravenous, cyclical ceftriaxone, in a patient with adult-onset Alexander's disease. We now provide evidence that the progression of the patient's signs/symptoms was halted and reversed with a 4-year-long extension of the trial.

The patient's clinical signs/symptoms were evaluated before the start and every 6 months for 6 years. For the early 2 years, without therapy, and for the following 4 years, after intravenous ceftriaxone 2 g daily, for 3 weeks monthly during the initial 4 months, then for 15 days monthly.

Gait ataxia and dysarthria were assessed clinically on a 0 to 4 scale. Palatal myoclonus and nystagmus/oscillopsia were monitored by videotape and a self-evaluation scale. The degree of disability, measured by a modified Rankin scale, and the brain MRI were periodically evaluated.

Before ceftriaxone therapy, in a 2-year period, gait ataxia and dysarthria worsened from mild to marked, palatal myoclonus spread from the soft palate to lower facial muscles, and the patient complained of oscillopsia. After 4 years of ceftriaxone therapy, gait ataxia and dysarthria improved, from marked to mild at clinical rating scales. The palatal myoclonus was undetectable; the patient did not complained of oscillopsia and declared a progressively better quality of life. Ceftriaxone was safe.

This case report provides Class IV evidence that intravenous cycles of ceftriaxone may halt and/or reverse the progression of neurodegeneration in patients with adult-onset Alexander's disease and may significantly improve their quality of life.

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Introduction

Alexander's disease (AxD) is a rare, usually fatal, primary disorder of astroglial cells in the central nervous system (CNS) related to dominant mutations in the gene encoding the type III intermediate filament protein, glial fibrillary acidic protein (GFAP) (Brenner et al. 2001; Mignot et al. 2004; Li et al. 2005). The prevalent pathophysiological hypothesis regarding AxD is based on the occurrence of a toxic gain-of-function of mutated GFAP, which causes intracytoplasmic aggregates in astrocytes (Rosenthal fibers), containing GFAP, αB-Crystallin, the heat shock protein 27, and ubiquitin (Mignot et al. 2004; Li et al. 2005), and on the occurrence of excitotoxicity related to impairment of the buffering capacity of dystrophic astrocytes and of their ability to metabolize extracellular glutamate (Mignot et al. 2004; Tian et al. 2010). Therefore, in AxD, lowering the production of GFAP and promoting its degradation and/or the enhancement of the astroglial glutamate uptake from the extracellular fluid may have potential therapeutic effects. No treatment is at present available for this neurodegenerative disorder, although recent data, in vitro and in animal models

of AxD, indicate that drugs such as the β-lactam antibiotic ceftriaxone and the tricyclic antidepressant clomipramine may have potential therapeutic effects (Bachetti et al. 2010; Cho et al. 2010). In particular, clomipramine lowers the production of GFAP in an animal model of AxD and delays the appearance of aggregates in astrocytes from the animal model (Cho et al. 2010). Ceftriaxone enhances the degradation of GFAP aggregates in a cellular model of AxD (Bachetti et al. 2010), and may also counteract neurodegeneration due to glutamatergic excitotoxicity, based on its ability to increase glutamate transporter subtype 1 (GLT-1) activity in astrocytes (Rothstein et al. 2005). Previously, we reported the successful clinical outcome related to a 20-month course of intravenous, cyclical ceftriaxone in a patient with an adult form of AxD and a rapidly progressive clinical course (Sechi et al. 2010a). Here we studied the tolerability and therapeutic effects of cycles of ceftriaxone in this patient at a 4-year follow-up.

Methods

The patient is a 44-year-old woman carrying the p.R70Q and p.D157N GFAP mutations with adult-onset AxD (Sechi et al. 2010a). The prominent clinical signs/symptoms were dysarthria, gait ataxia, and palatal myoclonus from the age of 38 years and evoked nystagmus/oscillopsia from the age of 40 years. We previously reported the effect of a 20-month long trial of ceftriaxone therapy. At that time, the patient declared an improvement of her clinical status, which was in agreement with clinical (ataxia, dysarthria) and electrophysiological (nystagmography recording) data (Sechi et al. 2010a). We presently report an extension of the trial encompassing a 6-year-long observation.

Gait ataxia, dysarthria, palatal myoclonus, and evoked nystagmus/oscillopsia were evaluated every 6 months over a 6-year period. For the first 2 years, without therapy, and for the following 4 years, after intravenous ceftriaxone 2 g daily, for 3 weeks monthly during the initial 4 months, then for 15 days monthly. Among the ceftriaxone cycles, a probiotic preparation was administered by mouth once daily: FlorVis-gg, containing 6 milliards of *Lactobacillus rhamnosus*. The patient's body weight was 60 kg. Her Mini-Mental State Examination score was 30. Intravenous ceftriaxone was administered by a chronic subcutaneously implanted port on the chest.

Gait ataxia and dysarthria were assessed clinically on a 0 to 4 scale (0 = no disturbance, 1 = mild, 2 = moderate, 3 = marked, 4 = severe). In particular, speech was assessed during normal conversation for at least 10 min, and the severity of dysarthria evaluated according to the following rating scale:

- 0: no disturbance.
- 1: mild. The speech is impaired, but all the words are understandable.
- 2: moderate. Occasional words difficult to understand.
- 3: marked. Many words difficult to understand.
- 4: severe. Only single words are understandable.

For evaluating gait ataxia, instead, the patient was asked to walk at a safe distance parallel to a wall, including a turn around to face the opposite direction of gait (half-turn), and to walk in tandem without support. The severity of gait ataxia was rated as follows:

- 0: no disturbance.
- 1: mild. Slight difficulties, only evident when walking in tandem.
- 2: moderate. Definite staggering, difficulties in half-turn but without support.
- 3: marked. Marked staggering, intermittent support of the wall required.
- 4: severe. Walking is only possible with strong support, stroller, or accompanying person.

Palatal myoclonus was monitored by videotape and the progressive involvement of contiguous muscles noticed. Videotape examinations were conducted in the same physical environment and conditions and with the same video equipment. Oscillopsia was monitored by a self-evaluation scale (severe, moderate, mild, no oscillopsia). The degree of disability was measured, every year, for 6 years, by a modified Rankin scale (van Swieten et al. 1988). The scale runs from 0–6, running from perfect health without symptoms to death. Brain MRI was evaluated about every 2 years. Periodic controls of hematological parameters, electrocardiography (ECG) and electroencephalography (EEG) were performed. The patient gave written informed consent. The study was approved by the local ethics committee.

Results

Before ceftriaxone therapy, during the first 2 years of observation, the patient's gait ataxia and the dysarthria worsened progressively, from mild to marked at clinical rating scales, and the palatal myoclonus spread, after about 10 months, from the soft palate to lingual and lower facial muscles. At the beginning of the study, eye movement recording (EMR) was normal and the patient did not complain of oscillopsia. After about 16 months, at EMR, bilateral hypometric horizontal saccades and horizontal left gaze evoked nystagmus with waveform-type pendular-like mixed were present, and the patient complained of severe oscillopsia. After 2 years without therapy, the gait ataxia, the oscillopsia, and the dysarthria prevented a completely

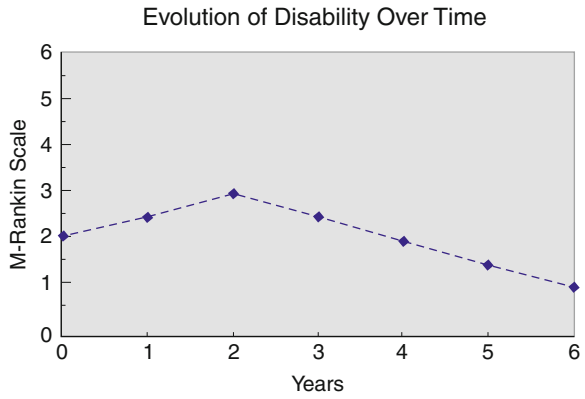

Fig. 1 Evolution of the degree of disability over time: 0–2 years, before treatment; 2–6 years, after ceftriaxone treatment

independent way of life (Rankin score, 3). Brain MRI revealed bilateral, high signal-intensity areas posteriorly in the periventricular white matter, pyramidal tracts of the medulla, and cerebellar white matter (Sechi et al. 2010a).

After about 2 years of ceftriaxone therapy, the severity of palatal myoclonus did not worsen; the gait ataxia and the dysarthria improved from marked to moderate at clinical rating scales. The evoked nystagmus showed a reduced amplitude in comparison with the previous recordings, with improvement in the ability to read or watch television. The patient rated her oscillopsia as mild. The patient rated herself as improved and declared a better quality of life (Rankin score, 1–2) (Sechi et al. 2010a).

After about 4 years of ceftriaxone therapy, the gait ataxia and the dysarthria showed a definite improvement from marked to mild at clinical rating scales. The palatal myoclonus, after about 3 years, disappeared from lower facial muscles and tongue, and after about 4 years it was undetectable (videos 1, 2). The patient did not complain of oscillopsia, rated herself as definitively improved, and declared a progressively better quality of life (Rankin score, 1) (Fig. 1). Importantly, different examiners blinded to the treatment, at the different time points, agreed for a progressive improvement of the gait ataxia, dysarthria, and palatal myoclonus in the patient. On follow-up examination, the signal-intensity changes on brain MRI did not reverse but did not progress further or show atrophy in involved areas (Fig. 2a–d). Ceftriaxone did not have obvious, major toxicity in the patient. After 7 months of therapy, the patient had a mycotic vaginal infection treated with miconazole. After 3.5 years, mainly during ceftriaxone cycles, the patient occasionally complained of single, right or left myoclonic jerks in upper limbs, about seven times a week. EEG showed a normal background rhythm without epileptiform activity. The patient refused any therapy for myoclonic jerks. Periodic controls, of white and red blood cell counts, glucose, creatinine, serum electrolytes, liver function tests, ECG, and EEG were normal.

Discussion

The available literature data indicate that in patients with adult-onset AxD, the clinical course is usually that of a progressive neurodegenerative disease without spontaneous remission (Schwankhaus et al. 1995; Balbi et al. 2010), as also shown by the evaluation of our patient during the first 2 years of observation without therapy. Thus, it seems very unlikely that the progressive improvement of the patient over a 4-year period, after ceftriaxone therapy, may be due to the natural course of the disease.

This case report provides Class IV evidence that parenteral cycles of ceftriaxone were able to halt and reverse the progression of the disease and to improve significantly the quality of life in a patient with adult-onset AxD and a rapidly progressive clinical course. Importantly, a definite improvement was achieved slowly for all the signs evaluated, from few months to 3 years, with a different timescale which seems related to the time necessary to the development and progression of the different signs, as exemplified from palatal myoclonus. On follow-up examination, brain MRI changes did not progress further, or show atrophy in involved areas. Because the available literature indicates that in patients with AxD the brain MRI signal intensities are not static but may vary in time and progress to atrophy in involved areas (Sawaishi 2009), this finding also may be indicative of a therapeutic effect of ceftriaxone in our patient with AxD. At the used dosages, cyclical administration of ceftriaxone was safe. Some adverse effects, as the self-limiting myoclonic jerks, indicate that ceftriaxone may lower the epileptogenic threshold. This adverse event needs long-term supervision, and an eventual lowering of ceftriaxone dosages, mainly in the infantile form of AxD, which is frequently characterized by the occurrence of epileptic seizures (Li et al. 2005).

Recent, essential advances in the understanding of the pathophysiology of AxD indicate that a dynamic and reversible aggregation of mutated GFAP may occur (Mignot et al. 2004; Mignot et al. 2007), and that in this pathology at least three main biochemical mechanisms play a fundamental role in promoting neurodegeneration: (1) The key initiating event is the expression and accumulation in astrocytes of mutant GFAP above a toxic threshold, with consequent, progressive astroglial damage (Messing et al. 1998). (2) The decrease of the cytoplasmic content of the small heat-shock proteins (sHPSs) HSP27 and αB-Crystallin in astrocytes, due to their sequestration in GFAP aggregates (Mignot et al. 2004; Der Perng et al. 2006). Indeed, it has been shown that overexpression of these sHPSs in astrocytes promotes dissolution of GFAP aggregates (Tang et al. 2010). (3) The impairment of the buffering capacity of dystrophic astrocytes and of their ability to metabolize extracellular glutamate, with chronic accumulation of extracellular glutamate and subsequent

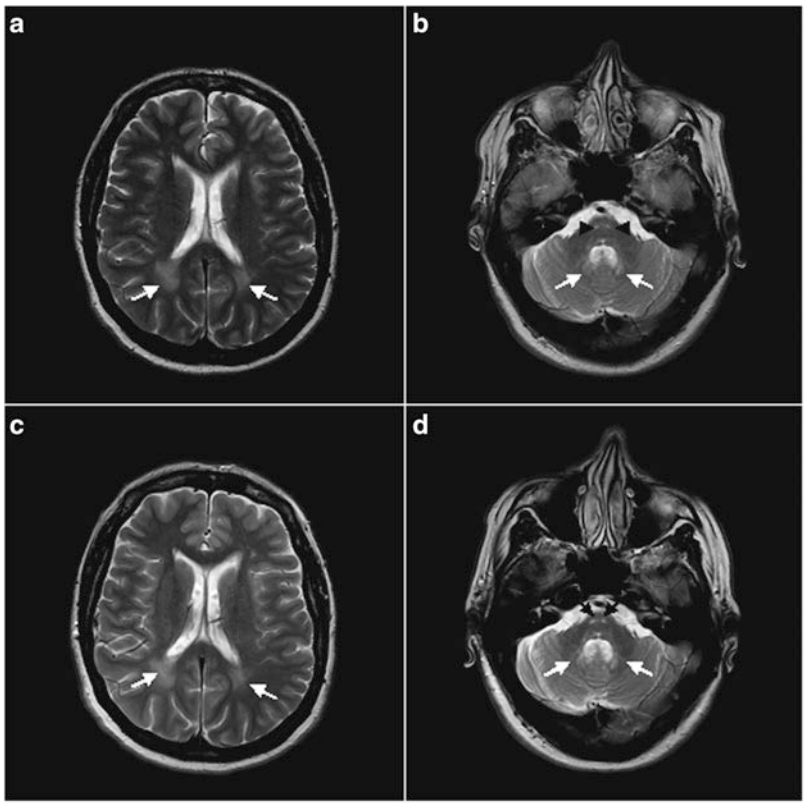

Fig. 2 **a**–**d**: Axial T2-WMR images, at the beginning of ceftriaxone therapy (**a**, **b**) and after about 4 years of ceftriaxone therapy (**c**, **d**). Bilateral high signal-intensity areas are seen posteriorly in the periventricular white matter (*arrows*) (**a**, **c**); MR scan obtained at a lower level shows involvement of the descending pyramidal tracts of the medulla (*arrowheads*) and deep cerebellar white matter (*arrows*) (**b**, **d**). On follow-up examination, the signal-intensity changes did not reverse but did not progress further, or show atrophy in involved areas

excitotoxicity, may play an important role in the pathogenesis of secondary oligodendrocyte and neuronal degeneration in AxD (Tian et al. 2010; Sechi et al. 2010b). Given these findings, it is conceivable that in AxD, for achieving an effective and long-term suppression of neurodegeneration, a chemical agent, or a combination of chemical agents that inhibit multiple biochemical injurious mechanisms in astrocytes may be needed. In such a scenario, ceftriaxone may be of particular interest. This β-lactam antibiotic is a safe and multipotent agent used for decades as antimicrobial (Nau et al. 1993). In 2005, ceftriaxone was suggested as a potential therapeutic agent in neurodegenerative diseases comprising glutamate-mediated toxicity, based on its ability to increase glutamate transporter subtype 1 (GLT-1) activity in astrocytes that are genetically impaired in their GLT-1 expression (Rothstein et al. 2005). In 2010, we showed that ceftriaxone is also able to successfully eliminate the toxic effects of misfolded GFAP in a cellular model of AxD by decreasing GFAP intracytoplasmic aggregates (Bachetti et al. 2010). Underlying mechanisms include mutant GFAP elimination, concurrent with upregulation of HSP27 and αB-Crystallin, polyubiquitination, enhancement of autophagy, and GFAP promoter downregulation (Bachetti et al. 2010). Interestingly, a recent study in an animal axotomy model showed that ceftriaxone was able to suppress, after 28 days of parenteral administration, the reactive increase of astroglial GFAP expression to control level, at 200 mg/kg, daily dose (Yamada and Jinno 2011). Another, very recent study in a mouse model of AxD documented that overexpression of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) dramatically reduced astrocytic GFAP content in all brain regions examined and restored body weights in R236H mice to near wild-type levels (Lapash Daniels et al. 2012). Since the astrocytic induction of Nrf2 is involved in the neuroprotective action of ceftriaxone (Lewerenz et al. 2009), this mechanism also may partly explain the therapeutic effect of this drug in AxD. Notably, in AxD the ceftriaxone activation of cellular mechanisms involved in the disposal of mutant GFAP acts in a synergistic way with its ability to counteract the chronic glutamatergic excitotoxicity in this pathology, with consequent rescue of the secondary oligodendrocytes and neuronal degeneration related to excitotoxicity (Sechi et al. 2010b).

Because AxD is rare and its presentation and course vary, evaluation of treatments in placebo-controlled trials is

very difficult (Sechi et al. 2010a). Instead, prolonged, longitudinal single patient studies may be a useful approach to identify new therapeutic strategies in this disorder (Sechi et al. 2010a). The successful clinical outcome related to ceftriaxone documented in our patient highlights the possibility that this safe drug may be useful for other AxD patients and puts the relevant question whether ceftriaxone may also be useful in treating other diseases caused by intracellular accumulation and aggregation of misfolded proteins (Sechi et al. 2011), particularly other neurodegenerative diseases with astrocyte involvement.

Synopsis

Long-term intravenous cycles of ceftriaxone have a therapeutic role in adult-onset Alexander's disease with a rapidly progressive clinical course.

GianPietro Sechi designed the study, analyzed and interpreted the data, provided the figures, wrote the manuscript, and obtained funding.

Isabella Ceccherini revised the manuscript for content.

Tiziana Bachetti revised the manuscript for content.

Giovanni A. Deiana analyzed and interpreted the data and revised the manuscript for content.

Elia Sechi revised the manuscript for content.

Pietro Balbi analyzed and interpreted the data and revised the manuscript for content.

GianPietro Sechi serves as guarantor for the article, accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

Isabella Ceccherini, Tiziana Bachetti, Giovanni A. Deiana, Elia Sechi, and Pietro Balbi report no disclosures.

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The study was approved by the local ethics committee.

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RESEARCH REPORT

Identification and Characterisation of a Novel Pathogenic Mutation in the Human Lipodystrophy Gene *AGPAT2*

C48R: A Novel Mutation in AGPAT2

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Abstract Loss-of-function mutations in *AGPAT2*, encoding 1-acylglycerol-3-phosphate-O-acyltransferase 2 (AGPAT2), produce congenital generalised lipodystrophy (CGL). We screened the *AGPAT2* gene in two siblings who presented with pseudoacromegaly, diabetes and severe dyslipidaemia and identified a novel mutation in *AGPAT2* causing a single amino acid substitution, p.Cys48Arg. We subsequently investigated the molecular pathogenic mechanism linking both this mutation and the previously reported p.Leu228Pro mutation to clinical disease. Wild-type and mutant AGPAT2 were expressed in control and AGPAT2-deficient preadipocyte cell lines. mRNA and protein expression was determined, and the ability of each AGPAT2 species to rescue adipocyte differentiation in AGPAT2-deficient cells was assessed. Protein levels of both p.Cys48Arg and p.Leu228Pro AGPAT2 were significantly reduced compared with that of wild-type AGPAT2 despite equivalent mRNA levels. Stable expression of wild-type AGPAT2 partially rescued adipogenesis in AGPAT2 deficient preadipocytes, whereas stable expression of p.Cys48Arg or p.Leu228Pro AGPAT2 did not. In conclusion, unusually severe dyslipidaemia and pseudoacromegaloid overgrowth in patients with diabetes should alert physicians to the possibility of lipodystrophy. Both the previously unreported pathogenic p.Cys48Arg mutation in AGPAT2, and the known p.Leu228Pro mutation result in decreased AGPAT2 protein expression in developing adipocytes. It is most likely that the CGL seen in homozygous carriers of these mutations is largely accounted for by loss of protein expression.

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Introduction

Congenital generalised lipodystrophy (CGL) is a rare autosomal recessive disease characterised by nearly global loss of adipose tissue from birth. Affected individuals are commonly severely dyslipidaemic, insulin resistant, and eventually diabetic and suffer a commensurately high rate of microvascular and macrovascular complications, as well as a high rate of severe fatty liver disease and pancreatitis. Like many other patients with extreme forms of insulin resistance, they may also exhibit acromegaloid soft tissue overgrowth without any evidence of growth hormone or IGF1 excess. Although the diagnosis of CGL is usually made based on the observation of absent adipose tissue on clinical examination, the severity of the complications and collateral clinical and biochemical features may serve to alert clinicians to the underlying diagnosis.

Defects in four genes – *AGPAT2, BSCL2, CAV1,* and *PTRF* – have been implicated in CGL to date (OMIM:

608594, 269700, 612526, and 613327, respectively) (Rochford 2010). *AGPAT2,* the first of these to be reported, was implicated first by genetic mapping of the disease locus to chromosome 9q34, followed by identification of the disrupted gene in 2002 (Agarwal et al. 2002). Patients with *AGPAT2* mutations fail to develop metabolically active adipose tissue in most subcutaneous, intra-abdominal, intra-thoracic, and bone marrow fat depots, as ascertained by whole-body magnetic resonance imaging. However, they often have preserved mechanical adipose tissue depots in the palms, soles, scalp, retro-orbital, and peri-articular regions (Agarwal et al. 2003).

AGPAT2 encodes a 278 amino acid acyltransferase, 1-acylglycerol-3-phosphate-O-acyltransferase 2 (AGPAT2) that converts lysophosphatidic acid (LPA) to phosphatidic acid (PA), a key step in the triacylglycerol (TG) biosynthesis pathway (Shindou et al. 2009). AGPAT2 is highly expressed in human adipose tissues, liver, pancreas, skeletal muscles, and small intestine (Shindou et al. 2009). Most reported *AGPAT2* mutations in CGL cause frame-shifts, insertions, deletions, or affect splicing, and are predicted to be functionally null alleles. Only a small number of pathogenic amino acid substitutions have been described to date (Hegele et al. 2007; Huang-Doran et al. 2010; Pelosini et al. 2011). In this report, we describe and characterise a novel missense mutation in the poorly characterised amino terminal domain of AGPAT2, p.Cys48Arg, identified in two female patients with CGL.

Methods

Mutational Analysis

Genomic DNA was extracted from peripheral blood leukocytes before PCR amplification of all coding exons of the *AGPAT2* and *BSCL2* genes plus 50 bp of flanking sequence at either end of the exons employing primers designed using ExonPrimer via the USC genome browser (http://genome.ucsc.edu/) and checked for common polymorphisms. The PCR products were purified using Agencourt® CleanSEQ® reagents (Agencourt Bioscience, Beverly, MA, USA) and sequenced in both directions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, PerkinElmer, Foster City, CA, USA) and an ABI 3730 DNA sequencer (Applied Biosystems). Sequence analysis was performed using Sequencher software (Gene Codes, Ann Arbor, MI).

Biochemical Assays

Insulin, leptin, and adiponectin were all assayed on a 1235 AutoDELFIA (PerkinElmer Lifesciences, Boston, MA) automatic immunoassay system using two-step time-resolved fluorometric assays as previously described (Semple et al. 2006). SHBG was determined using an IMMULITE 1,000 solid phase chemiluminescent enzyme immunometric assay (Siemens Medical Solutions Diagnostics). Testosterone was measured by a solid phase extraction RIA (Siemens Healthcare Diagnostics, Surrey, UK; formally DPC Coat-A-Count) using protocols provided by the manufacturer. Triglyceride concentrations were assayed in singleton on the Dimension RXL system (Dade Behring, Milton Keynes, UK). Plasma (total) IGF-I concentrations were measured in ethanolic extracts using aDSLELISA (DSL-UK Ltd., Upper Heyford, Oxon, UK) according to the manufacturer's instructions.

Cell Culture

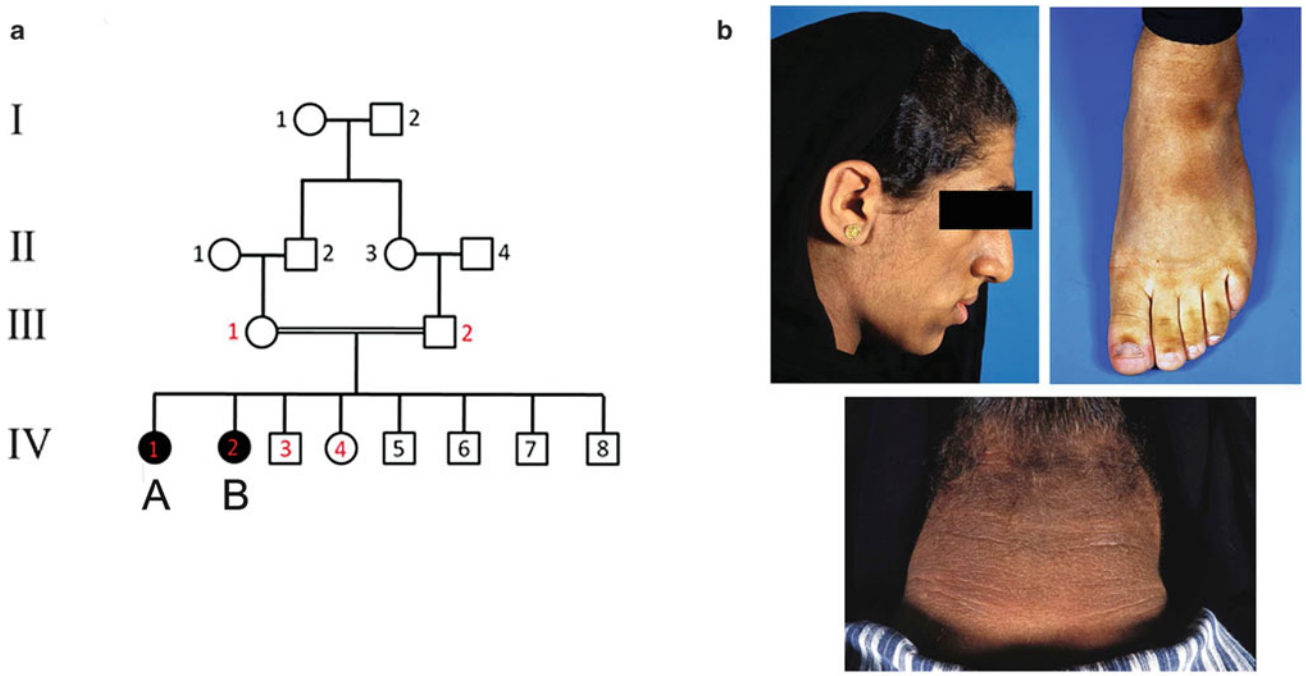
C3H10T1/2 murine mesenchymal stem cells and 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % newborn calf serum (NCS) as previously described (Payne et al. 2008). shRNA sequences targeting AGPAT2 were designed and cloned into the RNAi-Ready pSIREN-RetroQ vector (Clontech) and shRNA targeting luciferase used as a control. Wild-type human AGPAT2 was cloned into pcDNA3.1myc-HisA vector (Invitrogen) and p.Cys48Arg and p.Leu228Pro mutant forms created by site directed mutagenesis. Myc-tagged AGPAT2 constructs were subcloned into the pBabe retroviral vector. Retroviruses carrying shRNA or cDNA constructs were used to infect preadipocyte cell lines as previously described (Payne et al. 2008). Oil Red O staining of lipid accumulation was as described in (Payne et al. 2008).

Western Blot Analysis

Cells were scraped in lysis buffer (50 mM Tris pH6.8, 150 mM NaCl, 1mM EDTA, Complete Protease Inhibitor Cocktail (Roche), PhosSTOP Phosphatase Inhibitor Cocktail (Roche), and 50 mM n-β-D-glucopyranoside (Calbiochem)), sonicated, centrifuged at 14,000*g* for 10 min and protein concentration determined using a (BCA) kit (Bio-Rad). Samples containing equal amounts of protein were resolved on a NuPAGE® Novex® 4–12 % Bis-Tris precast gels (Invitrogen) and transferred using iBlot® (Invitrogen). Membranes were blocked in 5 % milk then probed with anti-myc (mouse monoclonal 4A6) (Milipore) or anti-calnexin antibodies. Secondary antibodies were from Thermo Scientific and signals were detected using ECL (GE Healthcare).

Real Time PCR

Cells were collected and RNA isolated using an RNAeasy kit (Qiagen). Purified RNA was reverse transcribed using



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	Patient		Reference Range
	A	B	
Age, years	16	19	
B.M.I., kg/m^2	19.6	20.1	18-25
Glucose, mmol/l	8.2	4.2	4 - 5
Insulin, pmol/l	1,700	393	18-173
C-peptide, pmol/l	6,900	1,400	380-665
IGF-1, μg/l	700	575	261-1,096
Triglyceride, mmol/l	25	14.8	<1.7
Testosterone, nmol/l	9.2	4.7	0.2-2.9
SHBG, nmol/l	9	27	18-114
Leptin, μg/l	0.4#	--	2.4-24.4
Adiponectin, mg/l	0.2#	--	4.4-17.7

Determined on a separate occasion

Fig. 1 (**a**) Family pedigree of the lipodystrophic patients in this study. Patients A and B are homozygous for the AGPAT2 p.Cys48Arg mutation, while family members in red are heterozygotes. (**b**) Appearance of patient A, showing acromegaloid overgrowth (*upper left image*) and acanthosis nigricans of the foot (*upper right image*) and nuchal acanthosis nigricans (*lower image*). (**c**) Biochemical profile of the patients studied

random hexamers and M-MLV reverse transcriptase (Promega). Gene expression was assayed by qPCR using either TaqMan® primers and probes (ABI Biosystems) or SYBR green and normalised to cyclophilin A.

