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

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Johannes Zschocke · K. Michael Gibson
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Managing Editor

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RESEARCH REPORT

Prevalence and Novel Mutations of Lysosomal Storage Disorders in United Arab Emirates

LSD in UAE

Fatma A. Al-Jasmi • Nafisa Tawfig • Ans Berniah • Bassam R. Ali • Mahmoud Taleb • Jozef L. Hertecant • Fatma Bastaki • Abdul-Kader Souid

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Abstract Lysosomal storage disorders (LSD) are rare entities of recessive inheritance. The presence of a "founder" mutation in isolated communities with a high degree of consanguinity (e.g., tribes in the Middle East North Africa, MENA, region) is expected to lead to unusually high disease prevalence. The primary aim of this study was to estimate the prevalence of LSD and report their mutation spectrum in UAE. Between 1995 and 2010, 119 patients were diagnosed with LSD (65 Emiratis and 54 non-Emiratis). Genotyping was performed in 59 (50 %) patients (39 Emirati from 17 families and 20 non-Emiratis from 17 families). The prevalence of LSD in Emiratis was 26.9/100,000 live births. Sphingolipidoses were relatively common (9.8/100,000), with GM1-gangliosidosis being the most prevalent (4.7/100,000). Of the Mucopolysaccharidoses VI, IVA and IIIB were the predominant subtypes (5.5/100,000). Compared to Western countries, the prevalence of fucosidosis, Batten disease, and α-mannosidosis was 40-, sevenfold, and fourfold higher in UAE, respectively. The prevalence of Pompe disease (2.7/100,000) was similar to The Netherlands, but only the infantile subtype was found in UAE. Sixteen distinct LSD mutations were identified in 39 Emirati patients. Eight (50 %) mutations were reported only in Emirati, of which three were novel [c.1694G>T in the *NAGLU* gene, c.1336 C>T in the *GLB1* gene, and homozygous deletions in the *CLN3* gene]. Twenty-seven (42 %) patients were clustered in five of the 70 Emirati tribes. These findings highlight the need for tribal-based premarital testing and genetic counseling.

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F.A. Al-Jasmi (✉) · A.-K. Souid
Department of Pediatrics, United Arab Emirates University, P.O. Box 17666, Al-Ain, UAE
e-mail: aljasmif@uaeu.ac.ae

N. Tawfig · M. Taleb
Department of Genetics, Latifa Hospital, Dubai, UAE

A. Berniah · J.L. Hertecant
Tawam Hospital, Al-Ain, UAE

B.R. Ali
Department of Pathology, United Arab Emirates University, Al-Ain, UAE

F. Bastaki
Department of Pediatrics, Latifa Hospital, Dubai, UAE

Introduction

Lysosomal storage disorders (LSD) are rare inherited entities with more than 50 distinct types. Individually, these diseases are rare, but collectively they are relatively common with a prevalence ranging from 12 to 25 per 100,000 live births (Pinto et al. 2004). Their prevalence in the Middle East North Africa (MENA) region, however, is unknown. These disorders are inherited as autosomal recessive (except for Fabry disease, Danon, and MPS II, which are X-linked recessive) and are more common in the MENA region when a "founder" mutation is present in the tribe (al-Gazali et al. 1997)." Thus, there is a need for accurate data about LSD prevalence and mutation spectrum in UAE and other Arab populations in order to design and implement preventative programs, such as premarital testing and neonatal screening. Such data are also required to assess the impact of treating these disorders on the public health care system.

UAE citizens (Emiratis) are ethnically diverse, with ancestries from Arabian Peninsula, Persia, Baluchistan, and

East Africa. The local society however remains tribal in nature and consists of at least 70 distinct tribes. Despite this ethnic diversity, inter-tribal marriages are less common than intra-tribal ones. Thus, the culture enforces appearance of rare recessive conditions. About 80 % of the current eight million UAE inhabitants are expatriates; in whom related marriages are also common (e.g., Palestinians and Pakistanis). In this report, all UAE citizens (including tribal living citizens) are termed Emiratis and all expatriates are termed non-Emiratis.

This study estimates the birth prevalence of LSD among Emiratis (in comparison to Western populations) since data from the MENA region are unavailable. It also describes the mutation spectrum in UAE citizens and expatriates.