Case Histories

This study was conducted in accordance with the Declaration of Helsinki and approved by the UK national research ethics committee. Written informed consent was obtained from both participants.

Patient A (14 years old at presentation) and her sister *patient B* (17 years old), both from Saudi Arabia, presented complaining of a male habitus and oligomenorrhoea. Their parents were first cousins (Fig. 1a). Both sisters were lean with marked hirsutism, androgenetic alopecia, deep voices, and muscle hypertrophy. They also had acanthosis nigricans with acrochordons (Fig. 1b), a markedly acromegaloid facial appearance (Fig. 1b), relatively large hands and feet, and although a paucity of subcutaneous fat was also noted, it was these features of overgrowth that were the focus of the initial diagnostic evaluation. Both patients had severe

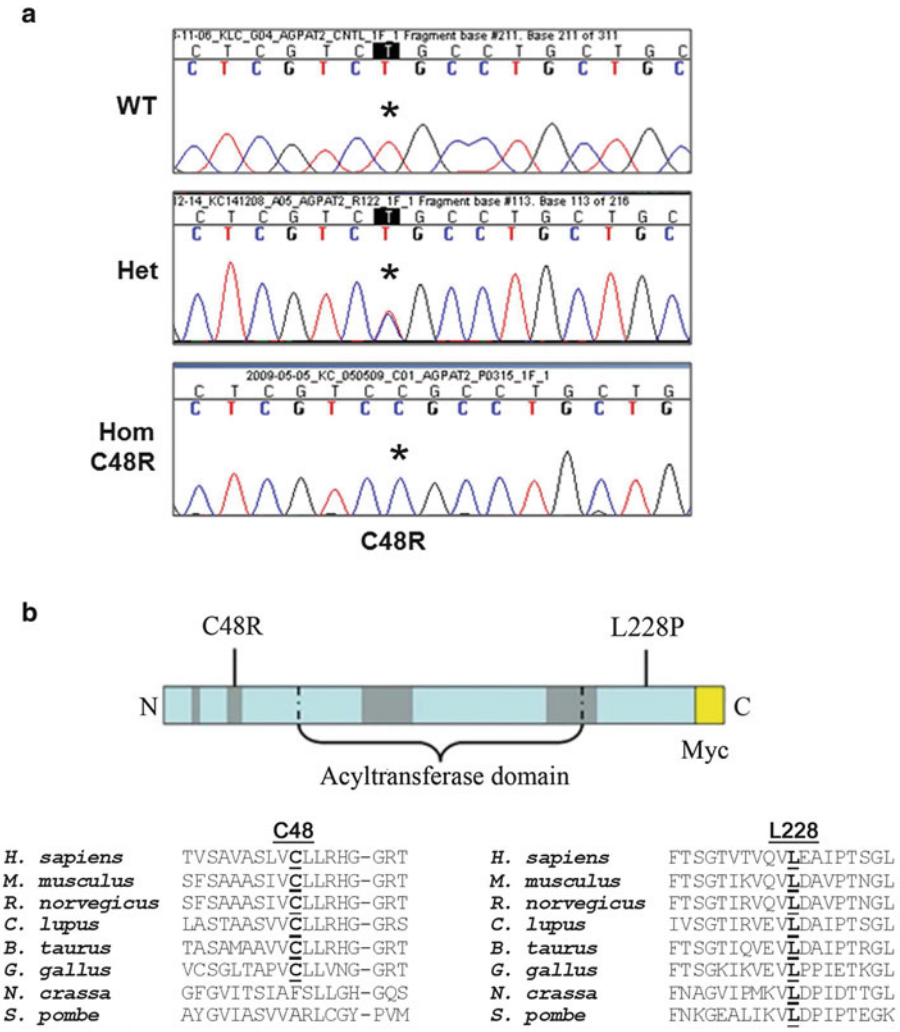

Fig. 2 (**a**) Representative chromatograms from sequencing genomic DNA of wild-type individuals (*upper panel*) or heterozygote (*middle panel*) or homozygote (lower panel) carriers of the AGPAT2 p.Cys48Arg mutation. (**b**) Schematic diagram showing the location of the acyltransferase domain and the p.Cys48Arg and p.Leu228Pro mutations within AGPAT2. Transmembrane domains are shown in *grey*. Phylogenic conservation of Cys48 and Leu228 are shown below

hyperinsulinemia, hypertriglyceridemia, and hyperandrogenemia with low sex hormone binding globulin (Fig. 1c). Serum IGF-1 and growth hormone response to 75 g oral glucose, and 17 hydroxyprogesterone response to 250 mcg synthetic ACTH were normal. At 15 years old patient A was found to be diabetic with an HbAIC of 16.4 %, and metformin, glyburide, gemfibrozil, and simvastatin were commenced. Subsequently insulin was introduced and titrated to 166 units/day, along with a maximal dose of pioglitazone. Despite these measures proliferative retinopathy, nephropathy, neuropathy, and severe hepatic steatosis developed. At that stage serum adiponectin and leptin were nearly undetectable, in keeping with near complete absence of adipose tissue. Patient B developed diabetes at 18 years old and is currently taking metformin and pioglitazone with no complications to date.

Results

No rare sequence variants were identified in the *BSCL2* gene; however, both affected probands were found to be homozygous for the *AGPAT2* c.142T>C transversion, producing the p.Cys48Arg missense change in the AGPAT2 protein (representative chromatogram Fig. 2a lower panel). Both parents, and two clinically unaffected siblings available for testing were heterozygous for the mutation (representative chromatogram Fig. 2a middle panel), which

was absent from more than 900 control patients from a panel of different ethnicities. In addition, the variant was not found in the NHLBI ESP Exome Variant Server, including around 4,000 people drawn from various cohort studies (NHLBI Exome Sequencing Project Exome Variant Browser (ESP), Seattle, WA, URL: http://evs.gs.washington.edu/EVS/) accessed October 2011). Cysteine 48 is highly phylogenetically conserved (Fig. 2b) and falls within the second transmembrane domain of AGPAT2.

To assess the effect of the AGPAT2 p.Cys48Arg mutation we generated 3T3-L1 murine preadipocytes stably expressing wild-type human AGPAT2, AGPAT2 Cys48Arg, or the previously described AGPAT2 Leu228Pro. The latter was selected as Leu228 is the most phylogenetically conserved of the residues reported to be substituted in CGL, being invariant in all species examined (Fig. 2b). This suggests that it may serve a particularly critical function in the mature AGPAT2 enzyme. Consistent with this AGPAT2 Leu228Pro has the lowest activity of any pathogenic single amino acid substitutions in AGPAT2 examined in a previous study (Haque et al. 2005). Western blotting revealed dramatically lower expression of the Leu228Pro and Cys48Arg mutant proteins compared to wild-type AGPAT2 in confluent preadipocytes (Fig. 3a). To investigate this further human AGPAT2 mRNA was determined using a quantitative real time PCR assay that did not detect murine AGPAT2 mRNA. As expected, no human AGPAT2 mRNA expression was detected in control cells whilst equal levels were present in cells expressing AGPAT2 wild-type, Leu228Pro, or Cys48Arg (Fig. 3b). The low expression of mutant AGPAT2 protein with normal mRNA levels suggests that both Leu228Pro and Cys48Arg AGPAT2 mutations most likely affect AGPAT2 folding and/or stability.

In order to model the in vivo situation in lipodystrophic patients more faithfully the effect of expressing wild-type or mutant AGPAT2 in murine preadipocytes with stable Agpat2 knockdown was examined. Murine Agpat2 was selectively knocked down in the C3H10T1/2 mesenchymal stem cell line using retroviruses expressing either control shRNA or mouse-specific shRNA against Agpat2. As shown in Fig. 3c, the low levels of Agpat2 present in undifferentiated cells were significantly reduced by expression of shRNA targeting murine Agpat2. When Agpat2 knockdown cells were induced to differentiate for 8 days in culture they showed significantly impaired expression of the key adipogenic transcription factor PPARγ and the insulin-sensitive glucose transporter Glut4, both well-characterised markers of adipogenesis (Fig. 3d). Human wild-type Leu228Pro or Cys48Arg AGPAT2 were then overexpressed stably using a second retroviral vector. As in 3T3-L1 cells, expression of Leu228Pro and Cys48Arg AGPAT2 proteins was significantly reduced compared to wild-type (Fig. 3e). This was despite equivalent expression of wild-type or mutant AGPAT2 mRNAs both as confluent preadipocytes and at various time points following induction of differentiation in these cells (Fig. 3f). Moreover the Leu228Pro and Cys48Arg AGPAT2 proteins remained undetectable at various time points tested following adipogenic induction (Fig. 3g). Consistent with this, expression of wild-type AGPAT2 partially rescued adipogenesis in AGPAT2 knockdown cells whereas neither Leu228Pro nor Cys48Arg mutant AGPAT2 constructs were able to do so when assessed by either light microscopy or oil red O staining of lipid accumulation (Fig. 3h) or by assessment of the induction of Pparγ mRNA expression (Fig. 3i).

Discussion

CGL is usually recognised in infancy due to severe lack of adipose tissue. However, these cases are a reminder that in older patients collateral features of lack of adipose tissue, including severe dyslipidaemia and fatty liver disease, and the hyperandrogenic and pseudoacromegalic features of severe insulin resistance, may instead dominate the clinical presentation. Thus, each of these should serve as 'red flags' alerting physicians to possible underlying lipodystrophy.

We investigated the effect of a novel pathogenic mutation in AGPAT2, p.Cys48Arg, and a previously described mutation, p.Leu228Pro, on the function of AGPAT2. Both mutations led to dramatically reduced protein expression suggesting that this may be the dominant reason for failure of adipose tissue formation in these patients. As the mutant forms of AGPAT2 were very poorly expressed it would not be trivial to assess their enzymatic activity accurately. As such we cannot formally exclude the possibility that, should these proteins be expressed in the affected individuals, they might also display reduced enzymatic activity. Whilst it may seem surprising that a single amino acid substitution so dramatically affects protein expression this has been observed with pathogenic mutations in other proteins such as steroid 5β-reductase (Mindnich et al. 2011). As the p.Cys48Arg mutation occurs in the putative second transmembrane domain of AGPAT2 it is possible that the mutation causes misfolding of this domain and altered membrane insertion, leading to degradation. However, a fuller understanding of the structure of AGAPT2 and further studies including modelling of this mutation would be required to determine this. A previous study of several pathogenic mutations in AGPAT2 including p.Leu228Pro (Haque et al. 2005) reported that Leu228Pro AGPAT2 exhibited significantly lower AGPAT activity than wild-type AGPAT2. However, critically, the expression of wild-type AGPAT2 was not directly assessed in parallel. Given our findings that

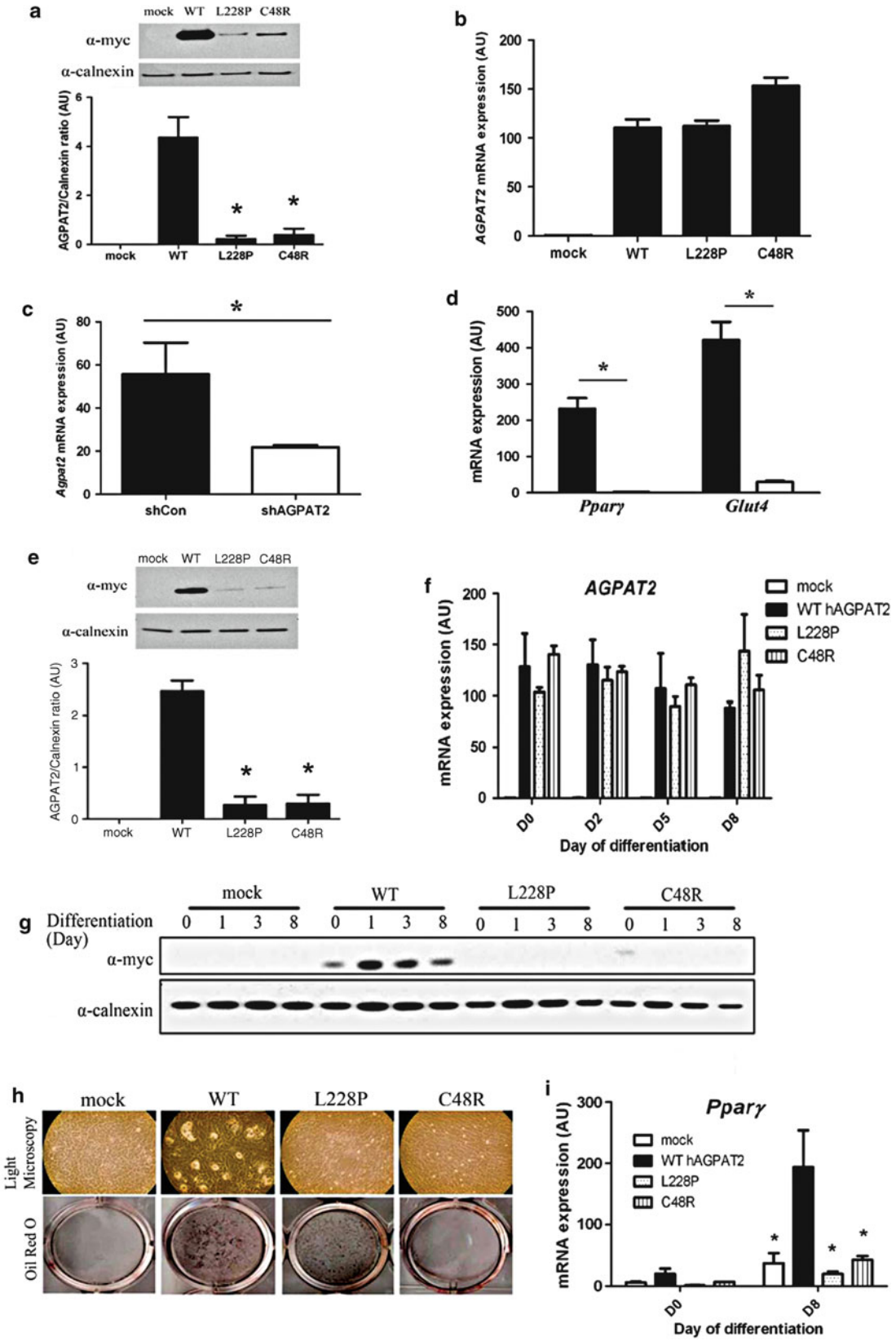

Fig. 3 (**a**) Protein levels of myc-tagged AGPAT2 determined by immunoblotting in 3T3-L1 preadipocytes stably expressing wild-type (WT) Leu228Pro (L228P) or Cys48Arg (C48R) AGPAT2 compared to mock infected cells. Data shown are mean +/− SEM of three independent experiments normalised to calnexin expression in the same samples as a loading control, * indicates a difference of $p < 0.05$ compared with expression of the wild-type AGPAT2. A representative blot is shown above. (**b**) AGPAT2 mRNA levels in the same cells as (A)

both p.Leu228Pro and p.Cys48Arg are expressed at significantly reduced levels, it will be interesting to determine whether this may also be so with other pathogenic mutants of AGPAT2 found in CGL patients.

The precise mechanism whereby AGPAT2 affects the complex process of adipogenesis remains unclear. However, it is evident that AGPAT2 deficiency does not merely result in the selective loss of triglyceride synthesis. Knockdown of AGPAT2 expression in cultured preadipocytes results in marked inhibition of adipogenic gene expression (Gale et al. 2006). The recent demonstration that, as in humans, Agpat2 deficiency in mice produces generalised lipodystrophy and severe insulin resistance provides an in vivo model for further investigations (Cortes et al. 2009). AGPAT2 loss may alter the generation of lipid species that can directly or indirectly play a role in modulating gene transcription. PA, the product of AGPAT2 activity, is a key intermediate in the production of several phospholipids which may influence both biogenesis and/or intracellular signalling. Interestingly, AGPAT2 inhibition in vascular smooth muscle cells suppresses the activation of PI3-kinase/AKT and MAPK pathways, both known to have roles in adipocyte differentiation (Coon et al. 2003). Evidently, further studies will be required to elucidate which, if any of these pathways, may be influenced by AGPAT2 during adipocyte development.

This study is the first to rescue adipogenesis in cultured cells lacking AGPAT2 by transfection with the wild-type enzyme and to demonstrate that expression of pathogenic mutants of AGPAT2 cannot do this. In the case of the mutants examined here this resulted from dramatically reduced expression of the mutants. However, it demonstrates that this system may be valuable for studying the underlying pathogenic mechanism of other mutants where protein expression is not affected.

In conclusion, we report the novel Cys48Arg pathogenic mutation in AGPAT2 and show that this and the previously described Leu228Pro mutation lead to dramatically reduced protein expression. Whilst we cannot exclude the possibility that Cys48Arg and Leu228Pro forms of AGPAT2 may also exhibit reduced enzymatic activity, we suggest that reduced expression of these mutated proteins may significantly contribute to the CGL phenotype seen in these patients.

Summary

Loss-of-function mutations in *AGPAT2*, encoding 1-acylglycerol-3-phosphate-O-acyltransferase 2 (AGPAT2), produce congenital generalised lipodystrophy (CGL). We screened the *AGPAT2* gene in two siblings who presented with pseudoacromegaly, diabetes, and severe dyslipidaemia and identified a novel mutation in *AGPAT2* causing a single amino acid substitution, p.Cys48Arg. We subsequently investigated the molecular pathogenic mechanism linking both this mutation and the previously reported p.Leu228Pro mutation to clinical disease. Wild-type and mutant AGPAT2 were expressed in control and AGPAT2-deficient preadipocyte cell lines. mRNA and protein expression were determined, and the ability of each AGPAT2 species to rescue adipocyte differentiation in AGPAT2-deficient cells was assessed. Protein levels of both p.Cys48Arg and p.Leu228Pro AGPAT2 were significantly reduced compared with that of wild-type AGPAT2 despite equivalent mRNA levels. Stable expression of wild-type AGPAT2 partially rescued adipogenesis in AGPAT2 deficient preadipocytes whereas stable expression of p.Cys48Arg or p.Leu228Pro AGPAT2 did not. In conclusion, unusually severe dyslipidaemia and pseudoacromegaloid overgrowth in patients with diabetes should alert physicians to the possibility of lipodystrophy. Both the previously unreported pathogenic p.Cys48Arg mutation in AGPAT2, and the known p. Leu228Pro mutation result in decreased AGPAT2 protein expression in developing adipocytes. It is most likely that the CGL seen in homozygous carriers of these mutations is largely accounted for by loss of protein expression.

(C) AGPAT2 mRNA levels in murine C3H10 T1/2 cells with (shAGPAT2) or without (Con) stable expression of shRNA targeting murine AGPAT2. (**d**) Pparg2 and Glut4 mRNA levels after 8 days of adipogenic differentiation in C3H10T1/2 cells stably expressing either control shRNA (*black bars*) or murine AGPAT2 shRNA (*white bars*) (**e**) Protein levels of AGPAT2 after stable expression of human wild-type or mutant AGPAT2 in C3H10T1/2 cells with stable murine Agpat2 knockdown determined by immunoblotting. Data shown are mean +/− SEM of 3 independent experiments normalised to calnexin expression in the same samples as a loading control, * indicates a difference of $p < 0.05$ compared with expression of the wild-type AGPAT2. A representative blot is shown above. (**f**) human AGPAT2 mRNA levels in the cells in (E) following induction of adipogenesis for the times shown. (**g**) Protein levels of myc-tagged wild-type and mutant AGPAT2 in the same cells as in (**f**) during adipogenic differentiation, assessed by immunoblotting, with calnexin as a loading control. (**h**) Triglyceride accumulation assessed by light microscopy (*upper panels*) and Oil Red O staining (*lower panels*) in C3H10T1/2 cells with stable Agpat2 knockdown and human wild-type or mutant AGPAT2 re-expression after 8 days of adipogenic differentiation. (**i**) Real time PCR analysis of Pparγ mRNA expression in C3H10T1/2 cells with stable Agpat2 knockdown and human wild-type or mutant AGPAT2 re-expression at day 0 and after 8 days of adipogenic differentiation. All real time PCR data are shown +/−SEM, $n = 3$, * indicates difference of $p < 0.05$, versus expression in cells re-expressing wild-type AGPAT2 at the same time point determined using two-tailed paired Student's T test. All western blots and cell images are representative of at least three independent experiments

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Synopsis

We have identified a novel mutation in the gene *AGPAT2* causing generalised lipodystrophy and characterised this in cultured models of adipocyte development which suggest that the mutation results in a failure to express the protein and a consequent lack of adipogenesis.

Conflict of Interest Statement

The authors declare that they have no conflict of interest relevant to the study reported in this manuscript.

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CASE REPORT

The Mild Form of Menkes Disease: A 34 Year Progress Report on the Original Case

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Abstract Classical Menkes disease is a neurodegenerative disorder caused by mutations in the copper-transporting ATPase *ATP7A* gene which, when untreated, is usually fatal in early childhood. A mild form of Menkes disease was originally reported in 1981 and clinical progress of the patient at 10 years described subsequently. The causative mutation is c.4085C>T in exon 21, causing an alanine to valine substitution in the highly conserved TM7 domain at the C-terminal end of the Menkes protein. Here we report his status at 34 years of age. Intellectual impairment is mild. Ataxia has nearly resolved but motor retardation, dysarthria and an extreme slow speech rate remain. In contrast to patients with the occipital horn syndrome, there have been no connective tissue complications of his mild Menkes disease. He has been under long-term copper therapy for more than 30 years and he continues to enjoy a good quality of life.

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Introduction

Menkes disease and the occipital horn syndrome are disorders at either ends of a spectrum caused by mutations in the copper-transporting ATPase *ATP7A* gene (OMIM #300011) (Kaler et al. 1994; Tumer et al. 1997). Additionally, specific mutations in *ATP7A* cause a distal motor neuropathy that is clinically distinct (Kennerson et al. 2010). Infant males with classical Menkes syndrome present in the first months of age with hypotonia, seizures and failure to thrive. Other manifestations include autonomic dysfunction, skin laxity, bladder diverticuli, pili torti hair changes and characteristic facies. Untreated boys with classical Menkes succumb in the first few years of life; however, early treatment with parenteral copper-histidinate may normalise the developmental outcomes in patients with residual enzyme activity (Kaler et al. 2008; Christodoulou et al. 1998). The occipital horn syndrome manifests with prominent connective tissue abnormalities as well as pathognomonic occipital exostoses; mild intellectual impairment may be seen (Tsukahara et al. 1994).

Between these two disorders lies the mild form of Menkes disease; the few described patients demonstrate developmental delay, variable connective tissue manifestations, pili torti and cerebellar ataxia but without seizures or childhood death (Gerdes et al. 1988; Proud et al. 1996; de Santos et al. 1984; Inagaki et al. 1988; Westman et al. 1988). Thus a clear distinction is drawn between classically affected patients with longer survival (including those treated with copper) and the mild Menkes patient.

Our patient was originally described by Procopis et al. in 1981 and his progress at 10 years of age was reported by David Danks (Procopis et al. 1981; Danks 1988). He presented with cerebellar ataxia, pili torti and vascular tortuosity. Follow-up demonstrated a predominantly motor delay with extreme dysarthria requiring a communication board until age 7. He commenced copper treatment at age 21 months and from 3 years has been on copper-histidinate injections. Here we describe his clinical status at the age of 34 years.

Case Report

When previously reported his major problems were cerebellar ataxia and dysarthria, both of which have improved, though not normalised, over time. He now mobilises independently without an ataxic gait and has a licence to drive a car. His movement is however not normal and is striking in the slowness with which he performs tasks. His speech remains extremely slow and dysarthric although the content is appropriate and his receptive understanding is not limited.

Formal intelligence testing has not been performed, but he is felt to function at the lowest end of normal. He underwent normal schooling, albeit with assistance from teachers, occupational therapists and speech pathologists. Long-term employment has been difficult to achieve, although he has worked periodically, predominantly due to his slowness of speech rather than intellectual impairment. Seizures have not occurred. Brain MRI was reported as normal at age 34.

Concurrent medical concerns have included nocturnal enuresis until the age of 20 years, subclinical hypothyroidism and frequent ear infections. Bladder ultrasound did not demonstrate diverticuli. He has long-standing and stable mild renal impairment with an estimated glomerular filtration rate of 57 ml/min/1.73 m^2 (>60 ml/min/1.73 m^2). There is stable microalbuminuria (urinary albumin:creatinine ratio 9.3 mg/mmol). Numerous investigations, but not including renal biopsy, have failed to demonstrate a cause and it is likely due to the damaging renal tubular effects of copper therapy (Lenartowicz et al. 2010).

His facial appearance in infancy had some features of the Menkes gestalt including pudgy cheeks and a carp-like upper lip. In adulthood these features have dissipated and he has a non-dysmorphic facial appearance. Pili torti was documented in previous reports; although his hair grows at a regular rate and he undergoes hair cuts every 6 weeks. Likewise his facial hair is normal and he sports a goatee beard (Fig. 1).

There was no orthostatic hypotension and his plasma catecholamines were normal (adrenaline <0.3 nmol/l, 0.0–1.5; noradrenaline 1.6 nmol/l, 0.1–6.3). There was no history of diarrhoea or abnormal sweating.

Connective tissue appears largely normal, without extraordinary skin elasticity, varicose veins, joint hypermobility or bony abnormalities such as scoliosis, radial head deformity or dislocation. Lateral skull X-ray did not show wormian bones or occipital horns. Arteriography at 21 months demonstrated vascular tortuosity; however, cerebral magnetic resonance angiography at age 34 years demonstrated only mild tortuosity of the cavernous portions of the internal carotid arteries.

Copper-histidinate injections were maintained at 2.4 mg every second day from his mid-teenage years until the age of 32 years when they were changed to 3 mg every third day. The decrease in injection frequency was at the patient's request as he found the injections increasingly difficult to comply with. Blood levels were monitored regularly via his local medical officer and most recently were slightly lower than usual with copper 9.1 umol/l (12–22) and caeruloplasmin 0.18 g/l (0.25–0.80).

The diagnostic biochemical parameters in blood, tissue and fibroblasts were reported in the original paper (Procopis et al. 1981), and since that time the family mutation in the *ATP7A* gene has been reported as c.4085C>T in exon 21, causing an alanine to valine substitution in the highly conserved TM7 domain at the C-terminal end of the Menkes protein that abrogates its copper-induced translocation to the plasma membrane (Ambrosini and Mercer 1999).

Discussion

In addition to our patient, a few other mild Menkes patients have been described and from this a phenotypic picture of variable intellectual impairment, ataxia and dysarthria, bladder diverticuli and vascular tortuosity emerges. Westman et al. described a 9 year old with near normal psychomotor abilities, bladder diverticuli and vascular tortuosity (Westman et al. 1988). Gerdes et al. described a 9 year old with borderline IQ and major problems of ataxia and bladder diverticuli (Gerdes et al. 1988). A more affected phenotype with prolonged survival comes from the family in which the responsible gene was cloned. These patients demonstrated various abnormalities across the spectrum of Menkes disease and Occipital Horn syndrome, with the 18 year old proband having mild intellectual disability, pili torti, bladder diverticuli, seizures, intracerebral haemorrhage, cutis laxa, vascular tortuosity, ataxia,

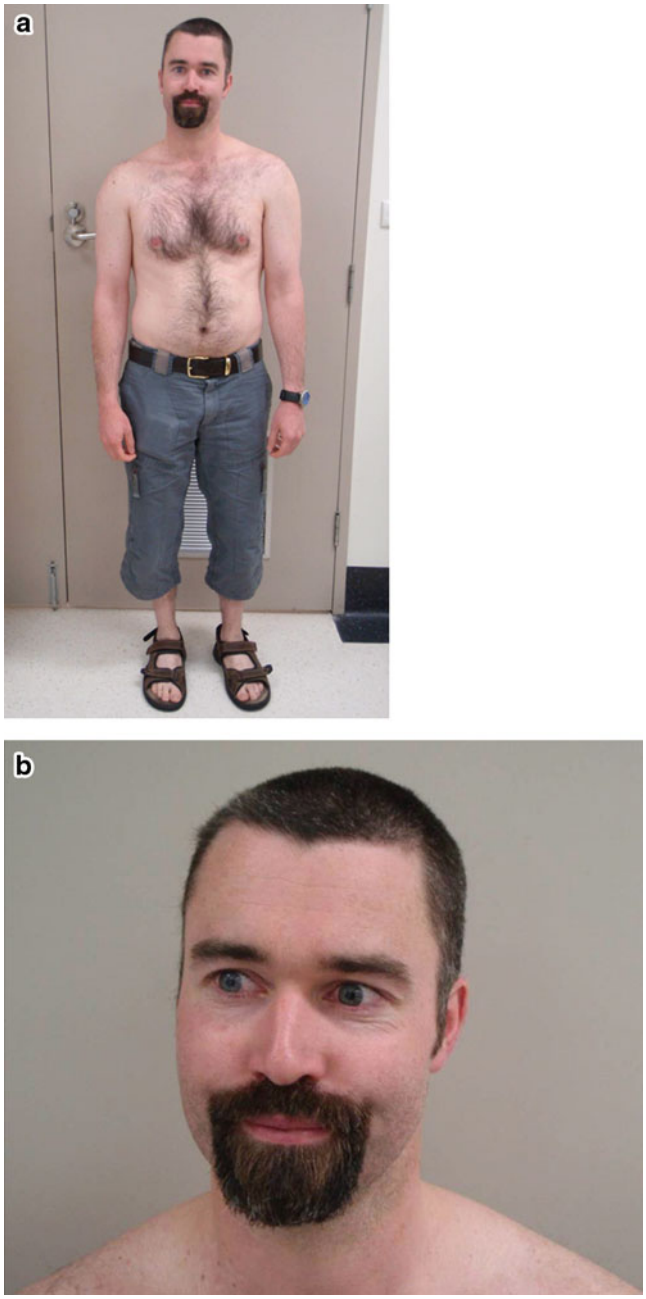

Fig. 1 Appearance of the patient at age 34

occipital horns, scoliosis, pectus and multiple joint dislocations (Kaler et al. 1994; Proud et al. 1996).

Our patient tolerated copper-histidinate injections for more than 30 years and has demonstrated a slow improvement in his dysarthria and ataxia. Additionally, cerebral vascular tortuosity has lessened over time. Dysarthria and motor retardation remain his biggest challenges. There have not been long-term complications and in general he enjoys a good quality of life.

Author Contributions

Michel Tchan: Wrote the draft article. Guarantor.
Bridget Wilcken: Revised the article.
John Christodoulou: Revised the article.

All three authors have shared clinical care of this patient.

Competing Interest Statement

None reported.

Details of Funding

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Ethics Approval and Patient Consent

Informed consent for publication of clinical information and photography was obtained from the patient and his mother.

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RESEARCH REPORT

Biochemical and Molecular Chitotriosidase Profiles in Patients with Gaucher Disease Type 1 in Minas Gerais, Brazil: New Mutation in CHIT1 Gene

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Abstract Chitotriosidase (ChT) is a human chitinase secreted by activated macrophages and its activity is used in therapeutic monitoring of Gaucher disease (GD), the most common lysosomal storage disease. About 6% of the population is homozygous for a duplication of 24 bp in exon 11 of the CHIT1 gene (dup24), which is the main polymorphism that results in the absence of ChT. As ChT enzyme activity can be used as a biomarker in GD, it is important to know the CHIT1 genotype of each patient. In this study, ChT activity and CHIT1 genotype were evaluated in 33 GD type 1 patients under treatment in the state of Minas Gerais, Brazil, and compared to healthy controls. As expected, the enzyme activity was found to be higher in GD type 1 patients than in healthy subjects. Four patients had no ChT activity. Their genotype revealed three patients (9%) homozygous for dup24 allele and one patient with two polymorphisms in exon 11: G354R and a 4 bp deletion at the exon-intron 11 boundary (g.16993_16996delGAGT), the later described for the first time in literature. Two other patients with lower ChT activity presented a polymorphism in exon 4 (c.304G>A, p.G102S), without dup24 allele. In conclusion, this study demonstrated that ChT activity can be used for therapeutic monitoring in 82% of GD patients of the state of Minas Gerais, Brazil.

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Introduction

Gaucher disease (GD; MIM 230800), the most prevalent of the sphingolipid storage disorders, is caused by a genetic defect that leads to glucocerebrosidase (β-glucosidade) deficiency (EC 3.2.1.45) and progressive accumulation of its substrate, glucocerebroside, in cells of monocyte/macrophage origin (Brady et al. 1965; Beutler and Grabowski 1995). Most patients present progressive visceral enlargement and gradual replacement of the bone marrow with lipid-laden macrophages, hematological abnormalities, and structural skeletal changes. Progressive neurologic deterioration develops in a minority (Beutler and Grabowski 1995).

Patients with GD type 1 are commonly treated by enzyme replacement therapy (ERT) with imiglucerase, only produced by Genzyme Corporation (Cambridge, MA, UK), a high-cost treatment provided by the Brazilian Ministry of Health. The parameter most frequently employed to monitor effectiveness of this therapeutic intervention is the activity of chitotriosidase (ChT) (Cox et al. 2000; Hollak et al. 1994, 2001; Mistry and Abrahamov 1997), a chitinase encoded by the chitotriosidase gene (CHIT1; MIM 600031). The CHIT1 gene is currently known to possess 13 exons with sizes ranging from 30 to 1055 bp (NG_012867.1, National Center for Biotechnology Information, 2012; http://www.ncbi.nlm.nih.gov/gene/1118).

The enzyme is synthesized by activated macrophages and is usually highly increased in serum from GD type 1 patients (Froissart 2006; Hollak et al. 1994). However, monitoring therapeutic response by measuring plasma ChT activity has a pitfall: the complete absence of enzymatic activity in approximately 6% of individuals (Boot et al. 1998). This observation can be explained by the homozygozity for a duplication of 24 bp in exon 11 of the CHIT1 gene (dup24, rs3831317) preventing formation of active enzyme (Boot et al. 1998). However, dup24, the main mutation described, is not the only one with implications for diagnosis and therapeutic monitoring of patients. Some polymorphisms, such as G102S (c.304G>A, p.G102S, rs2297950), G354R (c.1060G>A, p.G354R, rs9943208), and A442V (c.1325C>T, p.A442V, rs1065761) have been associated with reduced ChT activity (Bussink et al. 2009; Grace et al. 2007; Lee et al. 2007).

The relative scarceness of data regarding the incidence of GD in Brazil, the evidence that, as a group, Brazilian patients may have a more aggressive form of the disease (Sobreira et al. 2007), and the high cost of ERT to the public health budget prompted us to study the biochemical and molecular ChT profiles in an effort to assess treatment efficacy. Indeed, in 2007, about 500 patients with GD type 1 were treated in Brazil with 177,280 bottles of ERT being purchased at a cost of over US$ 125 million (Souza et al. 2010). The effective dose of imiglucerase is individually adjusted according to the clinical status and treatment response of the patient. The dose ranges from 15 to 60 U/kg and is supplied every 2 weeks (Martins et al. 2009). For this purpose we correlated the measure of plasma ChT activity with CHIT1 genotypes of GD type 1 patients from the Hospital das Clinicas of the Universidade Federal de Minas Gerais (HC-UFMG), and compared with healthy controls. Ultimately we reasoned that such studies could be valuable for the monitoring of ERT effectiveness and help physicians to devise the best treatment protocol strategies which would represent a more adequate use of treatment resources and improvement of therapy results for patients. We found that 9% of the patients analyzed (P1, P2, and P3) presented null or low ChT activity levels in spite of homozygous or heterozygous for the dup24 wild type allele.

Materials and Methods

Samples

Seventeen female and 16 male GD type 1 patients under treatment (32 in ERT with imiglucerase and one in substrate reduction therapy – SRT) at the reference center in HC-UFMG participated of this study. The median age of the patients was 27 years (range: 5–62 years) and the median treatment time was 11 years (range: 1–14 years). All patients had diagnosis confirmed by measurement of glucocerebrosidase activity in leukocytes (Beutler and Kuhl 1970) or cultured fibroblasts (Beutler et al. 1971). The control group consisted of 33 healthy volunteers of which 18 were females and 15 were males. The median age of the control group was 24 years (range: 8–52 years).

Ethics

The study was approved by the Ethics in Research Committee of Universidade Federal de Minas Gerais, under the numbers ETIC 625/07 and ETIC 0414.0.203.000-09, and informed consent was obtained from all participants.

CHIT1 Genotyping

Samples of 4 mL of peripheral blood were collected from GD type 1 patients in tubes containing EDTA and immediately subjected to DNA extraction according to the method described by Miller and coworkers (1988). DNA samples from 33 healthy controls were collected using buccal swabs and extracted with the BuccalAmp™ DNA Extraction Kit (Epicentre, Madison, WI, USA). The DNA samples were stored at −20 °C until molecular analysis.

Genotyping for dup24 in CHIT1 gene was performed by PCR using specific primers (ChTF: AGCTATCTGAAG-CAGAAG and ChTR: GGAGAAGCCGGCAAAGTC) as previously described (Boot et al. 1998). The 20 μL reaction contained 1x PCR Buffer (Sigma-Aldrich Co., St. Louis, MO, USA), 0.2 mM dNTPs, 0.2 mM of each primer, 1.25 mM $MgCl_2$, 0.5 U Taq polymerase (Sigma-Aldrich Co., St. Louis, MO, USA), and 50 ng of DNA template. The DNA was denatured at 95 °C for 2 min, and amplification was performed by 35 cycles at 95 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s. The amplified fragments were

Table 1 Primers used for analysis of the CHIT1 polymorphisms

Polymorphisms	Primers (5'–3')	Fragment Size	Annealing temperature	Reference
G102S	F: ACATAGGCACTTTCACACGTC R: AACAGCCTGGAGCAAAGCTC	380 bp	56 °C	Lee et al. (2007)
G354R*	F: GAATCTACAGCCACTCACAGG R: CAGGTAAGAGAGGAACAAGG	551 bp	56 °C	–
A442V*	F: GGGGAGCCTTGGTTGAATCT R: TGAGAGCAGAAAGCCTGGATA	423 bp	56 °C	–

* The primers for analysis of the G354R and A442V polymorphisms were based on CHIT1 genomic sequence from NCBI database (NG_012867.1)

separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Genotypes were determined by the presence or absence of wild type (75 bp) and/or mutant (99 bp) bands.

The three patients (P1, P2, and P3) who presented null or low levels of ChT activity in spite of being homozygous or heterozygous for the dup24 wild type allele were screened for three polymorphisms in CHIT1 that correlate with reduced ChT activity: G102S, G354R, and A442V (Bussink et al. 2009; Grace et al. 2007; Lee et al. 2007). PCR amplification of the regions containing the polymorphisms G102S (exon 4), G354R (exon 11), and A442V (exon 13) was performed using specific primers detailed in Table 1. The 40 μL reaction contained 1x PCR Buffer (Sigma-Aldrich Co., St. Louis, MO, USA), 1.5 mM dNTPs, 0.2 mM of each primer, 2.5 mM $MgCl_2$, 1 U Taq polymerase (Sigma-Aldrich Co., St. Louis, MO, USA), and 100 ng of DNA template. The DNA was denatured at 95 °C for 2 min, and amplification was performed by 35 cycles at 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s. The amplified fragments were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Fragments were purified from the agarose gel using the Promega Wizard® SV Gel and PCR Clean-up System kit (Promega Corporation, Madison, WI, USA) as recommended by the manufacturer. The purified samples were sequenced (forward and reverse direction) in the ABI 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA), using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, CA, USA) as recommended by the manufacturer. Sequences were analyzed and aligned to the reference sequence of CHIT1 gene NG_012867.1 using the CodonCode Aligner software (http://www.codoncode.com/aligner/).

To confirm heterozygosity for the G102S polymorphism in patient P2, the fragment containing the possible alteration was cloned. Cloning was performed on PCR®II-TOPO® plasmid using the TOPO TA Cloning Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA) as recommended by the manufacturer.

Plasma ChT Activity

Plasma was obtained by centrifugation from peripheral venous blood collected in EDTA and stored at –20 °C until enzymatic analysis at the Laboratory of Inborn Errors of Metabolism of HC-UFMG. The measurement of the ChT activity was performed as previously described by Hollak and coworkers (1994). Briefly, 5 μL of diluted plasma was incubated with 200 μL of a solution containing 0.022 mM of the artificial substrate 4-methylumbelliferyl-β-D-N-N′-N″-triacetylchitotrioside (Sigma-Aldrich Co., St. Louis, MO, USA) in citrate-phosphate buffer (0.1/0.2 M) (Sigma-Aldrich Co., St. Louis, MO, USA), pH 5.2, for 15 min at 37 °C. The reaction was interrupted by adding 2 mL of 0.3 M glycine buffer (Sigma-Aldrich Co., St. Louis, MO, USA), pH 10.6. The fluorescent 4-MU was measured with a spectrofluorometer Hitachi F-2500 (Hitachi High-Technologies Corporation, Tokyo, Japan), excitation (EX) at 366 nm and emission (EM) at 446 nm. The slit width was 5 nm, both EM and EX. The coefficient of variation (CV) between duplicates was 5%, which makes the test highly reproducible (Canudas et al. 2001; Comabella et al. 2009; Vellodi et al. 2005). ChT activity was expressed in nanomols of hydrolyzed substrate per hour per mL (nmol/h/mL).

Statistical Analysis

Analyses were performed using the statistical software package SPSS 14.0. Results are presented as median and range (minimum and maximum). Four GD type 1 patients and three healthy controls showed no detectable ChT activity and therefore were excluded from further statistical analysis (Canudas et al. 2001). The nonparametric Mann–Whitney test was used in order to evaluate differences in ChT activity between GD type 1 patients and controls, and also among patients and control individuals grouped by genotype. The same test was used to verify the effect of gender on enzyme activity. The relations between ChT activity and age were tested by the Spearman correlation. Chi-square test was used to evaluate differences in

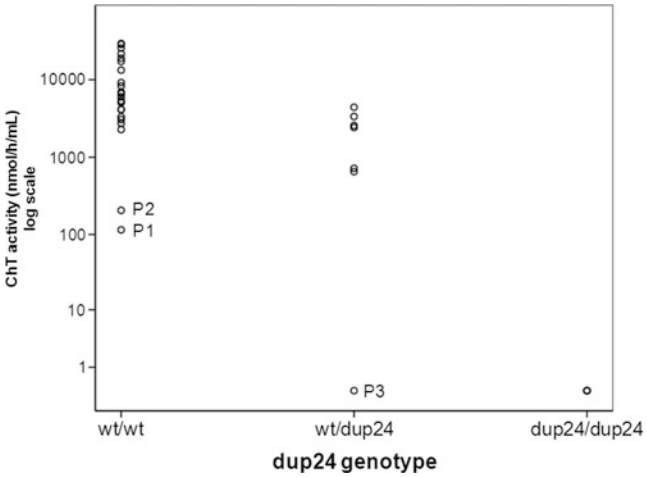

Fig. 1 Levels of ChT activity in 33 GD type 1 patients. Circles represent the ChT activity in each GD type 1 patient evaluated conform dup24 genotype. The enzyme activity was undetectable in patients with dup24/dup24 genotype ($n = 3$) and in patient P3 and reduced in patients with wt/dup24 genotype ($n = 7$). Patients P1, P2, and P3 are highlighted on the graph

frequency of dup24 genotype between genders in GD type 1 patients. In all tests, the level of significance was set at $p < 0.05$.

Results

Of the 33 GD type 1 patients, 23 (70%), seven (21%), and three (9%) were, respectively, wild type (wt/wt), heterozygous (wt/dup24), and homozygous (dup24/dup24) mutants for the allele dup24 (Fig. 1). Among healthy controls, 15 (45.5%), 15 (45.5%), and three (9%) showed, respectively, wt/wt, wt/dup24, and dup24/dup24 genotypes. The frequency of the wt and dup24 alleles among patients was 0.803 and 0.197, respectively, and 0.682 and 0.318 in the control group. The genotype distribution was in Hardy-Weinberg equilibrium.

As expected, plasma ChT activity was 176 times higher in 29 treated patients with GD type 1 (median: 5092, range: 115–29284 nmol/h/mL) than in control group (median: 29, range: 7–68 nmol/h/mL; $p < 0.0001$) (Table 2). Patients with wt/wt genotype (median: 6011, range: 115–29284 nmol/h/mL) showed ChT activity 194 times higher than healthy controls with the same genotype (median: 31, range: 17–68 nmol/h/mL; $p < 0.0001$). Patients with the genotype wt/dup24 (median: 2496 nmol/h/mL, range: 652–4417 nmol/h/mL) showed ChT activity 119 times higher than healthy wt/dup24 controls (median: 21, range: 7–52 nmol/h/mL; $p < 0.0001$) (Table 2). GD type 1 patients and controls with the genotype dup24/dup24 presented no ChT activity. Considering GD type 1 patients with different genotypes, the ChT activity was about twice lower in patients with the genotype wt/dup24 when compared to patients with the wt/wt genotype ($p < 0.05$).

No correlation was observed between ChT activity and age in both populations ($p > 0.05$). No relationship was noted between gender and ChT activity ($p > 0.05$) or gender and distribution of dup24 genotype ($p > 0.05$) in GD type 1 patients.

Two homozygous (P1 and P2) and one heterozygous (P3) patients for the dup24 wild type allele showed lower ChT activity levels than the average reported in their group (Fig. 1). Consequently, they were screened for three polymorphisms that have been shown to reduce ChT enzyme activity. We found that patient P1 (ChT activity of 115 nmol/h/mL) was homozygous for the polymorphism G102S, which is a guanine to adenine transition at position 304 of the cDNA (c.304G>A). Patient P2 (ChT activity of 206 nmol/h/mL) was heterozygous for the same polymorphism. Cloning confirmed the heterozygosity in this patient. Patient P3 (null ChT activity) presented two alterations in exon 11. This patient was found to be heterozygous for the polymorphism G354R, which is a guanine to adenine transition at position 1060 of the cDNA (c.1060G>A) and for a 4-base deletion in exon-intron 11 boundary (g.16993_16996delGAGT; rs143439055) (Fig. 2). This is the first report on this deletion to date.

None of the patients evaluated presented the polymorphism A442V, which is a cytosine to thymine transition at the position 1325 of the cDNA (c.1325C>T).

Discussion

To achieve a better monitoring of GD type 1 patients and correlate ChT activity with treatment efficacy we measured the enzyme levels and correlated them with the CHIT1 genotype of each of the 33 GD type 1 patients under treatment.

Our results showed that the ChT activity was higher in most GD type 1 patients analyzed than in the healthy controls, which is in accordance with previous studies (Hollak et al. 1994; Ries et al. 2006; Wajner et al. 2004, 2007). Wajner and colleagues (2004, 2007) reported that the ChT activity was around 600-fold greater in untreated GD patients of Brazilian origin than in healthy individuals. In the present study, GD type 1 patients also showed an elevation in the levels of enzyme activity; however, it was only 176 times higher. The divergence may be explained by the fact that all patients in our study were under treatment, whereas patients reported by Wajner and colleagues were receiving no treatment. We observed that ChT activity in GD type 1 patients homozygous wild type was approximately twice higher than in heterozygous patients. This result is in accordance with Schoonhoven and co-authors (2007), and

Table 2 Dup24 genotype and ChT activity in GD type 1 patients and controls

	N° of subjects	Genotype (%)	ChT activity (nmols/h/mL) Median (minimum–maximum)
GD type 1	33	100	5092 (115–29284)
wt/wt	23	70	6011 (115–29284)
wt/dup24	7	21	2496 (652–4417)
dup24/dup24	3	9	0
Control group	33	100	29 (7–68)
wt/wt	15	45.5	31 (17–68)
wt/dup24	15	45.5	21 (7–52)
dup24/dup24	3	9	0

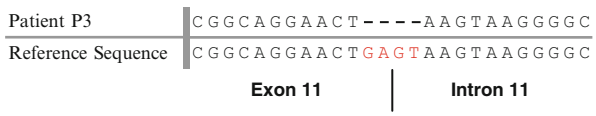

Fig. 2 4-base deletion (g.16993_16996delGAGT) at the exon-intron 11 boundary in patient P3. This deletion involves the last two bases of exon 11 (GA) and the first two positions of intron 11 (GT). The nomenclature g.16993_16996delGAGT was based on CHIT1 genome sequence from NBCI (NG_012867.1)

can be explained by the presence of the null allele dup24, which does not produce active enzyme (Boot et al. 1998).

The causes for this activity increase are not yet established. Some studies suggest that in GD type 1 patients the accumulation of the immunogenic components in macrophages, such as ceramide and sphingolipids, causes cellular activation and consequently ChT secretion, which may mediate the immune response involved (Ballou et al. 1996; van Eijk et al. 2005). Increased ChT activity was also recorded in several other diseases, such as Niemann-Pick (Brinkman et al. 2005), GM1 gangliosidosis (Malaguarnera et al. 2003), β-thalassemia (Barone et al. 1999), sarcoidosis (Boot et al. 2010), malaria (Barone et al. 2003), atherosclerosis (Artieda et al. 2003; Boot et al. 1999), and fungal and bacterial infections (Iyer et al. 2009; Labadaridis et al. 1998). The role of ChT enzyme is unclear, but a possible role in defense against chitin-containing pathogens and host immune response has been suggested (Choi et al. 2001; Di Luca et al. 2007; Di Rosa et al. 2005; Gordon-Thomson et al. 2009; Malaguarnera et al. 2005; van Eijk et al. 2005).

It is estimated that approximately 6% and 33–35% of the general population is homozygous and heterozygous for the mutation dup24, respectively (Boot et al. 1998). In our study, we found that 9% and 21% of GD type 1 patients present the genotypes dup24/dup24 and wt/dup24, respectively. Among healthy controls, 9% were dup24/dup24 and 45.5% were wt/dup24. The deviations observed can be attributed to the not large enough number of evaluated samples, and also to selection bias, since among GD type 1 patients there were six families, and twelve related individuals. The Hardy-Weinberg test revealed that the frequency of dup24 genotypes in GD type 1 patients and healthy controls is in equilibrium. However, the p value observed for the group of GD type 1 patients was $p = 0.06$, which is very close to the level of significance cut-off. This may also be due to the number of patients genotyped and/or selection bias.

Genotyping of polymorphisms in the CHIT1 gene for three patients (P1, P2, and P3) who presented divergent correlation between dup24 genotype and ChT activity revealed that patients P1 and P2 were, respectively, homozygous and heterozygous for G102S polymorphism. Grace and coworkers (2007) showed, using 4-MU-chitotrioside as substrate, that recombinant expressed G102S ChT had only 23% residual activity in relation to the wild type ChT. On the other hand, Lee and colleagues (2007) analyzing GD type 1 patients noted that this polymorphism was not associated with reduced enzyme activity. Bussink and co-authors (2009) reported that G102S affects differentially the ChT activity depending on the substrate used for enzyme measurement: when the substrate 4-MU-chitotrioside was used, the mutated ChT containing Ser102 showed reduced catalytic efficiency; whereas normal catalytic efficiency of the enzyme containing Ser102 was observed when the substrate 4-MU-deoxychitobioside (Aguilera et al. 2003) was used. In the present study, we used the substrate 4-MU-chitotrioside, and this may explain the low enzyme activity observed. Furthermore, the mentioned authors reported a considerable overlap of results and a relatively high frequency of the G102S allele in different ethnic groups. Thus, we cannot exclude the possibility that GD type 1 patients that were not genotyped do not present this polymorphism.

The patient P3 showed more complex changes in CHIT1 gene. Besides presenting the wt/dup24 genotype, this patient is heterozygous for the G354R polymorphism in exon 11, which has been previously associated with reduced ChT activity (Grace et al. 2007; Lee et al. 2007). Interestingly, this patient presented a 4-base deletion at the exon-intron 11 boundary (g.16993_16996delGAGT), first reported in this study. This deletion involves the last two bases of exon 11 and the first two positions of intron 11, altering the 5' donor splice-site. A similar genotype was described by Grace and colleagues (2007), whose patient with GD type 1 showed three alterations, called "complex E/I-10 allele": a G354R polymorphism, a silent base change (L385L) in exon 11, and a 4-base deletion in intron 11. The mutation described in the present study changes the 5'

donor splice-site, leading to a splice error which may underlie the absence of ChT activity observed in patient P3. This mutation may produce a truncated ChT enzyme, but future studies will be needed to confirm this hypothesis.

In conclusion, this study characterized the ChT activity in 33 GD type 1 patients from Minas Gerais state under ERT and SRT treatment and described a novel mutation in the CHIT1 gene with important implications for diagnosis and therapeutic monitoring of patients. We found that ChT activity cannot be used as biomarker in 18% of GD patients evaluated, since these patients had null (four patients) or low (P1 and P2) ChT activity. Such knowledge can improve the planning of treatment aiming at improving the response of GD type 1 patients to ERT and SRT.

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Synopsis

Biochemical and molecular chitotriosidase profiles in Brazilian patients of Minas Gerais state, affected by Gaucher disease type 1, including the discovery of a new mutation in CHIT1 gene, are reported.

Conflict of Interest

None declared

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CASE REPORT

A Young Adult with Sarcosinemia. No Benefit from Long Duration Treatment with Memantine

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Abstract Sarcosinemia is a rare inborn error of metabolism that is characterised by an increased level of sarcosine (N-methylglycine) in the plasma and urine. The enzymatic block results from a deficiency of sarcosine dehydrogenase (SarDH), a liver mitochondrial matrix enzyme that converts sarcosine into glycine. Although this condition may remain inapparent until later life, it has been reported in rare cases to lead to neurodevelopmental disability. A 19-year-old male with sarcosinemia presented with dystonia, developmental delay and cognitive impairment. Magnetic resonance imaging revealed vermian hypotrophy. A 2-year pharmacological treatment with memantine was negative on the clinical signs. In this case, it was concluded that the metabolic block leading to sarcosinemia was responsible of a pathologic condition with mental deficiency and complex neurological signs. A maternal isodisomy discovered in the vicinity of SarDH gene could contribute to this pathology. Deficit of SarDH may be considered as a differential diagnosis of growth failure during prenatal stages and respiratory failure at birth following a slowly progressive developmental delay.

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Introduction

Sarcosinemia (OMIM 268900) is a rare autosomal recessive inborn error of the one-carbon metabolism, with increased plasma level of sarcosine and high urine excretion. Sarcosine (N-methylglycine) is synthesised from dimethylglycine by dimethylglycine dehydrogenase and converted to glycine by sarcosine dehydrogenase (SarDH), within the mitochondrial matrix. Since the first case published in 1966 by Gerrsitsen and Waisman (1966), over around 30 patients were reported. The presentation is mostly by mental delay, but also cardiomyopathy, deafness and visual disturbance. Occasionally, deficit of SarDH might remain asymptomatic until later life (Christensen et al. 1989).

We report the case of 19 year old male in whom a sarcosinemia was diagnosed on a complex neurological syndrome developing progressively since childhood with a history of developmental delay. A follow-up of 18 months pharmacological trial is discussed.

Report of a Case

A 19 year young male was referred to our clinic because of the worsening of his neurological condition. From the parents, we learned that he had a long and progressive history of neurological impairment. Our patient was delivered prematurely at 34 weeks of uncomplicated gestation to non-consanguineous parents. The infant was

born small for date (weight 1,200 g, occipitofrontal circumference 28 cm, length 43 cm; –2 σ) but morphologically normal. He required a short ventilatory support because of mild respiratory difficulties and lethargy at birth. Because of low growth and reduced sucking he stayed in a continuous care unit during the first 2 months. Slight intermittent dystonic posture of the right leg and muscular hypotonia were noted in the first 14 months of his life. At the age of two, he started to walk but did not speak. Since the first school classes his education results were considered as weak but he learned to write. He was considered as awkward, with motor and speech disabilities (intelligence quotient of 56 in verbal and 57 in performance). Due to these difficulties, our patient had to attend a specialised school. At about the age of 9, gait progressively worsened due to dystonia and postural instability. He showed emotional lability with rare violent outbursts and behavioural problems. Around the age of 16, he progressively developed a dysarthria with blurred and slow speech and cerebellar ataxia. Otherwise, his neurological condition remained stable, without any acute exacerbation. At the age of 19, gait disturbance and dystonic movements became permanent, leading to hospitalisation. The physical parameters remained harmoniously low: occipitofrontal circumference of 53 cm (–2 σ), weight 43 kg (–3 σ) and height 163 cm (–1.7 σ). Other neurological symptoms included hyperreflexia without Babinski sign, hypotonia and dystonia exaggerated by stress and present both at rest and walking. There was a hollow foot with permanent internal rotation of the right leg. The dystonic posture of the body and the arms interfered with volitional movements and resulted in a clumsy gait, dropping and spilling, without losing ability to ambulate. Speech was slow, lacking normal prosody. Ophthalmological investigation revealed iris atrophy, congenital left eye cataract and poor saccadic adaptation.

Until this late period, he was considered as having late-onset atypical complications of a perinatal hypoxia, because of the history of respiratory distress at birth followed by complex neurological signs. However, since he was hypotrophic at birth, we considered that a developmental or metabolic illness should be considered. As such, we performed detailed laboratory and brain imaging analysis.

The MRI showed mild vermian atrophia without significant abnormality in the brainstem and the cerebrum or abnormalities in the myelination (Fig. 1a, b). The spectral profile within the pallidum was normal on a ^{1}H magnetic resonance spectrometry (^{1}H-MRS) (Fig. 1c, d).

Basic neuropsychological evaluation performed using standard test battery for intelligence, attention, visuo-spatial and executive functions showed a moderate dysexecutive syndrome with slow information processing speed, impairment of attention and planification processes. A 4 year follow-up showed a stable performance on global intelligence quotient assessment.

Ancillary blood tests including folic acid were within the reference intervals. A chromatography of urine showed an increased excretion of sarcosine at 1,214 μmol/mmol of creatinine. Excessive levels of sarcosine were found in plasma (319 μmol/L) and in CSF (7 μmol/L). All other amino acids were present at normal levels both in the plasma and urine except a slightly higher excretion of glycine considered as dependent of dietary factors (Table 1). Studies for inborn metabolic errors including organic acids, purines and pyrimidines, lactic and pyruvic acid were also normal in plasma, urine and CSF, thus excluding a possible glutaric aciduria type II. Thus, we raised the biochemical diagnosis of possible sarcosinemia. We found no abnormal blood and urine sarcosine levels in the other two siblings, the mother or the father.

Since sarcosine is a potent competitive inhibitor of glycine type 1 transporter (GlyT1) (Smith et al. 1992 May), we hypothesised that neurological and cognitive impairment in sarcosinemia could be related to an interference with NMDA receptors. Thus an NMDA receptor antagonist such as memantine could be considered as a symptomatic treatment option. With the informed consent of his parents, a memantine was given for 2 years (20 mg/day the first year, then 40 mg/day), in addition to a rehabilitation programme and orthopaedic treatment on the right leg. Loading tests with folic acid or riboflavin were not performed because of weak therapeutic efficiency reported (van Sprang et al. 1986).