Materials and Methods

Patients

All patients were diagnosed and followed in the only two metabolic referral centers in UAE, Latifa Hospital in Dubai and Tawam Hospital in Abu Dhabi. The LSD diagnostic center at Latifa Hospital was established in 1995 and at Tawam Hospital in 1992.

Between 1995 and 2010, 119 patients were diagnosed with LSD (65 Emiratis and 54 non-Emiratis). The entities spanned 26 distinct subtypes of LSD and the diagnosis was made by clinical presentation and biochemical analysis. Genotyping was performed in 59 (50 %) patients (39 Emiratis from 17 families and 20 non-Emiratis from 17 families).

Birth Prevalence

"Disease birth prevalence" in Emiratis, expressed as number of patients per 100,000 live births, was calculated using the method reported by Poorthuis et al. (1999). Briefly, the prevalence was set as the total number of Emirati patients with the specific disease divided by the total number of Emirati live births during the "birth period". The birth period was defined as the time interval between year of birth of the oldest patient and year of birth of the youngest patient. Live births per year were obtained from the National Bureau of Statistics (http://www.uaestatistics.gov.ae). Affected siblings and fetuses with GM1-gangliosidosis and α-mannosidosis were also included in calculating the disease birth prevalence.

The method of Pinto et al. (2004) was used to estimate disease birth prevalence when only a single patient was diagnosed with the disease, using the number of live births between 1995 and 2010 (Pinto et al. 2004). The overall prevalence was calculated by adding the prevalence of each phenotype.

Mutation Analysis

Direct genomic sequencing of the most known genes responsible for LSD was performed on samples from affected individuals by accredited genetic diagnostic laboratories. All novel variants were tested by damage-prediction assessment, using the softwares SIFT, PolyPhen-2, and Mutation Taster. For the *CLN3* gene, deletion/duplication studies were performed, using multiplex ligation-dependent probe amplification (MLPA).

Results

Table 1 shows the prevalence of LSD in UAE and compares it with five other countries. The prevalence of LSD among Emiratis was 26.9 per 100,000 live births. This rate was similar to Portugal, but twice that in The Netherlands, Czech Republic, or Australia. Sphingolipidoses were most common (9.8 per 100,000), with GM1-gangliosidosis being the most frequent (4.7 per 100,000, ~7.6-fold higher than Portugal). The remaining sphingolipidoses were comparable to other countries, with the exception of Gaucher disease which was ~6-fold lower in Emiratis. The single case of Gaucher disease in Emirati patient was the lethal type 2. By contrast, Gaucher disease type 1 is the most common one found worldwide.

In Emiratis, MPS IIIB, IVA, and VI are most common types were the most common subtypes of mucopolysaccharidoses, with a sum birth prevalence of 5.5 per 100,000. This value was ~2.9-fold higher than other countries. By contrast, MPS I and II are the most common ones worldwide. The only oligosaccharidoses noted in Emiratis were fucosidosis and α-mannosidosis, occurring ~40-fold and fourfold higher than the Western countries, respectively. Lipid storage disorders and mucolipidoses, on the other hand, were comparable to the other populations. The prevalence of Pompe disease was similar to that in The Netherlands, but only the infantile type was found in this study. Batten disease was ~7-fold higher than Portugal (Table 1).

Thirty-five distinct mutations were identified in UAE, 16 in Emiratis (Table 2) and 19 in non-Emiratis (Table 3). Eight mutations were documented only in Emiratis. Eleven mutations (three in Emiratis and eight in non-Emiratis) were novel, including eight missense, two frame-shift, and one large deletions. Most mutations resulted in severe infantile disease, except for c.499dupC in the *GNPTG* gene, which resulted in mild mucolipidosis type III.