No significant improvement of gait or cognitive performances was noted during the period of treatment with memantine. However, amino acid chromatography analysis of plasma, urine and CSF during the trial revealed some slight lowering of plasma levels and elevation in the CSF (Table 1).

Comment

We present a patient with movement disorder and developmental disabilities suggestive of brain damage where the biochemical analysis revealed sarcosinemia. The incidence of sarcosinemia is difficult to establish because as few as 30 cases were published during the last 50 years. No brain imaging data or follow-up information is available for the previously published cases. Since so few cases have been reported with sparse clinical descriptions, a detailed clinical phenotype could not be extracted from the literature. The only common feature was a low cognitive global performance. Although in some cases sarcosinemia was

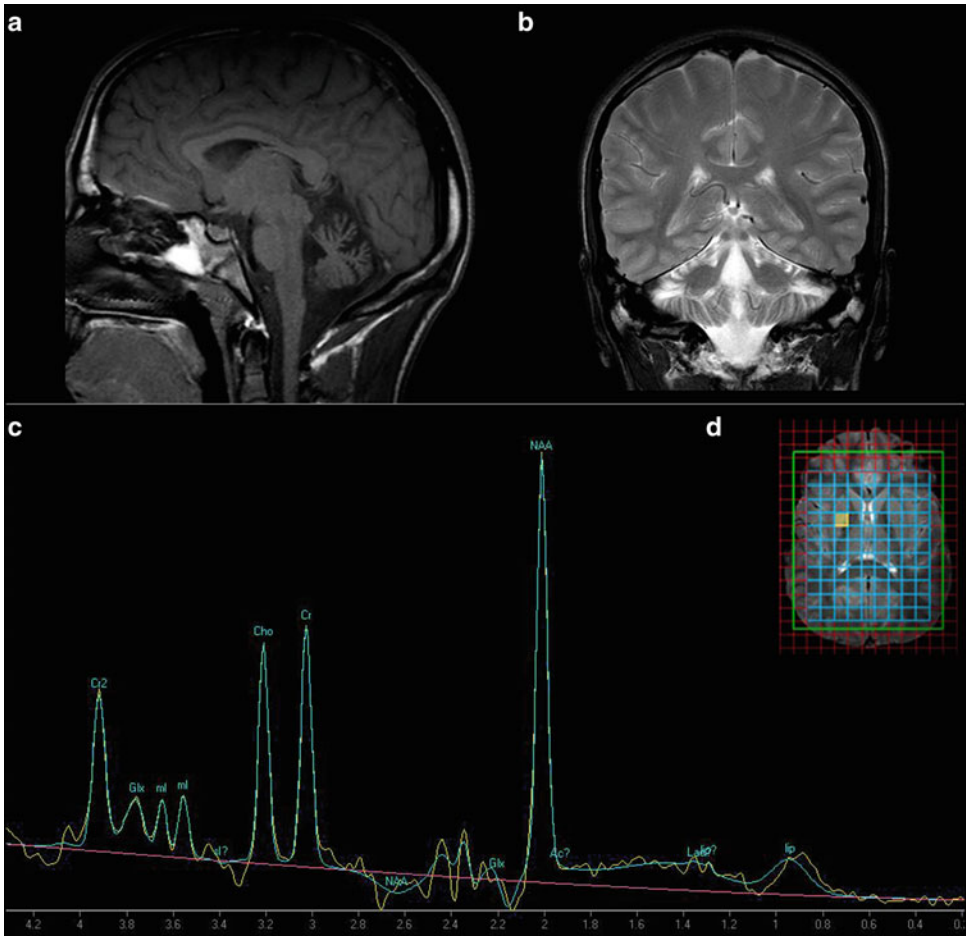

Fig. 1 A moderate antero-superior vermian atrophy on sagittal T1-weighted (TR/TE 425 ms/10 ms) (**a**) and on a coronal section on T2-weighted (**b**) brain MRI. The scan was performed before the memantine treatment. (**c**) Proton MR spectroscopy (1H-MRS) at the age of 21 (clinical system of 3T, TE: 30 ms, using commercially available software) reveals no significant changes at short TE. (**d**) Representative location of the MRS voxels on axial T2-weighted image within the basal ganglia

Table 1 Results of biochemical analysis. Nd: not determined.

Amino acids	Dec./ 07	Feb./ 08	Sept./08	Sept./09
Sarcosine (plasma) μmol/L (reference values <10)	319	326	379	238
Glycine (plasma) μmol/L (reference values =120–320)	254	255	291	310
Serine (plasma) μmol/L (reference values = 50–200)	145	135	116	133
Sarcosine (urine) μmol/mmol of creatinine(reference values < 50)	1214	655	631	nd
Glycine (urine) μmol/mmol of creatinine(reference values = 60–190)	419	236	198	nd
Serine (urine) μmol/mmol of creatinine(reference values = 20–50)	77	52	38	nd
Sarcosine (CSF) μmol/L(reference values = undetectable)	nd	7	12	12
Glycine (CSF) μmol/L (reference values = 7–11)	nd	6	7	7
Serine (CSF) μmol/L (reference values = 24–44)		32	28	29
Treatment with memantine	no	no	M7	M19

considered as a benign condition, usually a neurodevelopmental disability starts insidiously, before the brain is fully mature, and progresses slowly over several years. Our patient shares some symptoms (premature birth with respiratory distress, short stature and mental retardation) with previously reported cases of sarcosinemia. The neuropsychological

profile is not specific and is similar to the "cerebellar cognitive affective syndrome" (Schmahmann and Sherman 1998; Botez-Marquard et al. 1994). MRI revealed atrophy within the vermis and the anterior cerebellum. The lack of more obvious abnormalities in other parts of the brain suggests that myelination, dendritic arborisation and synaptogenesis are not impaired.

Other diagnostic options such as post-hypoxic encephalopathy or atypical neurodegeneration with brain iron accumulation (NBIA) were discussed. The mild respiratory difficulties at birth could hardly be responsible for significant hypoxic brain damage. None of the morphological or demyelinating sequelae usually reported in hypoxic brain damage (Rees and Inder 2005) was detected on repetitive brain imaging. In addition, we found no report of isolated cerebellar atrophy with slowly progressive late-onset ataxia occurring after brain anoxia in premature infants (Rees and Inder 2005). Furthermore, the low morphometric parameters at birth suggest that the clinical events were conditioned *intra uteri*, before the eventual perinatal hypoxia.

NBIA is a progressive extrapyramidal neurodegeneration, responsible for at least three different clinical forms always associated with abnormalities on MRI suggesting iron accumulation (Gregory et al. 2009). Considering the MRI images, an atypical NBIA due to *PLA2G6* mutation was suggested (A. Gregory, personal communication 2011). Further genetic studies on *PLA2G6* were not proposed with a view to the insignificant signal differences on the brain imaging in our patient compared to age-matched controls and the recent genetic data in our patient (Bar-Joseph et al. 2012).

The glycine levels in plasma and CSF in our patient were within the normal ranges except in the initial assay (Table 1). Still, we are not certain whether this assay is the appropriate measure of the potential pathological mechanism of sarcosinemia.

So far, the pathogenesis of sarcosinemia remains unexplained. The SarDH was initially identified in human brain but has high hepatic expression. The *SARDH* gene locus is on 9q34, which contains 21 exons and spans about 75 kb. A recent paper reports that our patient carries two maternal alleles in the vicinity of the 9q34 (Bar-Joseph et al. 2012). Even though no mutations were detected in the *SARDH* gene, this maternal isodisomy could be responsible for the sarcosinemia. These data may be helpful for early diagnosis and adequate nutrition early in life.

The clinical manifestations of our patient developed in a continuum of gradually impaired CNS dysfunctions of the structures involved in the movement control and global cognition rather than an acute perinatal event. We consider that the underlying cerebellar involvement may be involved in his cognitive profile. In light of these results, it seems that sarcosinemia may lead to harmoniously small antenatal size and premature birth, followed by slow evolution of cognitive and neurological signs that affect daily living functions and social integration. Our case adds some additional evidence that sarcosinemia may be associated to neurodevelopmental disability.

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Appendix

Relevant Conflicts of Interest/Financial Disclosures

Nothing to report. SB has been involved as main study coordinator in a clinical trial of memantine in Alzheimer's disease.

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RESEARCH REPORT

Amino Acid Profiles in Patients with Urea Cycle Disorders at Admission to Hospital due to Metabolic Decompensation

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Abstract Urea cycle disorders (UCDs) result from inherited defects in the ammonia detoxification pathway, leading to episodes of hyperammonaemia and encephalopathy. The purpose of this study was to answer the question, "what is the likely plasma amino acid profile of a patient known to have a UCD presenting with hyperammonaemia during acute metabolic decompensation", in order to support informed decisions regarding management.

We analysed the results of plasma ammonia levels and amino acid profiles taken simultaneously or within 30 min of each other during acute admissions of all patients with a UCD at the Royal Children's Hospital, Melbourne, over 28 years. Samples from 96 admissions (79, 9 and 8 admissions for OTC, CPS and ASS deficiencies, respectively) from 14 patients fulfilled these criteria. Amino acid levels were measured by ion exchange chromatography with post-column ninhydrin derivatisation and interpreted in relation to age-related reference ranges.

Plasma concentrations of all measured essential amino acids were low or low-normal in almost all samples. There was a strong positive correlation between low plasma branched-chain amino acids and other essential amino acids, and a negative correlation between ammonia and phenylalanine to tyrosine (Phe:Tyr) ratio in patients with OTC deficiency, and between glutamine and Phe:Tyr ratio in all patients, indicating protein deficiency.

Conclusion: At admission, protein deficiency is common in patients with a UCD with hyperammonaemia. These results challenge the current guideline of stopping protein intake during acute decompensation in UCDs. Supplementation with essential amino acids (particularly branched-chain amino acids) at these times should be considered.

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Introduction

Urea cycle disorders (UCDs) result from inherited defects in the hepatic ammonia detoxification pathway. The six urea cycle enzymes are carbamoyl phosphate synthetase I (CPS I) and its allosteric activator N-acetylglutamate synthetase (NAGS), ornithine transcarbamylase (OTC), argininosuccinic acid synthetase (ASS), argininosuccinic acid lyase (ASL), and arginase (ARG) (Leonard 2000). The clinical manifestations of these disorders are variable and UCD phenotypes range from neonates dying of the complications of hyperammonaemia in the first week of life to apparently asymptomatic adults. These disorders may lead to episodes of hyperammonaemia and encephalopathy during metabolic decompensation, which may occur at any age. Patients are treated with a normal-high calorie, low-protein diet and nitrogen-scavenging medications (sodium benzoate and/or sodium phenylbutyrate). Arginine, which becomes an essential amino acid in patients with a UCD (except for argininaemia), is also provided in sufficient amounts to overcome potential deficiency (Leonard and Morris 2002).

Table 1 Clinical details and number of samples from patients with UCD (*Patient found to have mosaic c.533 C>T (p.T178M) mutation. Mosaicism estimated at 50% based on hair root follicles and saliva specimen)

Patient	Disorder	Sex	Current age	Diagnostic confirmation	No. of samples (< 6 years)	No. of samples (> 6 years)
1	CPS I deficiency	Female	4 years 2 months	Mutation	1	0
2	CPS I deficiency	Male	6 years 2 months	Mutation	1	0
3	CPS I deficiency	Male	7 years 9 months	Mutation	1	0
4	CPS I deficiency	Male	15 years 4 months	Mutation	0	6
5	OTC deficiency	Female	15 years	Biochemical (cascade screening)	0	7
6	OTC deficiency	Female	12 years 4 months	Mutation	2	16
7	OTC deficiency	Female	7 years 10 months	Biochemical (cascade screening)	2	0
8	OTC deficiency	Female	9 years 1 month	Mutation	34	3
9	OTC deficiency	Male	8 years 9 months	Mutation*	11	1
10	OTC deficiency	Female	Deceased	Biochemical (cascade screening)	0	2
11	OTC deficiency	Female	2 years 9 months	Mutation	1	0
12	ASS deficiency	Male	Deceased	Biochemical	1	0
13	ASS deficiency	Male	30 years 2 months	Biochemical (cascade screening)	3	0
14	ASS deficiency	Female	Deceased	Biochemical	4	0

Amino acid profiles are a powerful determinant in the management of patients with a UCD and are considered a requisite in the follow-up of these patients (Leonard 2001; Summar 2001). However, there are very few published reports in which sufficient attention has been given to the timing of blood sampling (fasting hours), in order to obtain meaningful blood concentrations that can serve for therapeutic decision making. This study aimed to examine the relationships between plasma ammonia and amino acid profiles of patients with a UCD admitted during acute decompensation, in order to answer the question, "what is the likely plasma amino acid profile of a patient known to have a UCD presenting with hyperammonaemia during acute metabolic decompensation", and thus to facilitate a preferred therapeutic decision. We also wanted to identify possible correlations between ammonia and specific amino acids at the time of decompensation.

Methods

We reviewed the results of plasma ammonia levels and amino acid profiles of all patients with a known UCD admitted to the Royal Children's Hospital, Melbourne, due to metabolic decompensation, from January 1982 to December 2010. All patients have been treated in one centre under the same therapeutic protocol: protein restriction whilst providing sufficient energy intake, sodium benzoate and arginine (some patients with OTC deficiency were treated with citrulline for a short period). During acute admissions due to hyperammonaemia, protein intake is stopped for up to 24 h and is gradually re-introduced thereafter. Energy (calorie) intake is increased to >100% recommended daily intake. Sodium benzoate and arginine are given at a loading dose, followed by maintenance daily dose. The institutional ethics committee approved the study (HREC 32082A).

Inclusion criteria in this study were all acute admissions where plasma amino acid profiles and ammonia concentrations were undertaken simultaneously or within 30 min of each other. Information regarding the patients and the number of samples obtained from each is provided in Table 1. Seven patients with OTC (one male) provided 1–37 samples each; four patients with CPS I deficiency (one female) provided 1–6 samples each; three patients with ASS deficiency (one female) provided 1–4 samples. All samples for ammonia and amino acids were taken and analysed at the same site. Ammonia levels and plasma amino acid profiles were determined under the same protocols during these years. Amino acid concentrations were measured by ion exchange chromatography with post column ninhydrin derivatisation. Sequential models of Biochrom amino acid analysers have been used during the period in review. Quality assurance and validation testing were undertaken during transition between machines, using Sigma Aldrich standards and complying with external quality control programmes, as required for laboratory accreditation. Due to the fact that normal values of each

amino acid vary with age, correlations were performed in two age groups of less than 6 and greater than 6 years, as per the two age-related normal ranges of our laboratory, which are based on Armstrong and Stave (1973) and Applegarth et al. (1979). There were only four samples from infants <3 months old (three neonates) and their results were incorporated within the <6-year-old group of patients. Each plasma amino acid concentration was first compared to the relevant reference range and then correlated to other amino acids and to ammonia. Ratios of particular amino acids were then calculated to determine the extent of any correlation with ammonia or glutamine. Amino acids were also divided into groups: Branched-Chain Amino Acids (BCAA), Large Neutral Amino Acids (LNAA), Essential Amino Acids (EAA) and Non-Essential Amino Acids (NEAA). Due to the absence of some amino acids from the profiles provided by our laboratory, EAA comprised valine, isoleucine, leucine, phenylalanine, threonine, tyrosine, methionine, lysine and histidine, but not tryptophan.

Data analysis and graphing was performed in Excel 2007® (Microsoft, Seattle, WA, USA) using native correlation and regression analysis together with the Analyse-It® add-on (www.analyse-it.com). Descriptive statistics including medians and interquartile ranges were calculated. Standard statistical tests used included Spearman's rank, together with two-tailed p-value calculation. We used the Šidák Correction to correct for the large number of comparisons; thus a stringent p-value of 0.004 or less was deemed significant.

Results

Samples from 96 admissions (79, 9 and 8 admissions for OTC, CPS and ASS deficiencies, respectively; none from ASL deficiency) from 14 patients fulfilled the inclusion criteria.

Figure 1a and 1b depicts the concentrations of each amino acid in the OTC-deficient group (the largest in our cohort), compared with an age-specific reference range. Plasma concentrations of ammonia, glutamine and citrulline in patients with CPS I, OTC and ASS deficiencies are presented in Table 2 (a small number of samples from patients with OTC deficiency were taken at the period when these patients were treated with citrulline, leading to a wide range of plasma citrulline concentrations in this group but a low median concentration, as would be expected).

Plasma concentration of most amino acids, particularly the essential amino acids, was low-normal or low. All three BCAA were below the normal range in 35/96 (36.5%) of all samples and in 30/79 (38.0%) of OTC-deficient patients. Of the BCAA, valine concentration was most frequently low (in 57/96 (59.4%) of all samples and 51/79 (64.9%) of OTC-deficient patients), and by the greatest margin when compared with its reference range.

Glutamate concentration was above the normal range in 38/96 (39.6%) of all patients and 31/79 (39.2%) of OTC-deficient patients. Valine concentration was low in 27/38 (71%) and 24/31 (77.4%) of these samples, respectively. Leucine concentration was low in 25/38 (65.8%) and 21/31 (67.7%) of these samples, respectively, and isoleucine was low in 23/38 (60.5%) and 18/31 (58.1%), respectively.

Plasma alanine concentrations were above the normal range in 23/96 (24.0%) of all samples and 20/79 (25.3%) of OTC-deficient patients. The plasma concentration of other amino acids varied. Arginine concentration varied, as would be expected given that the patients may or may not have had a dose of arginine just prior to coming to hospital.

Ammonia concentration correlated with glutamine concentration in samples from all patients and in those with OTC deficiency, particularly those who were <6 years old ($\rho = 0.5$ $p < 0.0001$ and $\rho = 0.53$ $p < 0.0001$, respectively) (Table 3; Fig. 2), but not in samples from children >6 years of age. There was only a trend towards a correlation between glutamine and ammonia in the CPS deficiency group (all patients: $\rho = 0.70$ $p = 0.0347$; there were only three samples of patients <6 years, too few to calculate a meaningful Spearman's Rank; patients >6 years of age: $\rho = 0.61$ $p = 0.1997$). There was no correlation between glutamine and ammonia in the ASS deficiency group ($\rho = 0.17$ $p = 0.693$). Glutamine concentration was above the normal age-specific range in 87/96 (90.6%) of all samples and in 76/79 (96.2%) of patients with OTC deficiency. Given that there is a continuum of changes in amino acid concentrations in blood (and that the cut-off point between <6 and >6 is somewhat arbitrary), we explored the relationship between glutamine and age. A trend towards some correlation was found in OTC-deficient patients ($\rho = 0.28$; $p = 0.0122$) but not in other groups.

There was a trend towards a negative correlation between glutamine and glutamate in the >6 year old patients in the whole cohort ($\rho = -0.4$; $p = 0.0206$) and in the OTC-deficient patients ($\rho = -0.47$; $p = 0.0117$), but not in the younger group.

The Phe:Tyr ratio has been used as a marker for protein turnover and whole body protein balance (de Betue et al. 2011). There was a negative correlation between glutamine and Phe:Tyr ratio ($\rho = -0.34$; $p = 0.0008$), particularly in the <6 year old age group ($\rho = -0.45$; $p = 0.0003$), which was very similar in the young OTC-deficient patients ($r = -0.49$; $p = 0.0003$) (Fig. 3). There was a somewhat smaller negative correlation between ammonia and Phe:Tyr ratio in this age group ($\rho = -0.3$; $p = 0.0205$), which was stronger in the OTC-deficient patients ($\rho = -0.48$; $p = 0.0004$) (Fig. 4). These correlations were not noted in older children.

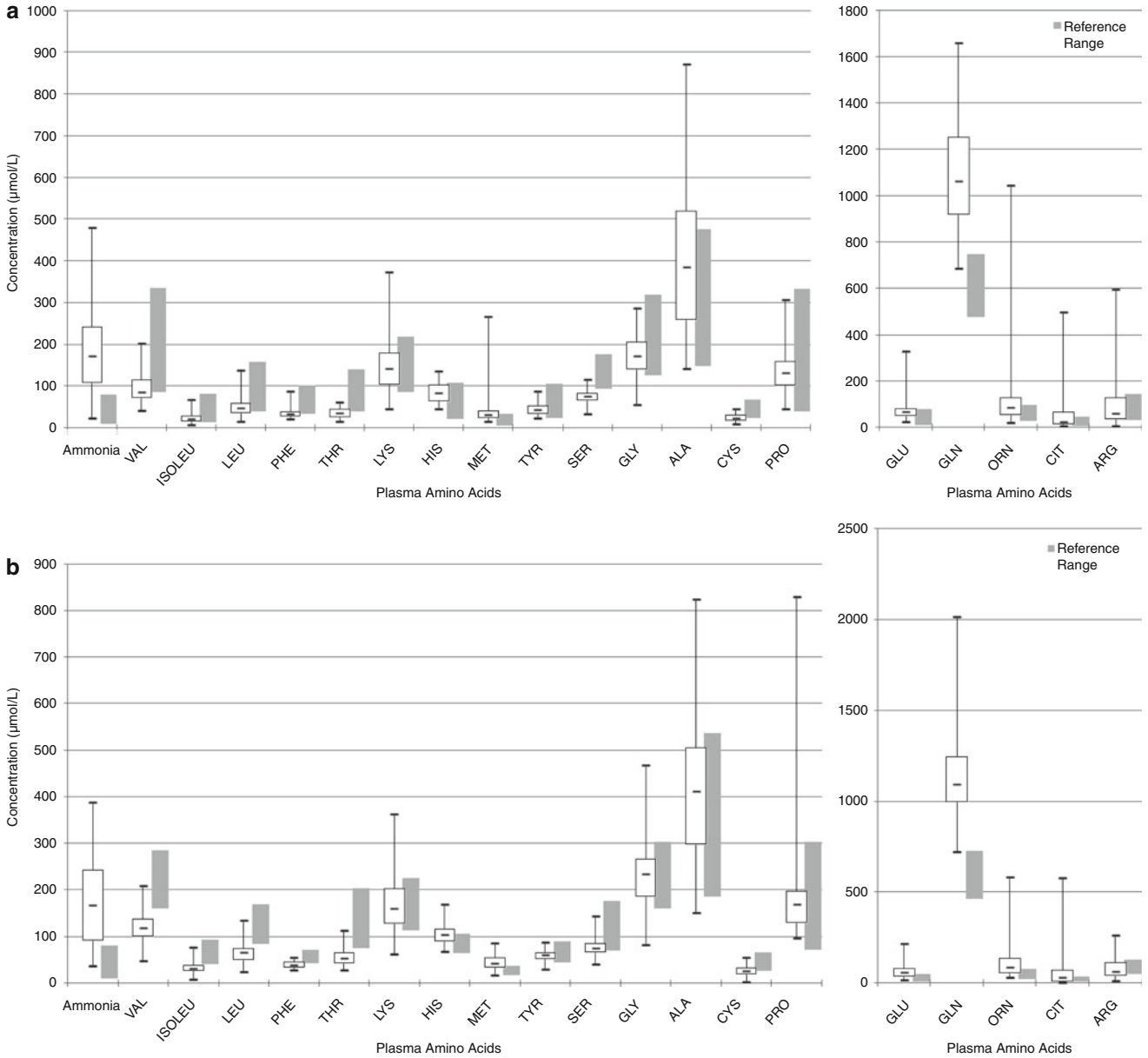

Fig. 1 Plasma amino acids concentrations in patients with OTC deficiency < 6 years old (**a**) and > 6 years old (**b**). *Boxes* represent interquartile ranges. *Horizontal bars* within boxes represent the median. *Vertical bars* represent the range of concentrations

There was a strong correlation between BCAA concentrations and those of other essential amino acids in the whole cohort, regardless of age (Table 3). There were only 4 out of 35 samples where the concentrations of all BCAA were low while those of the other essential amino acids (phenylalanine, tyrosine and threonine) were normal.

There was a strong correlation between plasma alanine and proline concentrations in all patients ($\rho = 0.6$; $p < 0.0001$), which was more obvious in patients of >6 years of age ($\rho = 0.74$; $p < 0.0001$) (Table 3). Plasma alanine concentrations did not correlate with ammonia concentrations (Table 3).

Discussion

Amino acid profiles are a powerful determinant in the management of patients with a UCD and are considered a requisite in the follow-up of these patients (Leonard 2001; Summar and Tuchman 2001). The rationale for this study was to provide information about the most likely plasma amino acid pattern in patients with a UCD at the time of decompensation and presentation to hospital, in order to guide appropriate treatment prescription. To this end, we reviewed the results of plasma amino acid profiles taken at the time of admission to our hospital due to

Table 2 Ammonia, glutamine and citrulline concentrations (in μmol/L) in blood samples from patients with CPS I, OTC and ASS deficiencies

	CPS I <6 years $n = 3$	CPS I >6 years $n = 6$	OTC <6 years $n = 50$	OTC >6 years $n = 29$	ASS <6 years $n = 8$	ASS >6 years $n = 0$
Ammonia	Median value = 86; range = 37–231; IQR = 62–159	Median value = 131; range = 17–171; IQR = 67–154	Median value = 172; range = 23–479; IQR = 109–241	Median value = 167; range = 38–389; IQR = 93–243	Median value = 209; range = 48–330; IQR = 175–286	No samples
Glutamine	Median value = 780; range = 662–933; IQR = 721–857	Median value = 846; range= 607–920.9; IQR = 710–897	Median value = 1061; range = 688–1658; IQR = 919–1,250	Median value = 1095; range = 723–2,015; IQR = 997–1,250	Median value= 917; range = 320–1,299; IQR = 553–1,050	No samples
Citrulline	Median value = 7; range= 4–9; IQR = 6–8	Median value = 7; range= 4–263; IQR = 5–167	Median value = 26; range=7–497; IQR = 14–66	Median value = 32; range= 5–578; IQR = 13–69	Median value = 3,297; range = 1,330–15,433; IQR = 2,300–4,090	No samples

IQR Interquartile range

Table 3 Spearman Rank correlations (ρ) between ammonia and amino acids and between amino acids themselves in all patients and in patients with OTC deficiency, based on age groups (* = $p < 0.004$)

			All patients (CPS, OTC, ASS deficiency)			OTC deficiency		
			All ages	<6 years	6–18 years	All ages	<6 years	6–18 years
Ammonia	GLN	ρ	0.33	0.5	0.08	0.27	0.53	−0.17
		p	*	*	0.64	0.01	*	0.37
Ammonia	PHE:TYR	ρ	−0.24	−0.3	−0.18	−0.4	−0.48	−0.25
		p	0.01	0.02	0.29	*	*	0.19
GLN	GLU	ρ	0	0.26	−0.4	−0.08	0.19	−0.47
		p	0.97	0.04	0.02	0.46	0.17	0.01
GLN	PHE:TYR	ρ	−0.34	−0.45	0	−0.43	−0.49	−0.1
		p	*	*	0.98	*	*	0.61
BCAA#	PHE	ρ	0.47	0.47	0.41	0.6	0.49	0.62
		p	*	*	0.01	*	*	*
BCAA#	TYR	ρ	0.59	0.5	0.59	0.75	0.68	0.71
		p	*	*	*	*	*	*
BCAA#	THR	ρ	0.53	0.44	0.57	0.7	0.57	0.77
		p	*	*	*	*	*	*
BCAA#	LYS	ρ	0.52	0.49	0.40	0.57	0.54	0.41
		p	*	*	0.01	*	*	0.03
BCAA#	HIS	ρ	0.24	0.33	0.13	0.43	0.39	0.36
		p	0.01	0.01	0.47	*	0.01	0.05
BCAA#	MET	ρ	0.32	0.48	0.03	0.47	0.49	0.23
		p	*	*	0.86	*	*	0.22
ALA	GLN	ρ	0.33	0.39	0.18	0.32	0.38	0.14
		p	*	*	0.30	0.05	0.07	0.46
ALA	PRO	ρ	0.6	0.54	0.74	0.65	0.59	0.85
		p	*	*	*	*	*	*
ALA	Ammonia	ρ	0.11	0.02	0.32	0.11	0.05	0.27
		p	0.27	0.87	0.05	0.31	0.74	0.15

#*BCAA* = Molar sum of LEU, ILE, VAL

hyperammonaemia, as a common denominator. To the best of our knowledge, this is the first study to document plasma amino acid profiles at times of decompensation, as opposed to chronic follow-up management. Statistical analysis of the results indicates that the correlations found when considering just OTC-deficient patients are stronger than when

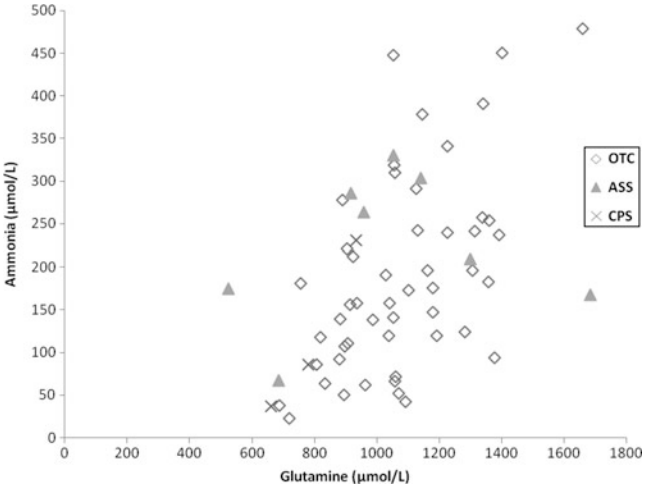

Fig. 2 Correlation between Ammonia and Glutamine in patients <6 years of age. *Diamond* = OTC deficiency. *Triangle* = ASS deficiency. *Cross* = CPS I deficiency

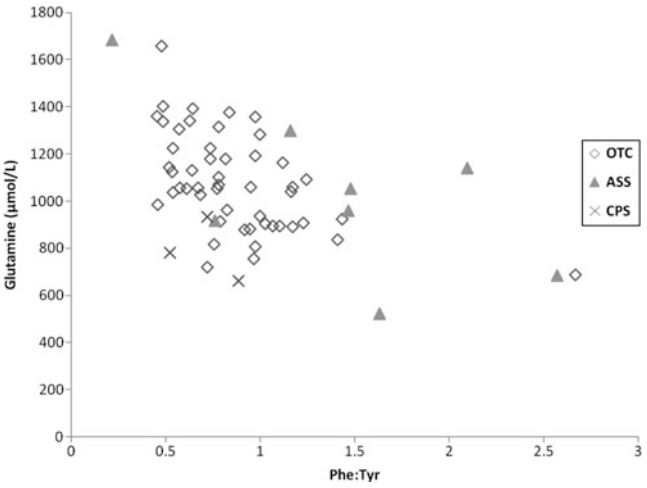

Fig. 3 Correlation between Glutamine and Phe:Tyr in patients <6 years of age. *Diamond* = OTC deficiency. *Triangle* = ASS deficiency. *Cross* = CPS I deficiency

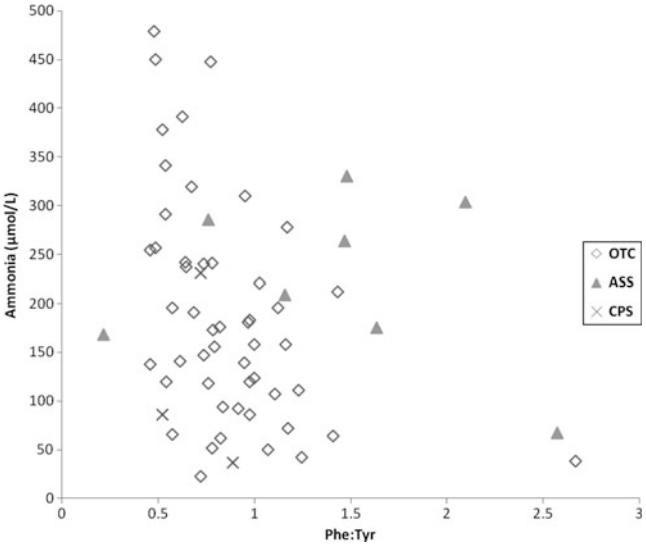

Fig. 4 Correlation between Ammonia and Phe:Tyr in patients <6 years of age. *Diamond* = OTC deficiency. *Triangle* = ASS deficiency. *Cross* = CPS I deficiency

considering the whole cohort. This could be incidental, but it raises the question as to whether there might be alternative significant correlations within the other UCDs. Further analysis of many more samples from more patients is required in order to answer this question.

Previous studies have demonstrated that patients with a UCD may have low plasma concentrations of BCAA. One reason for this finding is transamination of BCAA to form glutamate from alpha-ketoglutarate, as a pivotal step in the formation of glutamine and ammonia detoxification. As a result, there is enhanced oxidation of these amino acids in muscle during hyperammonaemia (Holecek et al. 2011). Treatment with sodium phenylacetate/phenylbutyrate has been shown to further deplete BCAA availability (Scaglia et al. 2004; Scaglia 2010). In our cohort, in which no patient is or has been treated with sodium phenylbutyrate, low concentrations of BCAA correlated with low concentrations of other essential amino acids, suggesting general protein deficiency at the time of decompensation. Of particular note is the strong correlation between the concentrations of BCAA and the concentrations of phenylalanine, tyrosine, threonine and other large neutral amino acids, demonstrating that plasma levels of these amino acids are proportionately low. Further support for the notion of protein deficiency at presentation in the emergency department is found in the disproportionately lower valine levels, compared with leucine and Isoleucine (Adibi 1976). These observations are not surprising, given that samples were taken at admission for hyperammonaemia, possibly following an intercurrent illness with low protein intake and a drive towards glutamine production (calorie intake could vary, depending on the patient's ability to consume high calorie formulae, as instructed when unwell). These results suggest that, unless reduced in parallel with other essential amino acids (suggesting a generalised protein deficiency), BCAA deficiency is likely to be the result of their enhanced disposal, such as with the use of sodium phenylbutyrate (Scaglia 2010).

The negative correlation between ammonia concentration and Phe:Tyr ratio in our samples, particularly in the <6 year old age group, lends support for the notion of a correlation between hyperammonaemia and protein deficiency (de Betue et al. 2011). Although plasma amino acid profiles measured at the time of decompensation may not

be appropriate for determining chronic protein deficiency, this finding can be extrapolated to highlight the risk of hyperammonaemia in chronic protein malnutrition due to a very restricted low-protein diet, as previously reported (Bachmann 2005). This notion should lead to careful individual monitoring of each patient, to avoid over-treatment, particularly given the documented protein aversion of patients with a UCD (Gardeitchik et al. 2012).

The observations of a correlation between age and metabolite concentration, and in particular the correlations between metabolites in one age group and not the other, may shed light on some of the previous findings reported in the literature. For example, Wilson et al. have shown that at high plasma glutamine concentrations, ammonia concentrations vary. They suggested that a possible reason for this was that glutamine synthetase, which converts glutamate and ammonia to glutamine, has a high affinity but low capacity for ammonia (Häussinger et al. 1992), and when it reaches its capacity for glutamine synthesis, ammonia levels can fluctuate wildly, due to the diminished effect of the glutamate/glutamine buffering system. Our results are in agreement with those of Wilson et al. (2001), but we found that the correlation between ammonia and glutamine is age dependent and appears to be limited to the <6-year-old age group (Table 1; Fig. 2). It should be noted that the number of samples from ASS-deficient patients available to us was too small to enable a comparison of the correlation between ammonia and glutamine in these samples and those from OTC-deficient patients (Wilson et al. 2001). Plasma concentrations of glutamine and glutamate do not necessarily reflect their concentrations in other compartments (such as muscle, hepatic periportal and perivenous zones, intestine and kidney), and therefore interpretation of these findings is limited. Nevertheless, these results suggest that pathophysiological mechanisms could be somewhat different in the two age groups, and therapeutic considerations may need to be age related.

We conclude that the majority of UCD patients are protein deficient at admission with hyperammonaemia, as shown by the low plasma levels of most amino acids, the disproportionately low valine concentration and the negative correlation between ammonia and Phe:Tyr ratio (de Betue et al. 2011). Current recommendations for the management of patients with a UCD who present with hyperammonaemia include cessation of protein intake for up to 24 h (Leonard 2000) while providing sufficient calories to prevent catabolism. The findings in this study challenge the notion of stopping all protein intake. Providing enteral BCAA, in particular leucine, has been shown to increase protein synthesis in the liver (de Betue et al. 2011) (reviewed in Bachmann (2008)). Thus, in order to enhance anabolism, supplementation of essential amino acids enriched with BCAA along with sufficient calories to patients with a UCD during acute decompensation and hyperammonaemia would seem prudent. Moreover, in view of the contribution of the splanchnic system to protein retention and metabolism (Newsholme et al. 2003), it would be advantageous to treat patients who present with metabolic decompensation through enteral supplementation, as previously suggested (Leonard 2001; Summar 2001), rather than intravenously.

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CASE REPORT

Non-syndromic Hearing Impairment in a Hungarian Family with the m.7510T>C Mutation of Mitochondrial tRNA$^{Ser(UCN)}$ and Review of Published Cases

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Abstract The m.7510T>C mitochondrial DNA (mtDNA) mutation is a tRNA$^{Ser(UCN)}$ alteration leading to matrilineal isolated hearing impairment. The current paper reviews the available reports on the m.7510T>C mtDNA mutation, with special attention to phenotypic variations and haplogroup background. A Hungarian family, the fourth family reported in the literature, is presented, in which analysis of three generations with bilateral isolated hearing loss revealed the m.7510T>C tRNA$^{Ser(UCN)}$ mutation in homoplasmic form in the affected members. Haplogroup analysis verified an unnamed subgroup of mitochondrial haplogroup H. Previously reported Spanish and North American Caucasian families belong to different subgroups of haplogroup H. Analyzing our biobank of Hungarian patients with sensorineural hearing loss, we did not detect this mutation in any other patient, nor was it found in Caucasian haplogroup H control samples. Comparing the cases reported so far, there is interfamilial variablity in the age of onset, accompanying symptoms, and haplogroup background. Our case adds further genetic evidence for the pathogenicity of the m.7510T>C mutation and underlines the need to include full mtDNA sequencing in the screening for unexplained hearing loss.

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Competing interests: None declared

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Introduction

Inherited hearing impairment is among a highly heterogenous group of disorders (Willems 2000). In the majority of cases it is non-syndromic, but it can sometimes be associated with abnormalities of other organ systems as part of a syndrome. Inherited as a matrilineal trait, sensorineural hearing loss (SNHL) can be attributed to mtDNA mutations. Isolated, non-syndromic hearing loss (OMIM #500008) has been associated classically with mitochondrial 12S rRNA (Prezant et al. 1993) and tRNA$^{Ser(UCN)}$ genes (Friedman et al. 1999), but recent genetic and functional studies suggest the role of further mitochondrial genes (Zheng et al. 2012), such as the ND1 of complex I of the respiratory chain (m.3388C>A), the tRNA^{Ile} (m.4295A>G), subunit COII of complex IV (m.8078G>A), the tRNA$^{Ser(AGY)}$ (m.12236G>A), the tRNA^{His} (m.12201T>C) (Yan et al. 2011), and Cytochrome B, subunit of complex III (m.15077G>A) (Gutiérrez Cortés et al. 2012). Certain mutations can cause syndromic or isolated hearing impairment, as in the m.7445A>G mutation, leading to hearing loss with or without palmoplantar keratoderma (Reid et al. 1994; Maász et al. 2008), or in the m.7472insC mutation, responsible for hearing loss sometimes associated with a neurological disorder (Verhoeven et al. 1999).

Accumulating data from affected families of different ethnic backgrounds, and analysis of control cohorts may gradually confirm the pathogenic role of other rare variants.

The m.7510T>C alteration in the tRNA$^{Ser(UCN)}$ was previously regarded as a rare variant, originally identified in three large families with matrilineal non-syndromic SNHL (Hutchin et al. 2000; Castillo et al. 2002; Labay et al. 2008). In the originally reported Caucasian family from the United Kingdom, the mutation was present in very high levels of heteroplasmy in blood with no available data on the haplogroup background (Hutchin et al. 2000). The second extended Spanish family carried the homoplasmic mutation on sub-haplogroup H1 (Castillo et al. 2002), while the third reported family, of North American origin, carried the mutation on an unnamed subgroup of mitochondrial haplogroup H (Labay et al. 2008). The m.7510T>C mtDNA mutation was not detected in normal Caucasian controls (Hutchin et al. 2000), nor in haplogroup- or sub-haplogroup-matched controls (Labay et al. 2008). Although it was not found in some large screening populations with non-syndromic SNHL (Jacobs et al. 2005; Lévêque et al. 2007), several families have already been described.

The current report reviews the available data on the m.7510T>C mutation with special attention to phenotypic variations and haplogroup background. Further, a Hungarian family is presented, the fourth family reported in the literature, in which the analysis of three generations with bilateral isolated SNHL revealed the m.7510T>C tRNA$^{Ser(UCN)}$ mutation.

Materials and Methods

This study was approved by our Institutional Review Board. Written informed consent was obtained from all adults, and parents or legal guardians of all children. Patients examined at our Department since 2001 with SNHL served as controls.

Genomic DNA was prepared from venous blood samples obtained from three members of the family, the index patient, his mother, and his maternal grandmother (Fig. 1). Sequencing for mtDNA mutations was done as described previously (Maász et al. 2008). The sequences were compared with the revised Cambridge reference sequence (http://www.ncbi.nlm.nih.gov/entrez; GenBank: #J01415) and haplogroup phylogeny from MITOMAP (http://www.mitomap.org). Heteroplasmy for m.7510T>C was assessed by *Hinf*I digestion (Fig. 3) (Hutchin et al. 2000; Castillo et al. 2002).

Patients

Phenotype of the Hungarian family

We examined a four generation Hungarian Caucasian non-consanguineous family with five affected family members.

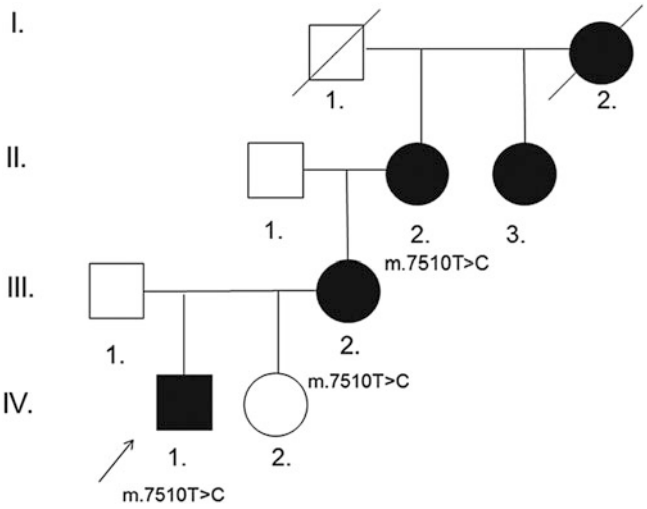

Fig. 1 Phenotypic findings of the Hungarian family. Filled symbols denote the members of the family with non-syndromic hearing impairment. II/2., III./2., and IV/1. were available for genetic analysis which showed the m.7510T>C mutation in the tRNA$^{Ser(UCN)}$ gene of the mitochondrial DNA. The index patient is indicated by an *arrow*

The segregation pattern of the hearing loss raised the possibility of matrilineal inheritance (Fig. 1). Our index patient (Fig. 1, IV./1.) is a 7-year-old boy with normal physical appearance, who was born by vaginal delivery at 40 weeks of gestation, weighing 3,200 g. The perinatal period was uneventful. His early psychomotor development was age-appropriate: he stood upright at 11 months of age, walked unaided by 12 months, started to utter words at 16 months and speak fluently at 2 years of age. At the age of 4.5 years he was investigated for hearing loss. At that age it was noticed that he had coordination problems and a delay in fine motor skills: he was very clumsy in drawing, he was unable to stand on one foot and ride the bike and was uneasy climbing the stairs. Detailed physical and neurological examination did not show any alteration apart from his clumsiness, laboratory investigations were all within the normal range (repeated lactate and pyruvate levels and kidney and liver functions were normal), opthalmologic and cardiologic investigation showed no pathologic finding, brain MRI and MR angiography as well as EEG were normal. Conductive hearing loss was ruled out by otoscopic examination, tympanometry with acoustic reflex testing, and the use of tuning fork tests. Pure tone audiometry confirmed bilateral, sensorineural, symmetrical hearing loss with an average loss of 40 dB HL in both ears (Fig. 4) at the age of 5 years. His hearing loss showed a slowly progressive and asymmetrical course, showing an average of 70 dB HL in the right ear, 80 dB HL in the left ear at the age of 6 years and 75 dB HL in the right ear and 90 dB HL in the left ear at the age of 7 years (Fig. 4). The patient received a hearing aid at the age of 5.5 years. The index patient has a healthy sister (Fig. 1, IV./2.) 3 years of age, no signs of hearing loss have been noted in her so far.

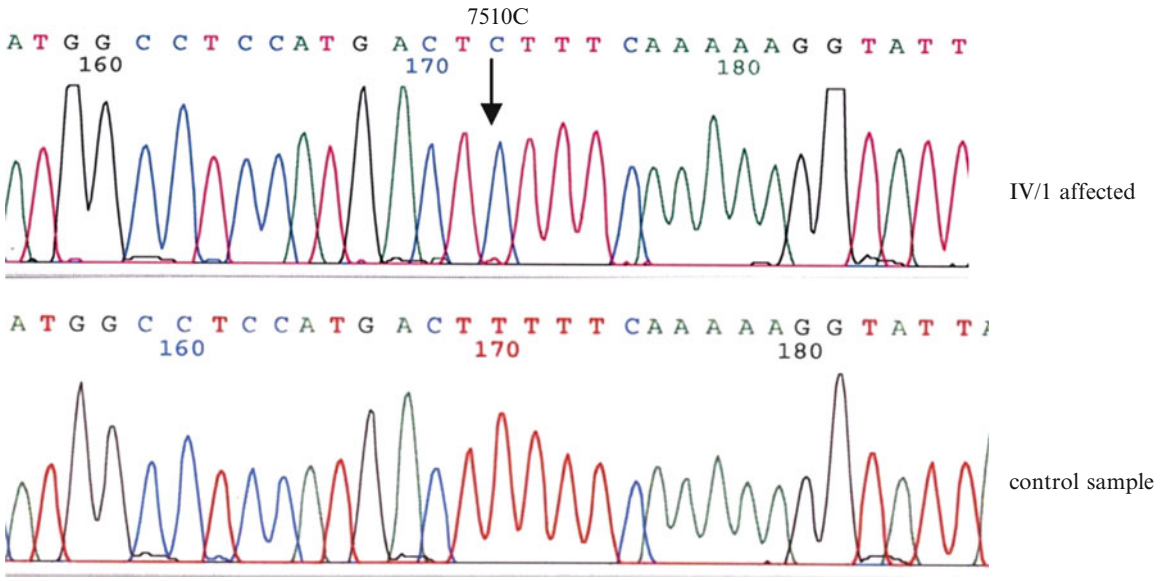

Fig. 2 Electropherograms of the index patient and an unaffected control. Sequence of the index patient and a control. The *arrow* shows the mutant 7510C and wild-type 7510T nucleotides of the mitochondrial $tRNA^{Ser(UCN)}$ gene in the affected proband and a control

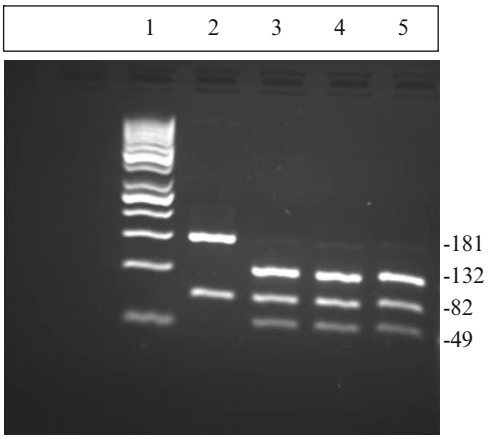

Fig. 3 *Hinf*I PCR-RFLP assay for m.7510T>C heteroplasmy in the members of the Hungarian SNHL family. Mitochondrial DNA was PCR amplified and digested with *Hinf*I as described in previous publications (Hutchin et al. 2000, Castillo et al. 2002). Digestion product sizes (bp) are shown on the right. **Lane 1**: Lambda DNA/EcoRI+HindIII Marker. **Lane 2**: Wild-type amplification product containing a single *Hinf*I cleavage site that results in 181 bp and 82 bp fragments after digestion. **Lane 3, 4, and 5**: the index patient, his mother, and his grandmother: the m.7510T>C mutation creates a *Hinf*I site within the 181 bp fragment that results in 132 bp and 49 bp products. Since no 181 bp fragment can be detected, all three family members harbor the mutation in homoplasmic form

The mother, the maternal grandmother, the maternal great-aunt and the maternal great-grandmother (Fig. 1) all developed severe symmetrical isolated sensorineural hearing loss by the age of 30 years, no audiograms were available from them. The mother (Fig. 1, III./2.) noticed a slowly progressive hearing loss in her early 20s, pure tone audiometry at the age of 30 years confirmed bilateral, sensorineural, symmetrical hearing loss with an average loss of 70 dB HL in both ears, she received a hearing-aid in her late twenties. Both of her pregnancies and especially the deliveries worsened her hearing deficit. Detailed physical and neurological examination did not show any other alteration, laboratory investigations were all within the normal range (repeated lactate and pyruvate levels and kidney and liver functions were normal), and opthalmologic and cardiologic investigation showed no pathologic finding. The maternal grandmother (Fig. 1, II./2.) and her sister (Fig. 1, II./3.) also claim to have first noticed their hearing impairment in their early 20s, no precise information was available about the maternal great-grandmother. Hearing loss was slowly progressive in the affected family members, no episodes of sudden hearing loss, of worsening of the symptoms after noise exposure, or episodes of vertigo were noted, the mother and the maternal grandmother reported to have intermittent tinnitus.

Genotype of the Hungarian family

The sequence analysis of the patient, his mother, and grandmother was performed on DNA extracted from blood leukocytes. No other tissues were available for investigation. During sequence analysis of the mtDNA in the index patient, we detected the m.7510T>C mutation in the $tRNA^{Ser(UCN)}$ (Fig. 2 all mtDNA sequence variants are listed in Table 2). We then screened the mother and maternal grandmother and confirmed the presence of the mutation in their blood samples. Unfortunately, the great-aunt and the sister of our index patient were not available for testing. A specified RFLP assay of all three investigated

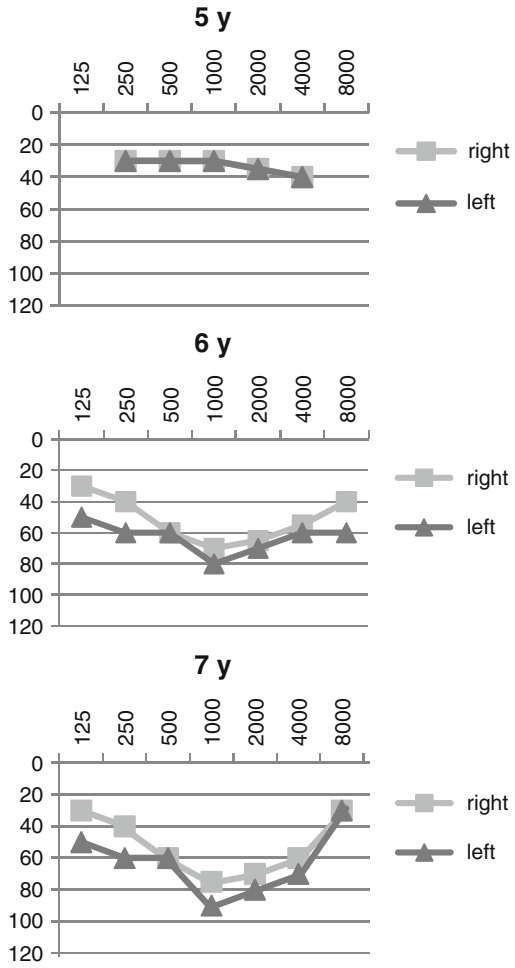

Fig. 4 Air conduction audiograms of the index patient carrying the m.7510T>C mutation at age 5, 6, and 7 years (y). *Squares*, right ear; *triangles*, left ear. Audiograms of the other affected family members were not available

family members confirmed the mutation in homoplasmic form (Fig. 3).

Haplotype analysis (Herrnstadt et al. 2002) of the index patient and his family members suggested that the family carries the mutation on an unnamed subgroup of the mitochondrial haplogroup H. We assigned the m.7510T>C mutation containing mitochondrial sequence in our Hungarian family to haplogroup H, based on the SNP 7028C, which is specific for all branches of haplogroup H (Herrnstadt et al. 2002). Our sequence contains 4793G, which defines a rare unnamed sub-haplogroup that is not among the major subgroups of H (Herrnstadt et al. 2002). In addition, we excluded the SNPs defining subgroups H1, H2, H3 and H4, and several other SNPs defining other rare unnamed subgroups of haplogroup H. We genotyped 60 Caucasian samples from our SNHL biobank but failed to detect the m.7510T>C mutation. Only the m.7445A>G mutation and the m.3243A>G mutations were detected previously among these samples (Maász et al. 2008). The m.7510T>C alteration was also absent in 30 Caucasian haplogroup H healthy controls from our biobank.

In addition, the common deafness associated nuclear alteration (35delG in the ***GJB2*** gene) was investigated, and gave negative results in the affected family members.

Phenotype and genotype details of the previously reported families with the m.7510T>C mutation are listed in Table 1.

Discussion

In this study, we describe a fourth family with matrilineal transmission of non-syndromic SNHL attributable to the m.7510T>C transition of the mitochondrial $tRNA^{Ser(UCN)}$ gene. Results of previous studies give functional evidence of an unfavorable effect of m.7510T>C on pre-$tRNA^{Ser(UCN)}$ processing (Yan et al. 2006), which may lead to decreased levels of the mature $tRNA^{Ser(UCN)}$ overall, thereby limiting mitochondrial protein synthesis and consequently respiration as demonstrated for other tRNA mutations (Yarham et al. 2010). One of these studies demonstrated that cells bearing the $tRNA^{Ser(UCN)}$ m.7445A>G mutation also have a decreased synthesis rate of some mitochondrial proteins, especially the sixth subunit of the NADH dehydrogenase (ND6), and a significant reduction in the amount of ND6 mRNA (Li et al. 2005). This observation suggests that the mRNA of the ND6 is derived from the same precursor as the $tRNA^{Ser(UCN)}$. The same mechanism may apply for the m.7510T>C variant, since it also disrupts a highly conserved base pair in the aminoacyl stem of the $tRNA^{Ser(UCN)}$ (Hutchin et al. 2000).

The m.7510T>C mutation has been described in three families so far (Table 1). In all published families, symptoms appeared mainly as isolated hearing loss. There were no reports of cardiac, ophthalmologic, or renal involvement or movement disorder in the affected family members (Hutchin et al. 2000; Castillo et al. 2002; Labay et al. 2008). Our index patient had a delay in fine motor skills and coordination at the age of 4.5 years when his hearing loss became evident, but no other organ involvement (renal, cardiac, opthalmologic, endocrine) could be detected. As accompanying neurological symptoms, episodes of sudden, severe, and self-limiting vertigo; chronic or intermittent spontaneous bilateral tinnitus; and worsening of the symptoms after noise exposure were reported in the third North American family (Labay et al. 2008). Penetrance and age of onset of the disease differed within the pedigrees reported so far (Hutchin et al. 2000; Castillo et al. 2002; Labay et al. 2008). In the second, Spanish, family, the largest reported so far, the mutation was confirmed in 26 members of the family and auditory impairment was shown in 21 out of 26 carriers. According

Table 1 Phenotype and genotype data of published families bearing the m.7510T>C mitochondrial mutation. The table gives a comparison of the so far published families bearing the m.7510T>C mutation, showing the proportion of the mutation, the haplogroup background, the age at onset of symptoms, the penetrance, the number of affected family members, and their accompanying symptoms. The numbers refer to the number of affected family members. The asterisk indicates the percentage of the mutation within the limits of detection

Families	Status of the mutation and haplogroup	Onset of the disease	Number of affected family members, penetrance	Clinical symptoms				References
				Hearing loss	Trigger factor	Tinnitus	Vertigo	
UK	Heteroplasmic* (>95% and 90%) haplogroup: n.a.	15 months to 70 years	**11** members **affected**, complete penetrance	2:bilateral asymmetrical mild 2: bilateral asymmetrical profound SNHL	No data available	No data available	No data available.	Hutchin et al. 2000
Spanish	Homoplasmic haplogroup: H1	First two decades of life	**26** members **carriers: 22 affected, 4 asymptomatic carriers,** incomplete penetrance	Progressive moderate to profound symmetrical SNHL 3: asymmetrical	1: measles 4 females: hearing loss worsened with pregnancy	Most affected family members	Not reported	Castillo et al. 2002
North American	Homoplasmic haplogroup: rare H variant 3333T	2 to 40 years of age	**16 affected**, complete penetrance	**16**: bilateral progressive SNHL	2: febrile illness 1:measles 5: blunt head trauma	7: chronic or intermittent	2: sudden, severe, self-limiting	Labay et al., 2008
Hungarian	Homoplasmic rare H variant 4793G	5 to 20 years of age	**5** members **affected**, no complete penetrance	5: bilateral mild to profound SNHL	Pregnancy, delivery	2: intermittent	No	Current report

n.a. *SNHL* sensorineural hearing loss

Table 2 Alterations in the mtDNA sequence of the index patient

mtDNA variation	Category	Interpretation
m.709G>A	Polymorphism	MT-RNR1 (12S rRNA)
m.4769A>G	Polymorphism	p.Met100Met in MT-ND2
m.4793A>G	**Polymorphism**	**p.Met92Met in MT-ND2 rare haplogroup H variant**
m.7510T>C	**Known mutation**	**MT-TS1 (tRNA Ser 1)**
m.8251G>A	Polymorphism	p.Gly222Gly in MT-CO2
m.11938C>T	Polymorphism	p.Leu393Leu in MT-ND4
m.15954A>G	Polymorphism	MT-NC10 noncoding

Apart from the m.7510T>C mutation, no other pathological alteration was detected in the index patient

to the symptoms and audiograms, four carriers were reported to be asymptomatic: at the age of 11, 15, 16, and 31 air conduction audiograms did not reveal an impairment (Castillo et al. 2002). Although no further data is available on the asymptomatic carriers, given the intrafamilial variable onset of the symptoms, it can be speculated that the carriers did not yet reach the age at which symptoms would appear. No complete penetrance can be demonstrated in our Hungarian family either. Although the so far unaffected, 3-year-old sister of the index patient was not available for testing, she might be just too young to show symptoms of hearing loss.

The wide range of age at onset, and the variability among individuals in the affected families, also suggests that other mitochondrial or nuclear factors as well as environmental agents may contribute to the symptoms in these carriers. The mutation was present in homoplasmic form in the Spanish and North American family (Castillo et al. 2002; Labay et al. 2008), and also in our Hungarian patients, while it was reported to be present in a high level of heteroplasmy (>95% and 90% respectively) in the original UK family (Hutchin et al. 2000). Only blood leukocytes were available in this work, and no other tissues were examined in previous reports. However, the proportion of the normal and the mutant alleles may vary in the auditory cells, explaining incomplete penetrance and the difference in the age of onset.

The m.7510T>C mutation has been reported to be a rare finding and was not found in some large screening populations with non-syndromic SNHL (Jacobs et al. 2005; Lévêque et al. 2007). It was originally interpreted as a benign polymorphism defining a deep branch in the mitochondrial DNA phylogeny (Labay et al. 2008). However, it was not detected in normal Caucasian controls (Hutchin et al. 2000), or in haplogroup- and sub-haplogroup-matched controls (Labay et al. 2008). Our own investigations demonstrate that it was absent in 60 other Caucasian patients with SNHL and in 30 Caucasian healthy controls of haplogroup H. Since several families with isolated SNHL have been described to harbor the m.7510T>C mutation, it can be regarded as a disease-causing mutation. Haplotype analysis in our Hungarian family suggested an unnamed subgroup of haplogroup H, delineated by the SNP m.4793A>G, which is different from the haplogroups of the previously reported Spanish and North American families, and the UK family with a sequence variant (m.4336A>G) not present in the other three families. The fact that the m.7510T>C mutation arose on different mitochondrial subgroups provides further evidence for the pathogenecity of this mutation. Evolutionary studies showed that deleterious mitochondrial mutations are influenced very strongly by selection and are eliminated rapidly, so their appearance should be the result of very recent mutational events and transmitted through very few generations; thus, founder events are not likely to be found (Torroni et al. 2003). The finding that all four so far described families carry the m.7510T>C mutation on different European mtDNA haplogroup backgrounds indicates that European haplogroups do not increase or reduce the risk of expressing the disease phenotype. Since there is no marked phenotypic variability among the families, the haplogroup data may support that phenotypic expression is not necessarily influenced by haplogroup background.

To our knowledge, this is the fourth family in the literature with the m.7510T>C $tRNA^{Ser(UCN)}$ mutation. Comparing the cases reported so far (Table 1), there is some variability in the age of onset, the accompanying symptoms such as tinnitus or increased sensitivity to noise, ototoxicity, and the haplogroup background. Given the fact that hearing loss resulting from mitochondrial mutations can exhibit incomplete penetrance and a broad phenotypic spectrum, screening for unexplained hearing loss should include full mtDNA sequencing (encompassing the $tRNA^{Ser(UCN)}$ mutations) in all cases in which maternal inheritance cannot be clearly ruled out.

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All authors declare no conflict of interest relating to the work in the manuscript.

Synopsis of the Article

We present the fourth family reported in the literature with bilateral isolated hearing loss due to the m.7510T>C $tRNA^{Ser(UCN)}$ mutation in an unnamed subgroup of

mitochondrial haplogroup H providing further evidence for the pathogenic role of the m.7510T>C mitochondrial mutation.

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CASE REPORT

Low-Dose Amitriptyline-Induced Acute Dystonia in a Patient with Metachromatic Leukodystrophy

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Abstract Acute dystonia is an abrupt event mainly related to toxicity of drugs such as antiemetics, antipsychotics, anti-acids, and, more rarely, tricyclic antidepressants. Use of amitriptyline in metachromatic leukodystrophy (MLD), a lysosomal storage disorder (LSD) due to arylsulfatase A deficiency, is suggested to control neurological pain and irritability. We describe a patient with MLD who experienced acute dystonia as a side effect of low dosage of amitriptyline. The distribution of psychotropic drugs, including antidepressants, depends upon lysosomal trapping which is inefficient in LSD. The defective lysosomal depot might raise cerebral levels of amitriptyline, thus enhancing its adverse effects.

Physicians caring for children with MLD treated with psychotropic drugs should be aware of such adverse events which are potentially related to lysosomal dysfunction. This experience raises a potential concern about the appropriate dose of amitriptyline in patients with MLD.

Introduction

Drug-induced dystonic reactions are the most common causes of acquired dystonia in childhood (van Harten et al. 1999). Side effects profile of amitriptyline includes sedation, dry mouth, impaired cardiovascular and central nervous system (CNS) function (Gore et al. 2006); among extrapyramidal manifestations, acute dystonia has been reported in cases of long-term or overdose of amitriptyline treatment (Ornadel et al. 1999; Finder et al. 1982; Lee 1988). In the literature, two cases of pediatric amitriptyline poisoning are described (Baysal et al. 2007; Doherty et al. 2012).

Metachromatic leukodystrophy (MLD) is an autosomal recessive lysosomal storage disorder (LSD) due to arylsulfatase A (ARSA) enzyme deficiency, resulting in rapid and progressive neurologic and cognitive deterioration. Irritability and neurologic pain are significant problems for the management of patients in advanced stages of the disease and evidence-based treatment options for these issues are lacking. Practical hints can be found in the document by the Evanosky Foundation (The Evanosky Foundation 2008). Listing of the suggested medications include: myorelaxant agents (Dantrium, Baclofen), anti-reflux, anti-seizure, and anti-inflammatory medications. Amitriptyline is suggested to control irritability and pain.

We report a 3.7-year-old girl affected by MLD who presented with an acute episode of dystonia related to amitriptyline which was given to control irritability and pain.

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Competing interests: None declared

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Case Report

A 3.7-year-old girl affected by the late infantile form of MLD, confirmed by both biochemical and molecular analyses, is regularly followed at the Department of Pediatrics of Federico II, University of Naples, Italy. Progressive spasticity had led to permanent plantar flexion of the feet at rest, despite baclofen treatment at the dosage of 2 mg/kg/day. Moreover, the girl began to

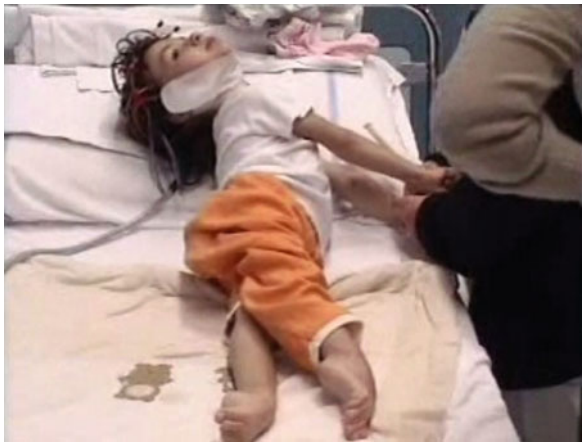

Fig. 1 Girl affected with MLD presenting with an acute dystonia following amitriptyline treatment. Note the opisthotonos with forced head and gaze deviation to the right, grimacing, and trismus

suffer from bouts of crying lasting for several hours during the night.

We used a modified Ashworth scale (Lee et al. 1989) to rate her severity of hypertonia: on the right side, passive movement was difficult and scored 3, on the left, range of motion and spasticity matched to a score of 4. After an extensive workup carried out in order to exclude treatable causes of pain, including gallbladder stone (Kim et al. 1996) dantrolene sodium (6 mg/kg/day) was added to therapy, and resulted in some improvements on the Ashworth scale: spasticity improved within 2 days to a score of 3 bilaterally.

Treatment with amitriptyline was started to improve crying and neurological pain, as suggested by the Evanosky paper (The Evanosky Foundation 2008). The starting dose was 0.2 mg/kg daily orally for the first 2 days, gradually raised to 0.4 mg/kg daily in the third and the fourth day, lower doses than those recommended for pediatric age (Fournier-Charrière 2010).

After 4 days of treatment, muscular tone further improved (bilaterally score 2 according to Ashworth scale). However, on the morning of the fifth day of amitryptiline treatment, the patient experienced generalized malaise, vomiting, profuse sweating, tachycardia (heart rate 190/min), tachypnea (respiration rate about 28/min), and mydriasis. A few hours later, she abruptly developed worsening of hypertonia with severe generalized muscle contractions and opisthotonos (score 4 bilaterally, according to the Ashworth scale). Furthermore, a forced head and gaze deviation to the right with horizontal nystagmus ensued, followed by trismus, and buccolingual automatisms (Fig. 1). Oxygen saturation was in the normal range (about 99–100%). The patient appeared unresponsive to her surroundings with an ill-defined state of consciousness. The video/EEG monitoring showed high amplitude, background activity of 3 to 4 Hz, with an excess of muscular artifacts. In view of the absence of epileptic abnormalities, a diagnosis of acute generalized dystonia was entertained and treatment with 0.16 mg/kg of diazepam intravenously was started.

As an amitriptyline-induced dystonic reaction could be reasonably foreseen, the drug was immediately discontinued. Common antidotes as anticholinergic benztropine were not used because of the tachycardia.

The patient showed a gradual reversion of the opisthotonos and resolution of all autonomic symptoms and signs. Video/EEG recording was repeated after 1 week from the acute episode and did not show any epileptic abnormalities.

The Naranjo scale (Naranjo et al. 1981) indicated that the adverse event was probably caused by amitriptyline (causality algorithm yielded a score of 7).

The chronic unexplained irritability in the little child has been treated subsequently with Gabapentin, 5 mg/kg per dose as previously described (Hauer et al. 2007), achieving successful results in decreasing irritability and crying, bettering nocturnal sleep.

Discussion

Drugs inducing extrapyramidal symptoms include anticonvulsants (Van Harten et al. 1999), antiemetic and antinausea drugs (Patel et al. 2011), atypical and typical antipsychotics, tricyclic antidepressants (TCAs) (Arnone et al. 2002), triptans (Garcia et al. 1994), antitussive drugs (Polizzi et al. 2001), histamine H2-receptor antagonists (Kapur et al. 1999), and antihistamine agents (Esen et al. 2008). Misdiagnosis is common, and video/EEG monitoring is useful to rule out epileptic fits.

Amitryptyline, a TCA, has pronounced side effects that tend to be most acute in the elderly because they frequently require multiple medications that increase the potential

drug-drug interactions (Gore et al. 2006). Tremor, dysarthria, and akathisia due to amitriptyline have been observed. Only few cases of acute dystonia due to amitriptyline have been reported in adults, ascribed to overdose and/or long-term therapy, drug interactions (Ornadel et al. 1999; Finder et al. 1982; Lee 1988).

List of medications potentially interacting with amitriptyline includes baclofen (Gore et al. 2006). In our young patient, concurrent administration of baclofen with the amitriptyline may have lowered the threshold for this unwanted adverse effect.

Among pediatric patients, acute neurological manifestations, like tonic-clonic seizures, due to amitriptyline overdose (47 mg/kg and 14 mg/kg respectively) have been described sporadically (Baysal et al. 2007; Doherty et al. 2012). In children, doses of 5 mg/kg are potentially toxic while severe toxicity is encountered at 20 mg/kg dose (Vernon and Gleich 1997). In the case herein reported, the patient presents with acute dystonia at dosage of amitriptyline within 0.2–0.4 mg/kg daily.

The causal relationship between acute dystonia and amitriptyline in our patient is based on (1) close temporal correlation between drug administration and onset of the dystonia, (2) improvement following drug withdrawal, (3) no recurrence of symptoms after the drug was discontinued, and (4) exclusion of other possible causes. In addition, according to the Naranjo adverse drug reaction probability scale, the event is assigned as probable (Lee et al. 1989).

Dystonia has been rarely described as a feature of MLD (Baumann et al. 2002). This may suggest a particular predisposition for dystonia episodes in MLD patients following centrally acting drugs.

A possible pathomechanism underlying this unexpected toxic effect of low-dose amitriptyline may depend upon lysosomal dysfunction of MLD. Tissue distribution of psychotropic drugs depends on phospholipid binding and lysosomal trapping, which play an important role, particularly for drug biodistribution within the central nervous system (CNS) (Daniel et al. 2001). A decrease in the concentration of psychotropic drugs in lysosomes, as a result of lysosomal dysfunction in LSDs, may result in altered intracellular distribution and/or drug uptake in neuronal and glial cells, which ultimately might lead to enhancement of the drug biological effect (Daniel 2003). Therefore, we speculate that impaired lysosomal trapping of amitriptyline in the patient reported might lead to increased drug CNS levels, which resulted in drug intoxication. This mechanism of drug toxicity could be responsible for the onset of extrapyramidal signs also in other previously reported patients with other LSDs, receiving psychotropic drugs.

In their review of 20 patients with mucopolysaccharidosis type III, treated with neuroleptic drugs (lamotrigine, risperidone, and olanzapine), Tchan and Sillence (2009) reported five patients with a clear relationship between drug assumption and extrapyramidal symptoms (included acute dystonia). Moreover, Shapiro et al. (2006) reviewed 44 adults with late-onset Tay-Sachs disease who experienced worsening of neurologic symptoms (weakness, incoordination, imbalance, tremor, dysarthria, cognitive decline, and dystonia) following treatments with medications such as haloperidol, risperidone, and chlorpromazine.

The above-mentioned psychotropic drugs have been proved to share lysosomotropic properties (Daniel et al. 2001). We suggest that adverse extrapyramidal manifestations reported in patients with LSDs taking psychotropic drugs could be related to defective lysosomal drugs uptake and disposal.

In summary, we report a patient with MLD who developed acute dystonia following amitriptyline therapy. Patients with a defective lysosomal depot might be prone to drug toxicity even at doses falling within or lower the recommended dose range. Treatment of irritability and neurological pain in patients with MLD is often difficult, since it may require the use of multiple medications (increasing potential drug-drug interactions), appropriate dosage, and monitoring for undesirable medical consequences.

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CASE REPORT

Considering Fabry, but Diagnosing MPS I: Difficulties in the Diagnostic Process

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Abstract *Introduction:* Recent studies have indicated that a proportion of patients with renal failure, left ventricular hypertrophy, or cryptogenic stroke have sequence variants in their aGal A gene (Fabry disease), which has resulted in an increase in diagnostic activities for this disorder. The diagnostic process for lysosomal storage disorders may result in findings of unknown clinical significance. Here we report such an unexpected outcome.

Case: A 32-year-old male presented at the emergency department because of a transient ischemic attack. Extensive investigations revealed no cause and an initial diagnosis of cryptogenic stroke was made. Subsequently, aGal A activity was measured in a bloodspot and was shown to be normal, but the activity of alpha-L-iduronidase (IDUA), used as reference enzyme, was unexpectedly low: 0.5 umol/L (ref = 1.7–14.3). A diagnosis of IDUA deficiency, mucopolysaccharidosis type 1S or Scheie disease was considered. IDUA gene analysis revealed two homozygous sequence alterations: a silent sequence change (979C > T) in exon 7 (N297N) and an unknown missense mutation 875A > T (R263W). Physical examination was completely normal, without clinical signs of mucopolysaccharidosis type I (MPS I). Leukocyte IDUA activity was also low: 2.1 nmol/mg prot/h (ref = 14–40 nmol prot/h), but higher than the patient range of <0.1 nmol/mg prot/h. Urinary glycosaminoglycan levels were normal both quantitatively and qualitatively. It was concluded that there was low IDUA activity without clinical symptoms and the diagnosis of mucopolysaccharidosis I was discarded.

Conclusion: The diagnostic process for lysosomal storage disorders may result in biochemical abnormalities of unknown clinical significance. Early evaluation by a specialist in inborn errors of metabolism may help to avoid anxiety in patients and unnecessary additional analyses.

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Introduction

Fabry disease (FD, MIM 301500) is an X-linked lysosomal storage disorder, caused by a deficiency of α-galactosidase A (alfa-Gal A; EC 3.2.1.22). This results in the accumulation of its substrates, glycosphingolipids with an 1,4 galactosyl moiety in various cell types

throughout the body. This ultimately causes the clinical presentation that in its typical (also called classical) phenotype presents with acroparesthesias, anhidrosis, and angiokeratoma in young male adults. Later in life, an increase in cardiovascular complications, including renal failure, occurs in males and to a lesser extent in females (Zarate and Hopkin 2008). As of 2001, enzyme replacement therapy (ERT) is available, but long-term studies have shown that ERT has limited efficacy in advanced disease (Lidove et al. 2010). This observation has resulted in efforts to increase awareness of Fabry disease. One way to detect Fabry disease is through aGal A activity analysis in high-risk populations. Patients identified through this method may subsequently be treated as Fabry patients, but more importantly, it may also lead to the identification of hitherto asymptomatic, yet affected, family members. Indeed, recent studies have shown that a small proportion of patients with renal failure, left ventricular hypertrophy or cryptogenic stroke have sequence variations in the gene encoding alpha-Galactosidase A (GLA, for review see (Linthorst et al. 2010)). Yet, it is unclear whether all patients with GLA sequence abnormalities should be considered as Fabry patients, as the clinical significance of their mutations is frequently unknown.

Both mutation analysis and the measurement of enzyme activity are performed in the diagnostic process. In males, detection of alpha-Galactosidase A deficiency remains the gold standard of diagnosing the disease (Gal et al. 2011). Chamoles showed in 2001 that bloodspots may serve as an alternative for the analysis of lysosomal enzyme activity in isolated leukocytes or lymphocytes (Chamoles et al. 2001). To correct for a variable leukocyte count per spot, the activity of a second lysosomal enzyme is generally used as reference value.

Here we report on an unusual outcome of the diagnostic process for Fabry disease.

Case

A 32-year-old Dutch man of non-consanguineous Moroccan parents presented in the emergency department with complaints indicating facial nerve palsy and possible hypoglossal nerve palsy. These symptoms resolved spontaneously within 24 h and it was considered to have been a transient ischemic attack (TIA). The patient had no risk factors for cerebrovascular disease. Extensive additional investigations were performed, but these revealed no cause: MRI brain, transthoracic and transesophageal echocardiography, and 24 h EKG registration were all normal. No coagulopathy, vasculitis, or carotid dissection was detected. A diagnosis of cryptogenic stroke was made and diagnostics for Fabry disease were initiated.

Analysis of a bloodspot revealed normal aGal A enzyme activity. Thus, Fabry disease was ruled out. Unexpectedly, activity of α-L iduronidase (IDUA; EC 3.2.1.76), which was used as a reference enzyme, was 0.5 μmol/h/L (reference range: 1.7–14.3 μmol/h/L). A second sample was requested, yielding similar results (IDUA activity 0.8 μmol/h/L). It was concluded there was low IDUA activity, possibly related to mucopolysaccharidosis type I (MPS I, Scheie phenotype).

Subsequently, a diagnosis of Scheie disease was considered and genetic analysis was requested. Analysis of the IDUA gene resulted in the identification of two unknown homozygous alterations of unknown clinical significance: a missense mutation c.875A > T (p.R263W) in exon 6, and a silent mutation, c.979C > T that does not result in an amino acid sequence alteration (p.N297N) in exon 7. The patient was told that a diagnosis of MPS I was likely and he was referred to an expert center in lysosomal storage diseases at another hospital.

Here, further assessment of the patient's history revealed that the patient had been healthy until the TIA, from which he had recovered completely. He had never experienced any complaints related to joint mobility, changes in facial morphology, or reduced exercise tolerance. Family history was negative for inborn errors of metabolism. Physical examination was completely normal and he had reached normal height (180 cm). In particular, there were no dysmorphic signs, cardiac and pulmonary sounds were normal and there was no organomegaly. The extremities did not demonstrate abnormalities and all joints had full range of motion. There were no signs of corneal clouding. Additional laboratory evaluations were performed. Glycosaminoglycan excretion in urine measured by means of a dimethylene blue test was normal 5 mg/mmol creatinine (reference range: 1–8 mg/mmol creatinine). In addition, two-dimensional electrophoresis of urinary glycosaminoglycans showed a normal pattern. IDUA activity in leukocytes was consistent with the results of the bloodspot assay: 2.1 nmol/mg prot/h (reference range: 14.0–40.0 nmol/mg prot/h). This is well above the patient range seen in our center (<0.1 nmol/mg prot/h).

Despite the low IDUA activity, the significant residual IDUA activity and the clinical presentation were considered not to be consistent with MPS I. The normal excretion of GAGs in urine was supportive of this assumption. The diagnosis of MPS I was discarded.

Discussion

A 32-year-old man was evaluated for Fabry disease because of a cryptogenic stroke. In bloodspot, no alfa-Gal A deficiency was found. IDUA was used as a reference

enzyme and repeatedly turned out to be low. After reference to a specialized center, this was confirmed in additional laboratory investigations, but not to such an extent as seen in mucopolysaccharidosis-I patients. The patient experienced no complaints compatible with MPS-I and there were no physical signs of the disease. Measurement of the storage product (glycosaminoglycans in urine) revealed normal levels.

Deficiency of α-L iduronidase leads to mucopolysaccharidosis type I. This disease has a wide phenotypic range and is often categorized in three subtypes. The severe form, Hurler syndrome (MPS IH; MIM 607014) presents in infancy and if left untreated will lead to premature death in childhood. It is characterized by mental retardation, hepatosplenomegaly, coarse facial features, corneal clouding, joint and bone deformities known as "dysostosis multiplex," macroglossia, valvular heart disease, obstructive airway disease, and inguinal or umbilical hernias. The intermediate phenotype Hurler/Scheie (MPS IH/S; MIM 607015) presents in childhood and has a slower progression without cognitive impairment. Survival into adulthood is common. Scheie syndrome (MPS IS; MIM 607016) is the attenuated form of MPS I and may present in late childhood or even adolescence. As in Hurler-Scheie, somatic disease is present without cognitive impairment (Neufeld and Muenzer 2007).

Although MPS may cause coronary artery insufficiency and myocardial infarction (Lin et al. 2005), a cerebrovascular event as presenting MPS I symptom has only recently been described in one individual (Fujii et al. 2012). In this particular case, the characteristic dysmorphology was the clue to the ultimate diagnosis of MPS I. The accumulation of glycosaminoglycans in (cells of) the vascular wall may induce reduced elasticity and atherosclerotic-like plaques in animal models and patients (Kelly et al. 2012; Lyons et al. 2011; Wang et al. 2011). These effects, however, are seen in advanced disease only and never in the absence of other disease-specific symptoms.In this light, it is very unlikely that a low IDUA activity may be explanatory for the cryptogenic stroke in this patient. Despite the reduced enzyme activity, we could not demonstrate an increase in GAG excretion in urine. The presence of two homozygous mutations is remarkable given the reported non-consanguinity of the patient's parents.

There are many examples in the literature where mutations in genes of lysosomal hydrolases may be accompanied by severely reduced enzymatic activities in vitro, without correlated clinical symptoms. This has been shown for Fabry disease (Froissart et al. 2003), Tay-Sachs disease (Triggs-Raine et al. 1992), Pompe disease (Nishimoto et al. 1988), and also in mucopolysaccharidosis type I, the disease suspected in the present case (Aronovich et al. 1996). These inconsistencies are usually explained by the fact that the in vitro diagnostic procedure to measure enzyme activity is performed by means of artificial substrates. Apparently, some coding region sequence variants may result in a reduced specificity toward artificial substrates, but not for natural substrates (Froissart et al. 2003).

Incidental findings such as described here, are a well-known phenomenon in the current practice of medicine, especially in radiology and genomics (Couzin-Frankel 2011; Soultati et al. 2010). As incidental findings are per definition unrelated to the patient's complaints, the question to what extent additional testing should be performed is part of both a medical and ethical discussion (Sijmons et al. 2011). Further research may burden the patient both mentally and physically, and may bring considerable extra costs (Ding et al. 2011; Stern 2012). To limit these aspects as much as possible, early consultation of clinical experts is recommended.

Synopsis

Interpretation of diagnostic tests for rare diseases by clinical experts is vital.

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CASE REPORT

Case Report of Argininemia: The Utility of the Arginine/Ornithine Ratio for Newborn Screening (NBS)

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Abstract We describe a case of Argininemia detected by Michigan Newborn Screening (NBS). The Secretary's Advisory Committee on Heritable Disorders in Newborns and Children recommends that every MS/MS newborn screening program include Argininemia as part of their uniform screening panel. While affected infants will be detected by this testing, Arginine levels may take time to accumulate. Thus, some infants may not be detected by this methodology and early sample collection. In Michigan, since initiating testing for Argininemia in 2006, there has been workup of 23 cases for elevated Arginine identified by NBS, with one case identified as affected. We report this affected case. Subsequently, the Arginine/Ornithine ratio was calculated for all cases and was found to be informative with respect to predicting whether a patient is affected by Argininemia.

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Introduction

The Secretary's Advisory Committee on Heritable Disorders in Newborns and Children recommends Arginemia be included in the NBS uniform panel (http://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/ accessed September 11, 2012). Hyperarginemia (OMIM 207800) has an estimated incidence of one in 2 million births (Scaglia and Lee 2006). Arginase is the enzyme involved in the last step of the urea cycle and converts L-arginine into L-ornithine and urea. The Arginase A1 gene is located at 6 q23. Arginemia is an autosomal recessive disorder. As opposed to other urea cycle defects, review of the literature suggests that this condition does not classically present in the newborn period with symptoms of hyperammonemia. Onset is typically between 2 and 4 years of age. (Crombez and Cederbaum 2005)

One review of 55 patients with hyperarginemia indicated that 52 of the patients were asymptomatic in early infancy (De Dyn et al. 1997). If untreated however, these individuals go on to develop symptoms of psychomotor deterioration, loss of developmental milestones, and spasticity. With appropriate adherence to dietary and medication treatment, these symptoms may be prevented.

In some individuals it may take time for Arginine to accumulate in the plasma, which may be in part due to the presence of mitochondrial Arginase(Arginase II) which can increase up to 40-fold if cystosolic Arginase I activity in liver is deficient. There have been reports based on siblings of known Argininemia patients that the Arginine level can

be normal in the neonatal period in patients who will ultimately be affected. Conversely, unaffected patients in the newborn period may have Arginine levels above 150 umol/L and have normal Arginase activity.

Biochemical abnormalities include accumulation of Arginine which is the hallmark of hyperargininemia. Arginine can be measured by tandem mass spectrometry. From a survey among participants of a LISTSERV for metabolic specialists, while a number of cases have been detected, at least one has been missed (Crombez and Cederbaum 2005). Regarding this missed case in California, at that time the Arginine cutoff level was 200 umol/L, and the infant's Arginine level was under this level. The cutoff has since been reduced to 50 umol/L and this patient would have been identified (Cederbaum, June 21, 2012, personal correspondence). However, lowering the cutoff may contribute to more false positives being identified.

It would be helpful to have a test which could reduce false positive results in screening for hyperargininemia, and thus conserve resources by identifying which patients have clinical elevations in Arginine. Such a test was suggested by Currier: December 2010 Regional Collaborative Update. Specifically a new ratio, Arg/Orn, was recommended (Rinaldo 2010). This ratio was calculated for our Argininemia case, the previous elevated False Positive cases, and the normal population during the period of time since Michigan has instituted MS/MS Newborn screening.

Case Report

We present a case of an infant with an elevated Arginine of 107 μmol/L (nml <68 μmol/L) with newborn screening of dried bloodspot collection at 25 h. The Arginine/Ornithine ratio was calculated to be 2.47 (Table 1). The infant was born at 39 weeks to a G2P2 mother. Family history was unremarkable for any consanguinity, and the patient was of Dutch and Irish ancestry. Birth weight was 8 pounds and birth length was 20.5 in. The postnatal course was uncomplicated and the infant was discharged at 3 days of age. The infant subsequently did well, feeding and growing appropriately. However, a repeat NBS Arginine at day 19 of life was 499 μmol/L (nml < 110 μmol/L) which prompted further evaluation at the Children's Hospital of Michigan. In the ED, the ammonia level was initially 107μmol/L but on repeat was 49 μmol/L. The patient's plasma amino acids showed an elevated Arginine level of 762 μmol /L (nml 40–148). The patient was subsequently started on a protein-restricted diet of 2.2 g/kg which included Cyclinex metabolic formula. The family was counseled regarding Arginase deficiency, and given an emergency management protocol outlining medical management in the event the patient had symptoms of illness. Diagnosis was later confirmed by DNA analysis which showed two mutations in the Arginase gene; c.807–811 deletion (inherited from the father), c.611A > G (p. D204G) (inherited from the mother). Both of these are novel mutations and are predicted to be deleterious, causing the disorder.

Table 1 Arginine results with calculated Arginine/Ornithine ratio for patients identified as having elevated Arginine level by Michigan newborn screening <180 h of age. Case report patient highlighted.

Age (hours)	Arg (μmol/L)	Orn (μmol/L)	Arg/Orn
25	107	43	2.47
36	68	94	0.72
0	75	106	0.71
6	83	120	0.69
26	77	121	0.64
24	76	123	0.62
34	75	125	0.60
26	75	144	0.52
27	76	164	0.46
25	71	187	0.38
25	70	192	0.37
24	71	193	0.37
168	74	219	0.34
36	79	279	0.28

Results and Discussion

This was the first Argininemia case identified by the Michigan NBS Program since initiating testing for Arginine in 2006 (>675,000 samples tested). Twenty-three cases with elevated Arginine (not due to TPN feeding) had been identified. For these 23 cases, 22 had confirmatory testing accomplished by a repeat Arginine level, which were all within normal limits. Our case highlights the challenge of identifying a patient with Argininemia, and how best to confirm clinical suspicion of a patient with an elevated Arginine value.

The December 2010 Regional Collaborative Update presented a new ratio: Arginine/Ornithine proposed by Bob Currier (Rinaldo 2010). This prompted the MI NBS laboratory to calculate the Arg/Orn ratio for this Argininemia case, the previous presumptive positive cases, and the normal population.

The initial Arginine/Ornithine ratio was 2.47 for our case as measured at 25 h of age (Table 1). For the period 2006–2012, for patients not on TPN or hospitalized in the NICU, the mean Arginine value was 11.8, median value was 10.6 (SD = 6.12, 99 % = 31.90). The mean Arginine/Ornithine ratio for this time period was 0.14, the median

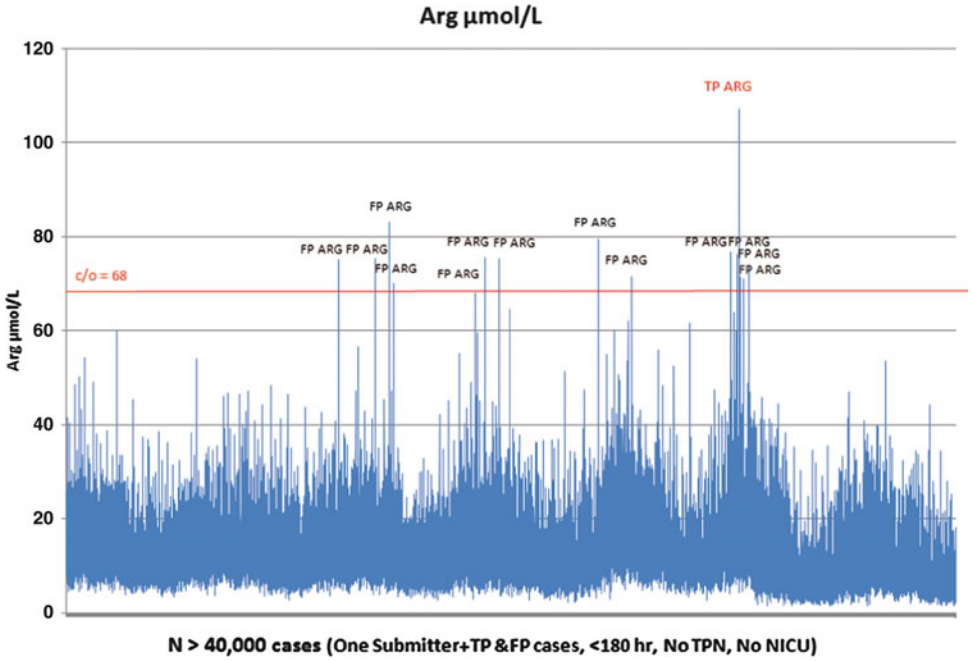

Fig. 1 Arginine Levels (umol/L) for newborn screening cases from one hospital in Michigan from 2006 to 2012 plus the MI false-positive cases and this Argininemia case. Arginine cutoff (c/o) = 68. FP = False positives, TP =True positives

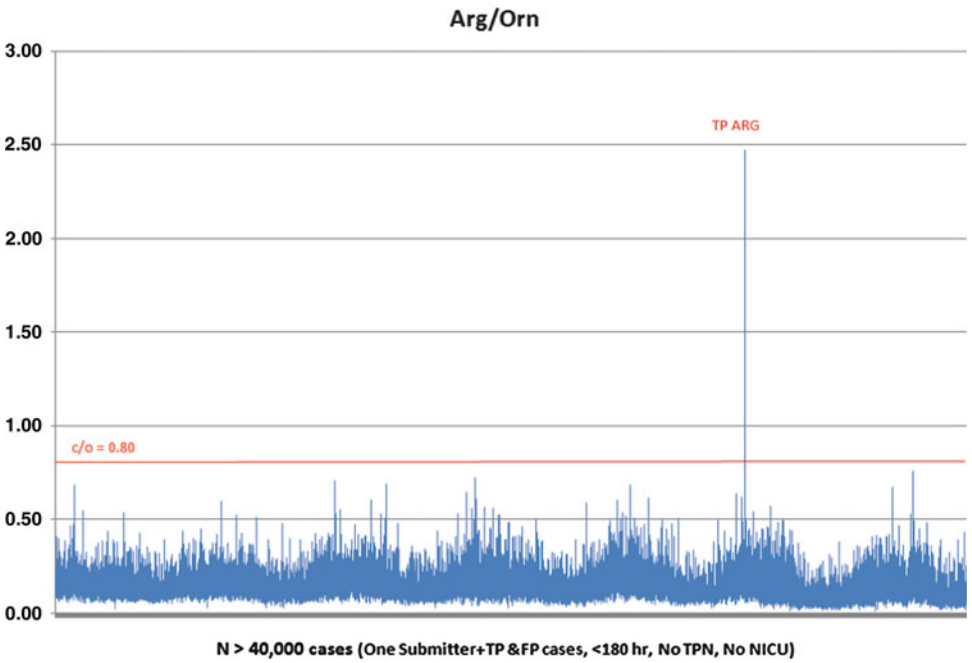

Fig. 2 Arginine/Ornithine ratios for newborn screening cases from one hospital in Michigan from 2006 to 2012 plus the MI false-positive cases and this Argininemia case, c/o = 0.80. TP = True positive

was 0.12 (SD 0.07, 99 % = 0.35). At present, the Michigan NBS Program has set the cutoff for Arginine/Ornithine ratio at 0.8 and is in the process of validating this ratio. To further illustrate the utility of this ratio, Fig. 1 represents the Arginine values for over 40,000 infants from one hospital in Michigan, plus the false-positive cases and this Argininemia case. For a number of infants, Arginine values were above the designated cutoff. Figure 2 represents the Arginine/Ornithine ratios for over 40,000 infants from one hospital in Michigan, plus the false positive cases and this Argininemia case. Only the infant that was a true-positive case for Argininemia had an Arg/Orn above 0.8.

This emphasizes the utility of the Arg/Orn ratio when screening for Argininemia patients.

Utilizing this ratio should both reduce the false positive rate for Argininemia and allow the Arginine cutoff to be lowered. It can serve as a tool to improve selectivity and sensitivity for detecting patients with this condition. In addition, if this ratio is not available to the clinician, this case illustrates the importance of repeat testing in the event of an elevated Arginine value above the cutoff. Plasma Arginine may be on an upward trend, as was the case with our patient.

Take-Home Message

The Arginine/Ornithine ratio may be an important tool in predicting which patients are affected by Argininemia.

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RESEARCH REPORT

Leptin Levels in Children and Adults with Classic Galactosaemia

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Abstract Among the long-term complications of Classic Galactosaemia (Gal) is premature ovarian insufficiency (POI) in female patients with subtle abnormalities of reproductive function also reported in male patients. Leptin is a circulating hormone which reflects body energy stores and which affects the neuroendocrine reproductive axis and pubertal development.

We measured serum leptin in 28 children (10 girls, 18 boys; mean age 7.6 years, range 0.5–17.9 years) and in 22 adults (10 females, 12 males; mean age 23.9 years, range 18–37 years) with Gal on a strict galactose-restricted diet in comparison with control data.

Leptin levels (expressed as SDS for gender and pubertal stage) were lower in Gal children than controls (mean leptin-SDS = –0.71 for girls, $p < 0.05$, –0.97 for boys compared with SDS = 0 for controls, $p < 0.05$). In an age-related analysis, leptin levels did not correlate with age in children with Gal for both sexes as it did for matched controls.

As expected, females had higher leptin levels than males in either group. In adults with Gal, leptin concentrations were within normal limits for both sexes when adjusted for gender and BMI. There was a linear relationship between log-leptin and BMI in children with Gal and in controls. For Gal women, log-leptin was also associated with BMI. However, for Gal men, and hence for the entire group of adult Gal patients, this association between log-leptin and BMI was not detectable. Our findings suggest that leptin dysregulation may play a role in fertility issues in individuals with Gal from an early age.

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Abbreviations

AMH	Anti-Mullerian hormone
BMI	Body mass index
FSH	Follicle-stimulating hormone
Gal	(Classic) Galactosaemia
GALT	Galactose-1-phosphate uridyltransferase
HPO	Hypothalamic-pituitary-ovarian
HRT	Hormone replacement therapy
LH	Luteinising hormone
n.s.	Not significant
SD	Standard deviation

Introduction

Classic Galactosaemia (Gal; OMIM 230400) is an autosomal recessive disorder which is characterised by a deficiency of galactose-1-phosphate uridyltransferase (GALT). Mutation p.Q188R in the *GALT* gene is the most common

mutation in patients with Gal. Despite newborn screening and early treatment with a strict galactose-restricted diet, a high percentage of patients experience long-term complications such as speech deficits, tremor, cognitive and/or behavioural impairment, and osteopenia in both sexes as well as primary or premature ovarian insufficiency (POI) in females (Hughes et al. 2009; Fridovich-Keil et al. 2011). POI is a spectrum disorder of ovarian insufficiency which may range from subfertility to primary amenorrhoea or demonstrate a fluctuating course of ovarian dysfunction. POI can be detected by measuring follicle-stimulating hormone (FSH), luteinising hormone (LH), oestradiol and anti-Mullerian hormone (AMH) in blood of females with Gal, but given the inactivity of the hypothalamic-pituitary-ovarian (HPO) axis in childhood, these levels may be not sensitive enough to identify ovarian dysfunction in a presymptomatic state.

Although it is well known that females with Gal have a high risk of hypergonadotropic hypooestrogenic ovarian dysfunction along with infertility, the underlying mechanisms for POI in galactosaemic patients remain unknown (Rubio-Gozalbo et al. 2010). They may comprise, for example, toxic effects of accumulated galactose and its metabolites, disturbances in glycoprotein and glycolipid synthesis, altered cell signalling pathways and dysregulated gene expression (Coman et al. 2010; Coss et al. 2012a). FSH levels are often found to be elevated in females with Gal but biologic activity of gonadotropins was described as essentially normal (Kaufman et al. 1981; Berry 2008). Along these lines, FSH isoform patterns did not differ between patients with Gal and postmenopausal controls (Gubbels et al. 2011a). Data on ovarian histology in galactosaemic females is scarce, but hypoplastic or streak gonads with decreased numbers of primordial follicles along with reduced or absent intermediate and Graafian follicles were found, similar to other genetic diseases which directly affect the ovary (Rubio-Gozalbo et al. 2010; Berry 2008). Hormone replacement therapy (HRT) with oestrogen and progesterone as appropriate along with clinical monitoring are reasonable interventions at the current time. However, spontaneous pregnancies may occur in women with Gal, and a predicting factor for the possibility to conceive is spontaneous menarche (Gubbels et al. 2008).

Males with Gal spontaneously reach puberty but onset of puberty may be delayed (Rubio-Gozalbo et al. 2010). Recently Gubbels and colleagues have reported a higher-than-predicted prevalence of cryptorchidism in Gal males with subtle decreases in testosterone, inhibin B, semen volume and sperm count which might indicate mild defects in Sertoli and Leydig cell function (Gubbels et al. 2011b).

Among the endocrine and metabolic key molecules which directly modulate the HPO axis is leptin. This is a 146 amino acid non-glycosylated protein which is secreted predominantly by adipocytes but which is also expressed in other tissues such as stomach, kidney and liver (Meissner et al. 2005). Leptin is involved in body weight regulation and provides information on body energy stores (Knerr et al. 2006). Leptin is involved in regulation of food intake and energy expenditure at the hypothalamic level as well as in reproductive maturation and fertility. It is capable of increasing oestrogen production through the stimulation of aromatase expression and activity in luteinized granulosa cells and adipocytes (Catalano et al. 2003). The leptin receptor is expressed in the brain and several other tissues such as liver, stomach, kidney and immune cells (Denver et al. 2011; Xu et al. 2012). Leptin acts on multiple brain regions including the brain stem, hypothalamus, hippocampus and ventral tegmental area by activating the cytokine type 1 leptin receptor which is coupled to the JAK2 signalling mechanism (Trinko et al. 2011). As we have reported dysregulation of multiple central cell signalling pathways in Gal (Coman et al. 2010), we proposed to study circulating leptin levels in children and adults with Gal.

Patients and Methods

Patients

Our study was approved by the Ethics Committee of Children's University Hospital, Dublin. Informed consent was obtained prior to enrolment. We recruited 28 children and 22 adult Irish Gal patients, including 10 pairs of siblings, who were being treated at the National Centre for Inherited Metabolic Disorders, Dublin, and the Metabolic Unit at The Royal Belfast Hospital for Sick Children. Our paediatric cohort comprised of 10 girls (age 0.6–17.9 years, mean 7.7 years.) and 18 boys (age 0.5–16.7 years, mean 7.6 years). All children with Gal were diagnosed on newborn screening (day 5 of life) or selective screening (day 1) in the case of a positive family history for Gal and immediately commenced on a lactose-free diet. The adult study cohort comprised 10 females (age 19–37 years, mean 25.4 years) and 12 males (age 18–28 years, mean 22.4 years). The mean age at diagnosis in our adult patient group was 12 days (median 7 days, range day 1–day 45 of life). Mean age at menarche in female adults was 14.1 years. (range 12–16 years) and 15.3 for adolescent girls (range 13–17 years); only four female patients had had spontaneous puberty and menarche. At the age the blood sample was taken, all women except one and all but one adolescent girl were on HRT. Adequate intake of calcium and vitamin D was also recommended along with physical exercise. All patients were on a strict galactose-restricted diet for life. No female patient in this study group had given birth and no male patient had fathered a child at the time of the study.

In the entire Gal cohort, 46 patients (92%) were homozygous for the *GALT* mutation *p.Q188R*; two siblings

were compound heterozygous for *p.Q188R/p.R333W* and *p.Q188R/p.K285N*, respectively; and two patients were not genotyped. Clinical data and serum samples were compiled during routine outpatient visits. Weight and height were measured, and BMI calculated as body weight (kg) divided by height (m) squared.

Leptin Assay

Serum leptin was measured using a human leptin immunoassay. A microtitre plate-based DELFIA assay was applied, and antibodies and standards were purchased from R&D Systems (R&D Systems Europe, Abingdon UK). The intra- and inter-assay CVs (%) were between 3.9 % and 7.1 %. The lower limit of detection was 0.1 ng/ml. Samples were analysed in duplicate.

Control Data

The control data for our paediatric cohort was obtained by recruiting apparently healthy age- and sex-matched control children and adolescents (age range 1–17.0 years). The control data for adults were obtained from 1,670 healthy young adults under 45 years of age and stratified according to gender and BMI. We obtained data from 340 men and 558 women with a BMI less than 25 and 456 men and 316 women with a BMI of 25–29.9 kg/m^2. These volunteers have been recruited for ongoing population-based studies in the UK after gaining ethical approval in 2004. Exclusion criteria for our control subjects were chronic diseases such as obesity, diabetes or cancer, and details are published elsewhere (Kilpeläinen et al. 2011).

Statistical Analyses

Accounting for the logarithmic distribution of leptin levels, leptin standard deviation (SDS) according to gender, BMI and pubertal stage was calculated using a standard equation based on published normative data (Blum et al. 1997). In adults, a base-10 logarithm transformation of serum leptin concentrations was used to ensure normal distribution of data. Statistical analyses were performed using Prism 5 Software (GraphPad, La Jolla, CA, USA), including Pearson correlation, linear regression analysis, and Student's *t*-test. A significance level of $p < 0.05$ was chosen for all comparisons.

Results

In our cohort, we found lower leptin levels and leptin-SDS in children with Gal than in controls (mean leptin-SDS = –0.71 for girls, –0.97 for boys compared with SDS = 0 for controls, $p < 0.05$ for each analysis, Figs. 1, 2). In general, girls had higher leptin levels than boys (4.4 ± 4.1 for Gal girls vs. 0.9 ± 0.5 for Gal boys, $p < 0.01$, and 8.1 ± 3.2 for control girls vs. 2.1 ± 1.4 for control boys, $p < 0.05$, Fig. 1).

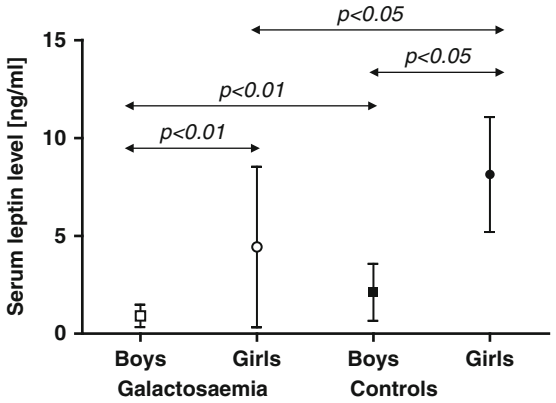

Fig. 1 Serum leptin levels for children with Gal and age- and gender-matched controls are presented as mean values ± SD

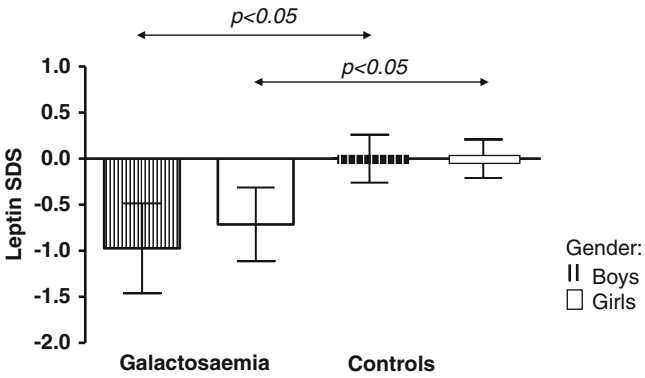

Fig. 2 Leptin standard deviation scores (SDS) for gender and pubertal stage are given for children with Gal and age- and gender-matched controls as mean values ± SD

In an age-related analysis, serum leptin levels did not correlate with age in the entire paediatric Gal group (r = 0.0598, n.s.) or for both sexes (r = 0.1326, n.s., for girls with Gal, r = –0.0877, n.s., for boys with Gal).

In detail, we also found no significant correlation between leptin and age when data of prepubertal boys with Gal was tested separately to exclude effects of rising testosterone levels ($r = –0.4690$, n.s.). Conversely, leptin was found positively correlated to age in our paediatric control group ($r = 0.6546$, $p < 0.01$). In detail, leptin correlated positively with age in our female paediatric controls ($r = 0.8188$, $p < 0.01$). For our male paediatric controls, we found a correlation between leptin and age only in the subgroup of prepubertal boys ($r = 0.6655$, $p < 0.05$). We found higher leptin levels along with higher BMI in women compared to girls with Gal ($p < 0.001$), and

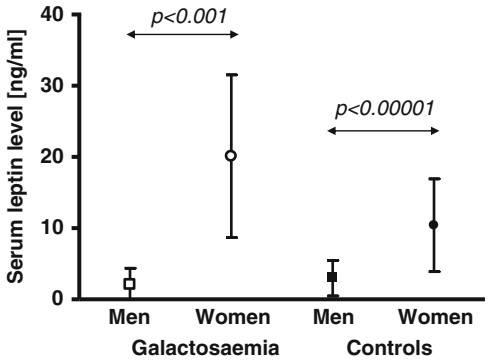

Fig. 3 Illustrative data set (mean values ± SD) for serum leptin concentrations for men and women of normal weight with Gal as well as controls

individuals with normal body weight (BMI 19–24.9 kg/m^2, $p < 0.001$ for Gal patients and $p < 0.00001$ for controls, respectively, Fig. 3).

A linear regression model was then fitted to determine the relation between log-leptin and BMI for each gender and cohort. We found a significant linear relationship between log-leptin and BMI in children with Gal ($r^2 = 0.9942$, $p < 0.0001$ for girls, $r^2 = 0.9939$, $p < 0.0001$ for boys and $r^2 = 0.9888$, $p < 0.0001$ for the entire group of children with Gal) and also for our control subjects ($r^2 = 0.8791$, $p < 0.01$ for girls, $r^2 = 0.8287$, $p < 0.01$ for boys, and $r^2 = 0.8681$, $p < 0.001$ for the entire paediatric control group, Fig. 4a). Among female adults with Gal, BMI was strongly

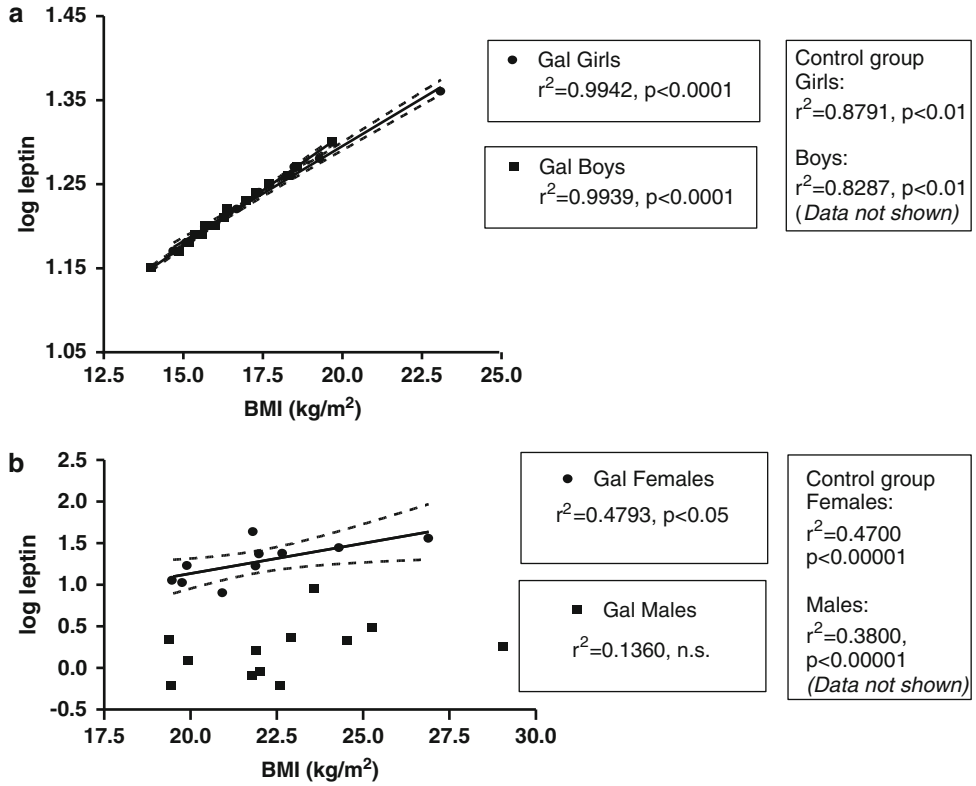

Fig. 4 Linear regression analysis between log-leptin concentration and BMI in children (**a**) and adults (**b**) with Gal. Linear regression r^2 and p values for control subjects are also given but data is not shown in this diagram

lower leptin levels despite higher BMI in men compared to boys with Gal ($p < 0.01$). Essentially, serum leptin concentrations were within normal limits for women and men with Gal when adjusted for gender and BMI (Fig. 3). As expected, leptin and log-leptin concentrations in male adults were lower than in female adults in the entire cohort ($p < 0.0001$ for Gal patients, $p < 0.00001$ for control subjects, for both comparisons) and also in the subgroup of associated with log-leptin values ($r^2 = 0.4793$, $p < 0.05$). This was also found in healthy controls of both sexes ($r^2 = 0.4700$, $p < 0.00001$ for females, and $r^2 = 0.3800$, $p < 0.00001$ for males, Fig. 4b). However, in male adults with Gal and, therefore, in the entire group of Gal patients, this association between log-leptin and BMI was no longer detectable ($r^2 = 0.1360$, $p = 0.2382$, n.s. and $r^2 = 0.0070$, $p = 0.7104$, n.s., respectively, Fig. 4b).

Discussion

Our data demonstrate that children with Gal had lower serum leptin levels than controls, expressed as SDS for gender and pubertal stage. In an age-related analysis, serum leptin levels did not correlate with age in our paediatric Gal group as it did in our control group and peak leptin levels were lower in children with Gal than in controls. As expected, females had higher leptin levels than males in either group. Although leptin concentrations were essentially within normal limits for women and men with Gal when adjusted for gender and BMI, there was a lack of the physiological association between BMI and log-leptin in males and, therefore, also in the entire adult Gal cohort. In females with Gal on HRT, this strong association between BMI and log-leptin was preserved.

Fertility issues in female patients with Gal are complex and multifactorial. Possible mechanisms which may underlie POI in females with Gal may comprise (1) increased oocyte loss, e.g. due to direct toxic effects of galactose and its metabolites or due to hypoglycosylation; (2) altered dynamics of follicle development, e.g. due to dysregulated balance of apoptosis and survival pathways in the folliculogenesis; (3) perturbated gene expression pattern along with metabolic dyshomeostasis; and (4) altered follicle maturation due to reduced biopotency of nurturing factors such as FSH or others (Coman et al. 2010; Rubio-Gozalbo et al. 2010; Fridovich-Keil et al. 2011; Coss et al. 2012a). We have described earlier that, consistent with the international experience, 91.2% of females with Gal suffer from hypergonadotropic hypogonadism along with pubertal delay and fertility issues (Coss et al. 2012b).

Leptin is a key molecule for pubertal development in many mammalian species. It has been proposed that leptin may exercise immediate action in the function of the reproductive glands and leptin receptors have been identified in ovaries and testes (Goumenou et al. 2003). Essentially, leptin levels increase in early pubertal stages but to a higher extent in girls than in boys due to an increase in testosterone in the latter (Garcia-Mayor et al. 1997). These differences and age-dependence of leptin concentrations become especially evident after appropriate logarithmic transformation (Blum et al. 1997). Essentially, there is a significant correlation of leptin, or log-transformed values of leptin, and BMI in healthy males and females of a wide age range (5–77 years) (Isidori et al. 2000; Gómez et al. 2003; Falorni et al. 1997). Contrary to what is expected, there was a lack of a correlation between log-leptin and BMI in adult males with Gal and in our entire adult Gal cohort. Along these lines, one might explain the differences between our paediatric and adult Gal cohort, at least partly, by the chronic nature of this disorder. Ninety percent of women in our study have been treated with HRT at the time the sample was taken which might contribute to subsequent normal leptin levels in these individuals. Therefore, our findings do not rule out partial leptin deficiency in galactosaemic women who do not use HRT, or an individual susceptibility to low leptin levels that may act as a 'second hit'. It has been shown, for example, that leptin serum levels declined in women who underwent bilateral ovariectomy for benign reasons and that treatment with oestrogen and progesterone prevented a decrease in leptin concentrations (Messinis et al. 2000).

The essential role of leptin in fertility is highlighted by the fact that mice lacking leptin (ob/ob mice) or leptin receptor (db/db mice) are typically infertile as they fail to enter puberty (Donato et al. 2011). Leptin deficiency in mice is associated with impaired folliculogenesis and increased follicular atresia (Hamm et al. 2004), findings reminiscent of those seen in ovarian tissues obtained from females with Gal. Female offspring of pregnant rats placed on a high galactose diet show altered germ cell migration, increased rate of follicular atresia, attenuated FSH bioactivity and a higher galactose-incorporation of serum proteins compared with control animals (Banerjee et al. 2012). Among the follicular survival factors are gonadotropins and integrin (Hussein 2005). Isolated galactose-treated rat granulosa cells demonstrated increased expression of p53, a protein which can facilitate apoptosis and cell cycle arrest. In principle, apoptosis takes place predominantly in oocytes during foetal life and in granulosa cells of secondary and antral follicles during adult life, and there are subsequent mechanisms which eventually lead to apoptosis in these cells (Hussein 2005). It is unclear at present whether females with Gal experience disturbed development or a progressive loss of ovarian tissue or, most likely, both, as the ovarian tissue in humans is vulnerable throughout life (Fridovich-Keil et al. 2011).

An association between galactose absorption and leptin has been demonstrated; leptin inhibits galactose uptake in the small intestine (Lostao et al. 1998) by acting on the Na (+)/glucose cotransporter (Barrenetxe et al. 2004). We have shown that glycoprotein processing defects persist in treated Gal patients which could potentially affect many cell signalling mechanisms to include leptin-mediated cell signalling (Coss et al. 2012a). Essentially, patients with Gal consume considerable amounts of soy products from birth onwards. These soy products contain isoflavones (phytooestrogens) which can function as oestrogen agonists or selective oestrogen receptor modulators. Infants fed with soy formula have higher concentrations of phytooestrogens in plasma and urine than breastfed babies; however, it cannot be concluded that these soy isoflavones are biologically active (Vandenplas et al. 2011). Along these lines, human studies did not detect any clinical significance of the amounts of nutritional phytooestrogens in infants fed with

soy formula, but animal studies with phytooestrogens provide conflicting data, including an association between the intake of a related compound, coumestrol, and decreased fertility (Vandenplas et al. 2011). In a group of 40 children with Gal who had decreased body weight and height, body fat mass (FM) has been shown to be correlated with soy intake (Panis et al. 2005). In this group of patients, body composition was abnormal, possibly due to their metabolic defect, decreased levels of IGF-I and/or soy nutrition as speculated by the authors (Panis et al. 2005). However, daily isoflavone intake (up to 130 mg/day) had essentially no effect on leptin levels in healthy premenopausal and postmenopausal women (Phipps et al. 2001). Oestrogen increases leptin production, but it has been demonstrated that even high levels of isoflavone consumption do not alter leptin concentrations in females studied over 3 months (Phipps et al. 2001).

However, data on galactosaemic individuals are lacking and difficult to obtain given a long-term 'lactose-free' diet and high soy intake. It thus remains speculative whether a lower FM and higher intake of phytooestrogens may ultimately lead to pseudo-normal leptin levels.

The relative increase of leptin with BMI during puberty is comparable in both sexes but the absolute values at a given BMI are lower in boys in late puberty (Blum et al. 1997). However, leptin is positively correlated with BMI in healthy boys (El-Eshmawy et al. 2010). Early puberty might be a particular vulnerable period in individuals at risk of decreased reproductive capacity. Along these lines, reduced leptin levels were found in adolescent boys with constitutional delay of growth and puberty (El-Eshmawy et al. 2010). Additionally, decreased leptin and its impact on the reproductive axis may help to explain delayed pubertal development in adolescent boys with Gal and perhaps also cryptorchidism in some cases (Gubbels et al. 2011b).

Recent results from our group, as well as others, have demonstrated abnormal *N*-glycan processing and assembly in individuals with Gal (Coss et al. 2012a; Coman et al. 2010). This might affect G-protein-coupled receptors, including the insulin-like peptide relaxin receptors LGR7 and LGR8 which have roles in reproductive tissue remodelling. Hypothalamic KiSS-1 neurons, which express leptin receptors, execute a prominent role in the neuroendocrine and metabolic control of fertility, including regulation of the gonadotropic axis and its full activation at puberty (Tena-Sempere 2006). We speculate that hypoleptinaemia may, therefore, contribute to a delay in pubertal maturation and dysregulation of reproductive function in individuals with Gal at a neuroendocrine level from an early age. In addition to gonadal function loss, e.g. POI in women with Gal, hypoleptinaemia may be an aggravating factor in individuals with Gal at risk for reproductive dysfunction. Moreover, gonadal function loss may further decrease circulating leptin levels independently from age and BMI (Benetti-Pinto et al. 2010).

In summary, we present data which suggests that leptin dysregulation may play a role in delayed pubertal maturation and fertility issues from an early age in Gal patients. Further studies are now needed, including the application to animal models, to study the impact of altered leptin levels in Gal.

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Synopsis

Leptin is an interesting key molecule for reproductive development and fertility and its dysregulation might play a role in individuals with Gal.

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CASE REPORT

CRIM-Negative Pompe Disease Patients with Satisfactory Clinical Outcomes on Enzyme Replacement Therapy

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Abstract Pompe disease, especially in its infantile form, is a fatal disease. Most of the patients with this disease synthesize a nonfunctional form of the enzyme alpha glucosidase (GAA), the deficient enzyme in this disease. Patients producing some amount of this protein are labeled as cross-reactive immunologic material (CRIM)-positive. Few of them are unable to synthesize it and are labeled CRIM-negative. The clinical course of the disease has changed with the advent of enzyme replacement therapy (ERT) with recombinant alpha glucosidase enzyme (rhGAA). However, CRIM-negative patients have always been known to have poor outcome on ERT due to the development of anti-rhGAA antibodies in their bodies that neutralizes ERT efficacy. Here, we describe two CRIM-negative siblings on rhGAA ERT with unusually low anti-rhGAA antibody titer and good clinical outcome. Up to our current knowledge, this is the first report that describes such a good response to ERT in CRIM-negative patients.

Abbreviations

CRIM	Cross reactive immunologic material
ERT	Enzyme replacement therapy
GAA	Acid alpha glucosidase
rhGAA	Recombinant human acid alpha glucosidase

Introduction

Pompe disease (glycogen storage disease type II) is an autosomal recessive lysosomal storage disease caused by deficiency of acid α-glucosidase (GAA). As a consequence, its substrate glycogen accumulates in cardiac, skeletal, and smooth muscle tissues. The infantile form, which is the most severe phenotype, presents clinically with cardiomyopathy, hypotonia, generalized muscle weakness, feeding difficulties, failure to thrive, and respiratory insufficiency.

GAA protein is called cross-reactive immunologic material (CRIM) because anti-GAA antibodies recognize it on Western blot analysis. Most Pompe patients still have some residual GAA protein production and are labeled as CRIM positive while those with total absence of that protein are labeled CRIM negative (Amalfitano et al. 2001). Without treatment, death typically occurs with the first 1 to 2 years secondary to cardiorespiratory failure (Van den Hout et al. 2003). Enzyme replacement therapy (ERT) with recombinant acid α-glucosidase (rhGAA) has been shown to prolong these patients' lives (Kishnani et al. 2007, 2009; Nicolino et al. 2009). However, the response of these

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patients to rhGAA treatment depends on their CRIM status. CRIM-negative infants generally have a poor prognosis, where they have been found to have an initial improvement followed by a clinical deterioration. Their deterioration has been attributed to the high levels of anti-rhGAA IgG antibodies their bodies develop against the recombinant enzyme. Conversely, CRIM-positive infants have a more favorable prognosis and their anti-rhGAA antibodies are not as significantly elevated as CRIM-negative ones (Kishnani et al. 2010; Banugaria et al. 2011).

In this case report, we describe two siblings, brother and sister, who are CRIM negative on a relatively long GAA ERT with good outcome.

Patients and Methods

The patients are two African American CRIM-negative siblings. The first is a 4.5-year-old boy while the second is 2-year-old girl.

CRIM status was determined as described previously (Kishnani et al. 2006). Anti-rhGAA IgG antibody titer measurements and CRIM status were performed at Genzyme Corporation (Cambridge, MA). Leukocyte GAA activity assays were performed at Emory University Biochemical Genetics Laboratory. GAA mutation analysis was performed at Emory University Molecular Genetics Laboratory.

Results

The boy was a product of full-term pregnancy delivered via cesarean section secondary to abnormal heart rate deceleration. Soon after delivery, he was noted to be mildly cyanotic and was given a diagnosis of transient tachypnea of the newborn (TTN). A chest x-ray showed left ventricular enlargement. A follow-up echocardiogram showed hypertrophic obstructive cardiomyopathy with mild left ventricular outflow tract obstruction and mild mitral regurgitation. He was started on propranolol, which lead to improvement in the mitral regurgitation and left ventricular outflow tract gradient. He was discharge on propranolol 2 mg every 6 h to be followed bimonthly by the cardiologist. Because of the progressive worsening of his cardiomyopathy, his cardiologist referred him to our genetics department to investigate for a possible genetic cause of his condition, specifically to rule out Pompe disease. When he was first seen in our genetics clinic, he was 6 months old and his investigations showed total creatine kinase (CPK) level of 2902 IU/L (normal: 60–305 IU/L) and CK MB fraction of 19.9 ng/mL (normal range: 0.0–3.3 ng/mL). Pompe disease was suspected and diagnosed on the basis of decreased leukocyte GAA activity (0.3 nmoles/mg protein/h; normal: 6.01–37 nmoles/mg protein/h) and confirmed by mutation analysis that showed a deletion mutation in one allele (IVS2 +2_ +5 delTGGG deletion in intron 2) and an insertion mutation (c.1650_1651 dupG in exon 12) in the second. Western blot testing on a skin fibroblast sample showed him to be CRIM-negative.

Since his diagnosis was confirmed, he has been receiving 20 mg/kg rhGAA every 2 weeks, now for a total of 4 years on treatment. His clinical course was complicated by one hospital admission at the age of 1.5 years for pneumonia for which he was intubated, but is now completely recovered. Since that hospitalization, he has been followed annually with a pulmonologist who prescribed him Levalbuterol and Albuterol nebulizers. He has regular follow up with his cardiologist. He still has mild intraventricular conductive delay and mild hypertrophy. His echocardiogram showed significant improvement since he was started on rhGAA ERT; he currently has a ventricular septum of 7 mm, which has been stable for the last 12 months, and is significantly decreased compared to his highest measurement of 18 mm just before the start of his ERT (Fig. 1a and b). His anti-rhGAA antibody titers over the course of his ERT are summarized in Fig. 2. During the course of his treatment, he never had any infusion-related events even at the time of his highest anti-rhGAA antibody titer.

He has mild developmental delay. He walked at the age of 17 months. He receives physiotherapy and can run and walk up and down stairs. He wears ankle-foot orthotic braces now on a daily basis due to a development of foot drop in the last year. He has been found to have low vitamin D level, low borderline calcium, and elevated parathyroid hormone. He is now on supplemental vitamin D and calcium. His receptive language is normal but expressive skills are delayed for which he is receiving speech therapy. He has passed all his hearing tests so far.

Based on the positive family history, his sister's diagnosis was reached prenatally via mutational analysis on amniocyte-isolated DNA where she was found to have the same mutations as her brother. Her leukocyte GAA activity was 1.02 nmoles/mg protein/h (normal: 6.01–37 nmoles/mg protein/h). At the age of 2 days, her CPK level was found to be 1350 IU/L. Her echocardiogram was consistent with severe obstructive cardiomyopathy for which she was transferred to pediatric intensive care unit. She has been receiving 20 mg/kg rhGAA every 2 weeks since the age of 6 days, now on ERT for over 2 years. She still has mild intraventricular conductive delay and T-wave inversion in the anterolateral leads with features of ventricular strain. Her last echocardiogram showed a ventricular septum thickness of 5 mm, which has been persistently decreasing from her highest, i.e., 11 mm at

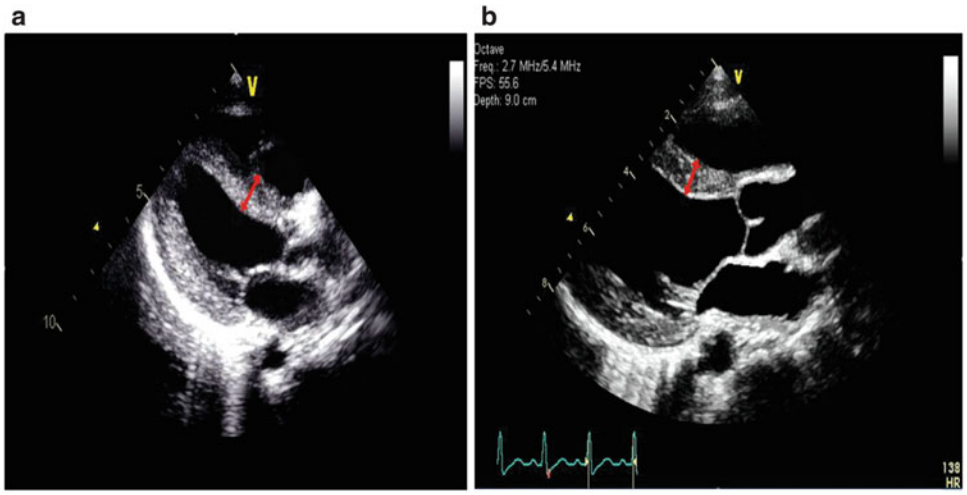

Fig. 1 Echocardiogram images showing the first patient's interventricular septum thickness (*red bidirectional arrow*) before (**a**) and after (**b**) ERT

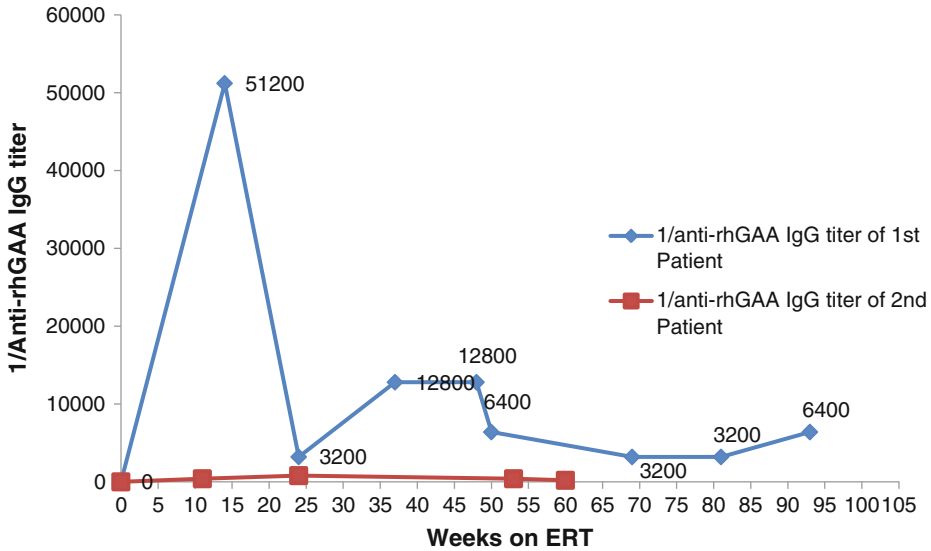

Fig. 2 Patients' anti-rhGAA antibody titers over their corresponding duration of treatment

birth (Fig. 3a and b). She passed all her hearing tests so far and has been developing normally with no hospitalization or decompensation. Her anti-rhGAA antibody titers over the course of her ERT are summarized in Fig. 2. Unlike her brother, she had multiple infusion-related reactions in the forms of hives that respond very well to 11 mg oral dose of diphenhydramine hydrochloride and infusion time extension. None of these reactions were life threatening or severe enough to stop the infusion or decrease its frequency.

Discussion

Since its approval for commercial use in 2006, ERT with rhGAA has yielded significant change in the course of infantile Pompe disease. It has been found, particularly in CRIM-positive patients, to reverse cardiomyopathy, delay or stabilize motor disease progression characterizing this disease, and prolong ventilator-free survival (Banugaria et al. 2011). Because of the high and sustained titers of neutralizing anti rhGAA antibodies produced in their bodies, CRIM-negative patients are known to benefit the least from such therapy and they are either ventilator dependent or deceased by age 27.1 months (Abbott et al. 2011). The longest CRIM-negative ventilator-free survival was described in a single case report (Rohrbach et al. 2010) in which the patient was treated with omalizumab (anti-IgE mAb) for IgE-mediated anaphylactic episode related to ERT. The elder CRIM-negative patient in our case report has so far reached the age of 54 months, which is

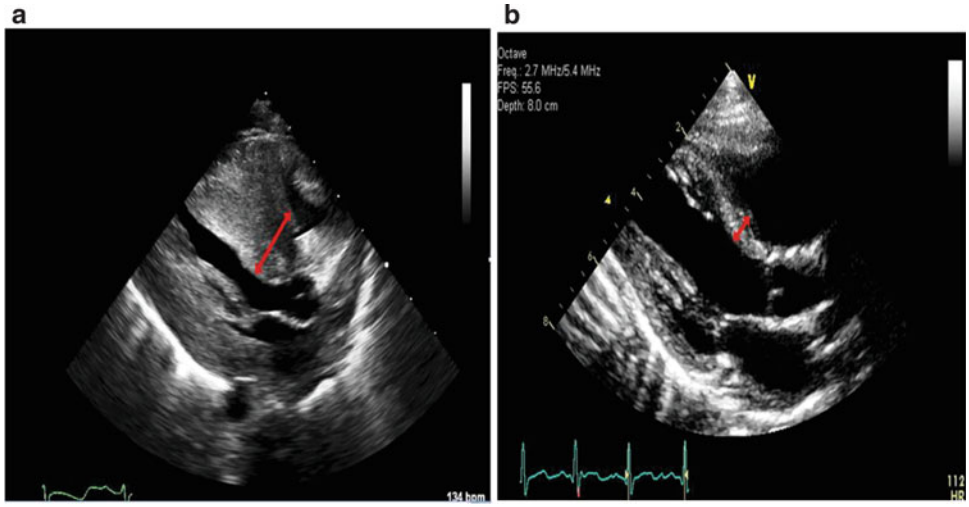

Fig. 3 Echocardiogram images showing the second patient's interventricular septum thickness (*red bidirectional arrow*) before (**a**) and after (**b**) ERT

the longest ever ventilator-free survival reported for a CRIM-negative patient. In addition, he completed this ventilator-free period of time without having any immunomodulatory intervention.

The nature of the mutation in these two siblings is a candidate reason behind their favorable outcome to enzyme treatment compared to other CRIM-negative patients. Both mutations have recently been reported and associated with CRIM-negative status (Bali et al. 2012). To our knowledge, no study to correlate the type of mutation in CRIM-negative patients with their response to rhGAA ERT was published. In these two siblings, it is possible that the product of the first mutation, a splice mutation, might produce a quantity of the enzyme that is too small to be detected by western blot and yet enough to desensitize the patient's immune system so that it would not produce high titer of anti-rhGAA antibodies. Since the second mutation is a frame shift mutation with nonesense mutation 85 base pairs downstream, we do not expect a protein to be produced by this mutation especially when we take into consideration the nonesense-mediated messenger RNA decay that occurs with such mutations.

The fact that the second sibling started her enzyme therapy earlier than the first one, explains her lower anti-rhGAA antibody titers compared to her brother who carries the same mutations and points toward the importance of early enzyme treatment.

This case report does not only describe a new pattern of immunological response to rhGAA ERT but emphasizes the fact that our understanding of that immunological process is limited. It also proves that CRIM status is not the sole immunological determinant of Pompe patients' prognosis on such treatment and reinforces the need to study the genotype-phenotype correlation between CRIM-negative mutations and the response to enzyme treatment.

With the availability of immunomodulatory regimens (Joseph et al. 2008; Mendelsohn et al. 2009; Rohrbach et al. 2010; Messinger et al. 2012) to combat immunological reaction against rhGAA that CRIM-negative patients usually develop, it is clear from these two cases that not all CRIM-negative patients need them; relying solely on CRIM status to decide who should receive such immunomodulatory treatment might subject some CRIM-negative patients to unnecessary immunosuppression. This issue holds further importance as Pompe disease is being proposed to be included on the newborn screening panel. If added, consensus guidelines will be needed to address timing of ERT initiation in CRIM-negative patients and if immunomodulatory regimen should be used.

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Synopsis

Some CRIM-negative patients can have good outcome on enzyme replacement therapy

Authors' Contribution

Hamoud H. Al Khallaf contributed to this work by gathering some data, drawing a figure, and participating in writing the manuscript.

Jennifer Propst contributed to this work by summarizing patients' clinical data and revising the manuscript.

Eleanor Botha helped in summarizing patients' clinical data and revising the manuscript.

Serge Geffrard performed echocardiogram for the patients and revised the manuscript.

M. Ali Pervaiz planned the study, obtained the clinical data from the patients, and edited the entire manuscript.

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Ethics Approval

Was not required.

Patients' Consents

Was obtained from the mother of the two subjects.

Conflict of Interest

None.

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CASE REPORT

Partial Pyridoxine Responsiveness in PNPO Deficiency

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Abstract *Objective:* Autosomal-recessive pyridox(am)ine phosphate oxidase (PNPO) deficiency causes pyridoxal-5-phosphate (PLP)-dependent epilepsy. We describe partial PNPO deficiency with a transient response to pyridoxine (B6).

Methods: CSF neurotransmitter metabolites, PLP, and amino acids were analyzed while the patient was receiving pyridoxine. PNPO gene sequencing was performed by standard techniques.

Results: A full-term 3,220 g male with refractory neonatal seizures became seizure free for 6 weeks on pyridoxine (B6). Breakthrough seizures followed. These stopped upon the first dose of PLP although episodes occurred as a dose became due. An unidentified peak was detected on the chromatographic system used to measure CSF PLP. PNPO gene sequencing identified a homozygous mutation in a highly conserved area in exon 3: c.352G>A p.G118R, predicting substitution of arginine for glycine. At age 28 months the child has hypotonia and developmental delay, both mild in severity.

Conclusions: Transient pyridoxine responsiveness may be seen in partial PNPO deficiency. A CSF metabolite peak, likely pyridoxine phosphate, is identifiable in patients with PNPO deficiency who are taking supplemental pyridoxine. Partial B6 responsiveness is an indication for possible PNPO deficiency and trial of PLP.

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Introduction

Pyridoxine (B6)-dependent epilepsy was first described in 1954 (Hunt et al. 1954). The biological defect in this syndrome was recently identified as antiquitin mutations resulting in alpha aminoadipic semialdehyde (AASA) dehydrogenase deficiency (Mills et al. 2006). This enzyme is in the lysine degradation pathway and its deficiency leads to accumulation of intermediates that include pipecolic acid, AASA, and piperideine 6′-carboxylate. The latter sequesters pyridoxal 5′-phophate (PLP), leading to PLP deficiency within the central nervous system.

Pyridoxine (B6) is not itself an active cofactor. It is converted, together with the pyridoxal and pyridoxamine vitamers, to the active PLP via phosphorylation through the action of a kinase followed by oxidation by pyridox(am)ine 5′-phosphate oxidase (PNPO) (Surtees et al. 2006). Autosomal-recessive PNPO deficiency leads to a deficiency of PLP and subsequently to PLP-dependent epilepsy that, in general, is not thought to respond to pyridoxine (Mills et al. 2005). Without rapid detection and specific intervention, this causes catastrophic neonatal encephalopathy. In this report, we describe partial PNPO deficiency in an infant initially suspected to have pyridoxine responsiveness.

Methods

CSF neurotransmitter metabolites, pyridoxal-5-phosphate, and amino acid quantification. PNPO gene sequencing was performed by standard techniques on the proband followed by the parents.

Results

A full-term 3,220 g newborn male delivered following an uncomplicated gestational course and maternal labor presented with irritability, inconsolable crying, erratic eye movements with eyelid twitching, and grunting sounds 12 h following birth. EEG showed bilateral sharp discharges and episodic background suppression, although without a constant temporal correlation with the clinical symptoms. MRI was unremarkable. Following trials of phenobarbital and levetiracetam, the patient became seizure free for 6 weeks on pyridoxine. Breakthrough tonic seizures then occurred at 3.5 months of age, followed by myoclonic seizures, leading to hospitalization and trials of topiramate and prednisolone which were ineffective. EEG monitoring showed intermittent paroxysms of diffuse sharp electrographic activity associated variably with clinical facial grimacing, abnormal eye movements, and erratic multifocal myoclonias of the extremities which sometimes resembled thrashing type movements (Fig. 1). These were separated by periods of relative background suppression, accompanied by an initial tonic spasm and then quieting until the next paroxysm. This sequence persisted 25 min until intravenous lorazepam administration led to temporary resolution. The seizures stopped upon the first dose of PLP, but breakthrough events occurred as a dose became due. The patient achieved complete seizure control on PLP 10 mg/kg/dose given every 6 h.

CSF was sent for neurotransmitter metabolites, PLP, and amino acid analysis during the patient's hospitalization with concomitant pyridoxine therapy. CSF PLP level was 23 nmol/L (normal range of 23–64 nmol/L). CSF amino acids showed a slight increase in threonine. Neurotransmitter metabolites were normal. An additional, previously unidentified, peak was detected on the chromatographic system used to measure PLP.

Subsequent PNPO gene sequencing identified a homozygous mutation in a highly conserved area in exon 3: c.352G>A p.G118R, predicting a substitution of arginine for glycine. Both parents were identified as heterozygous for this mutation. The patient has had sustained seizure control and developmental progress with ongoing dosing of PLP 10 mg/kg/dose administered four times daily with follow-up out to 2 years of age. Breakthrough clinical events tend to appear with intervals approaching 7–8 h after a prior dose, and examination shows mild symmetrical hypotonia and language delay. Developmental assessment at age 28 months indicated these levels: gross motor 21–24 months, fine motor 20–22 months, cognitive 20 months with scatter to 27 months, social-emotional 21–24 months, receptive language 24–28 months, expressive language 20–23 months, and self-help skills 21–24 months. His overall vocabulary is approximately 40 words, with the ability to place a few words together. However, much of his speech is repetition.

Discussion

The neonatal onset neurotransmitter disorders include both pyridoxine and PLP-responsive epilepsies (Pearl 2009). PLP, the biologically active form of B6, is a cofactor in approximately 120 enzymatic reactions, many of which involve amino acid and neurotransmitter metabolic pathways (Stockler et al. 2011). PNPO deficiency represents an epileptic encephalopathy resistant to pyridoxine but responsive to PLP.

The PNPO mutation identified, c.352G>A, has not been reported before. It is predicted by Polyphen to be probably damaging and by SIFT to be damaging. Our proband demonstrated an initial response in seizure control to oral pyridoxine dosing following refractoriness to standard antiepileptic drugs. When this control was lost after 6 weeks, CSF analysis was undertaken for neurotransmitter metabolite and PLP analysis. An unidentified compound was detected on the fluorescence chromatogram which has been noted in other patients with PNPO deficiency supplemented with pyridoxine. This peak is not detected in CSF samples derived from individuals with alpha-AASA dehydrogenase deficiency while on pyridoxine supplementation and is not the same peak seen in the chromatograms of folinic acid-responsive patients. We suspect this represents pyridoxine phosphate as this compound has been shown to accumulate in plasma in the absence of the PNPO (Footitt et al. 2012).

The seizure semiology in our patient is notably similar to the recently reported signs in four patients with pyridoxine-responsive epilepsy and one patient with PNPO deficiency (Schmitt et al. 2010). The features of neonatal and infantile irritability, inconsolable crying, facial grimacing, abnormal eye movements, eye twitching, multifocal myoclonias and erratic movements, and tonic seizures are characteristic of both diagnoses. The intermittent periods of EEG background suppression in our patient are remarkably similar to the PNPO-deficient patient in that series, as is the clinical observation of breakthrough events approximately 7 h after the last dose of PLP. The inconstant ictal EEG findings, absence of consistent clinical correlation with otherwise nonspecific paroxysmal EEG changes, and difficulty distinguishing interictal from ictal EEG background

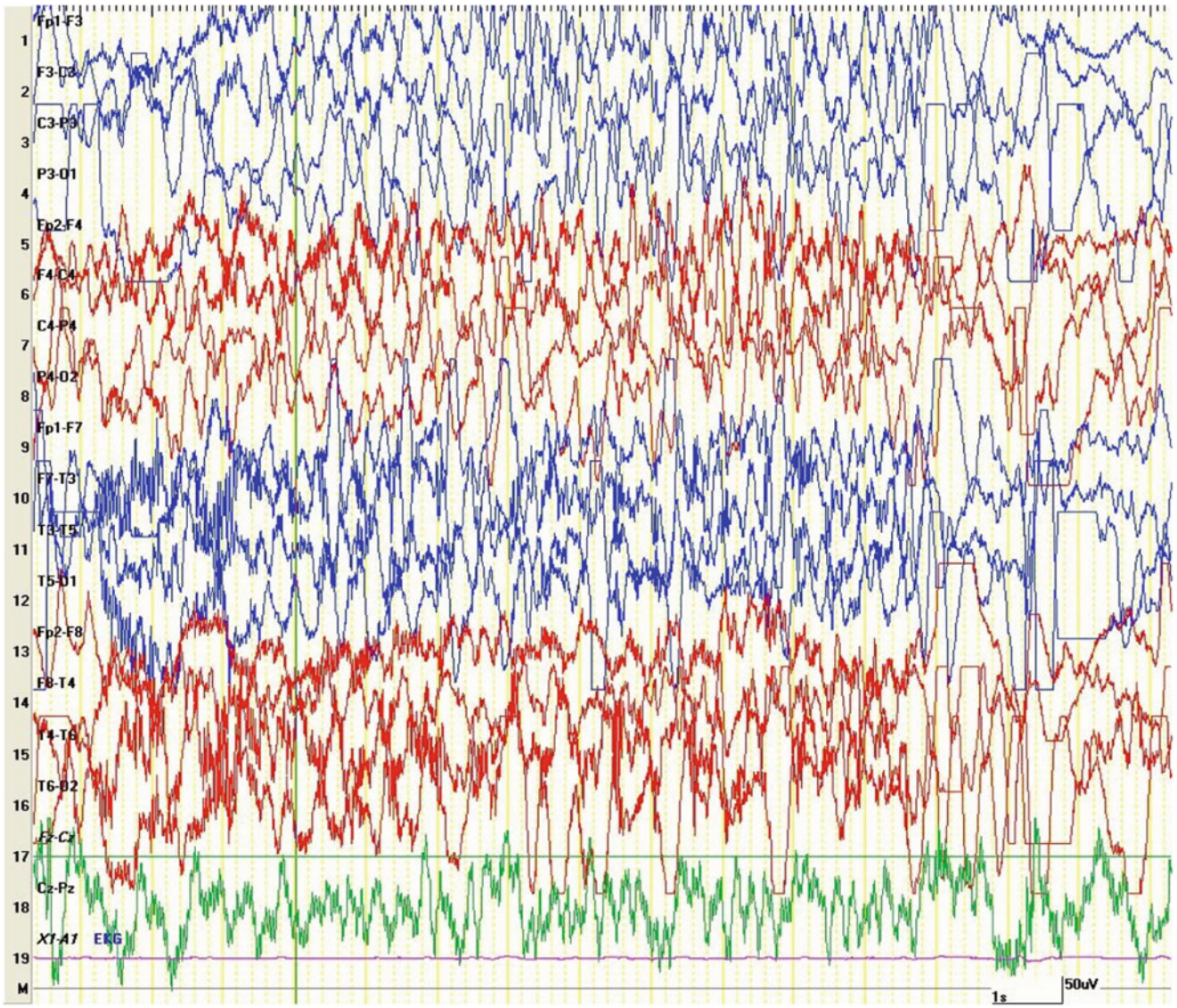

Fig. 1 EEG of patient with PNPO deficiency, age 5 months, showing paroxysms of diffuse spike-wave discharges accompanied by multifocal myoclonic and erratic movements. Settings: 20 s epoch, sens 10 uV/mL, tc 0.1 s, HFF 70 Hz (Reproduced with permission from Alduligan and Pearl 2012)

reported in both pyridoxine-responsive epilepsy and PNPO deficiency were also seen in our patient.

Patients without a defect in PNPO who receive pyridoxine generally have high-normal to above normal CSF PLP levels (KH – personal observation). The borderline low CSF PLP seen in our patient, while on pyridoxine, may indicate that the c.352G>A p.G118R mutation is "leaky" and allows for some oxidation of pyridoxine phosphate. This may explain the partial clinical response to pyridoxine. The pyridoxine dose was not increased, and it is plausible that a static pyridoxine dose was not sufficient to maintain efficacy as the child grew. Enzyme studies of the expressed mutant protein would be required to verify this hypothesis.

Our patient demonstrates that an initial response to pyridoxine that fails to materialize into permanent seizure control may be an indication of an underlying PNPO deficiency. In this situation, a trial with pyridoxal-5-phosphate is warranted.

Synopsis

We describe a novel case of partial pyridox(am)ine phosphate oxidase (PNPO) deficiency and pyridoxal-5-phosphate-dependent epilepsy with a transient response to pyridoxine (B6) treatment.

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CASE REPORT

Erratum to: Non-syndromic Hearing Impairment in a Hungarian Family with the m.7510T>C Mutation of Mitochondrial tRNA$^{Ser(UCN)}$ and Review of Published Cases

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RESEARCH REPORT

Erratum to: Identification and Characterisation of a Novel Pathogenic Mutation in the Human Lipodystrophy Gene *AGPAT2*

C48R: A Novel Mutation in AGPAT2

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