Table 1 Prevalence of lysosomal storage disorders in UAE and other countries

UAE								
		Emiratis		Other countries				
Disorders	Total No. of patients	No. of patients	Prevalence (per 100,000)	NL[1] Prevalence (per 100,000)	Portugal[2]	Czech[3]	Australia[4]	Turkey[5]
Mucopolysaccharidoses (MPS)								
MPS I	4	1	0.25	1.19	1.33	0.72	1.14	–
MPS II	2	0	–	0.67	1.09	0.43	0.74	–
MPS IIIB	6	5	1.05	0.42	0.72	0.02	0.47	–
MPS IIIC	1	1	0.25	0.21	0.12	0.42	0.07	
MPS IVA	4	2	1.41	0.22	0.60	0.71	0.59	–
MPS VI	13	2	2.51	0.15	0.42	0.05	0.43	–
Oligosaccharidoses								
α-Mannosidosis	6	5	1.51	0.09	0.12	0.38	0.1	–
β-Mannosidosis	0	0	–	0.13	0.12	0.16	0	–
Fucosidosis	3	2	2.02	0.05	0	0	0	–
α-*N*-acetyl-galactosaminidase defic.	0	0	–	0.2	0	0	0	–
Aspartylglucosaminuria	0	0	–	0.13	1.72	0	0.05	–
Sialidosis	0	0	–	–	–	–	–	–
Galactosialidosis	0	0	–	–	–	–	–	–
Sphingolipidoses								
GM1 gangliosidosis	20	14	4.66	0.41	0.62	0.26	0.26	0.54
Tay-Sachs	7	3	0.74	0.41	3.13	0.30	0.5	0.23
Sandhoff	10	4	1.21	0.34	1.49	0.19	0.26	0.95
Galactosialidosis	0	0	–	0.04	0.77	0	0	–
Metachromatic leukodystrophy	3	2	1.50	1.42	1.85	0.69	1.09	1.43
Niemann-Pick A	0	0	–	–	–	0.18	–	–
Niemann-Pick B	1	1	0.25	–	–	0.15	–	–
Gaucher (all types)	5	1[a]	0.25	1.16	1.35	1.13	1.75	0.45
Fabry	1	1	0.25	0.21	0.12	0.52	0.86	0.015
Krabbe	2	0	0	1.35	1.21	0.4	0.71	1
Farber	2	2	0.96	–	–	–	–	–
MSD	1	0	0	0.05	0.48	0.26	0.07	–
Lipid storage disorders								
Niemann-Pick C	3	1	0.25	0.35	2.2	0.91	0.47	–
Wolman	1	0	–	0.19	–	0.27	–	–

(continued)

Table 1 (continued)

UAE								
		Emiratis		Other countries				
Disorders	Total No. of patients	No. of patients	Prevalence (per 100,000)	NL[1] Prevalence (per 100,000)	Portugal[2]	Czech[3]	Australia[4]	Turkey[5]
Mucolipidoses								
Mucolipidosis I	0	0	–	0.05	0	0.07	0.02	
Mucolipidosis II/III	11	7	1.35	0.24	0.81	0.22	0.31	–
Mucolipidosis IV	0	0	–	–	–	0.02	–	–
Other disorders								
Cystinosis	1	1	0.25	–	–	–	–	–
ISSD	0	0	–	0.07	0	0.02	0.19	–
Pompe disease	10	8	2.66[b]	2.0	0.17	0.37	0.69	–
Batten disease	2	2	3.54	–	0.48	0.27	–	–
Total	**119**	**65**	**26.87**	**11.75**	**20.92**	**9.12**	**10.77**	

Note: The diagnosis was based on enzyme analysis and not all patients had genotyping.

NL The Netherlands, *MPS* mucopolysaccharidoses, *MSD* multiple sulfatase deficiency, *ISSD* infantile sialic acid storage disease

[a] Gaucher type 2

[b] All cases are the infantile type

[1] Poorthuis et al. 1999

[2] Pinto et al. 2004

[3] Poupetova et al. 2010

[4] Meikle et al. 1999

[5] Ozkara and Topcu 2004

Table 2 Genotypes and phenotypes of LSD in Emiratis

Disorders	Phenotype	Gene	Nucleotide change	Amino acid change
Mucopolysaccharidoses (MPS)				
MPSIIIB(MIM 252920)	HSM, coarse face, hyperactivity, aggressive behavior, MR	*NAGLU*	c.1694G>T[a,b]	p.R565L
MPS IVA(MIM 253000)	Early-onset obstructive sleep apnea, HSM, atlantoaxial dislocation	*GALNS*	c.319G>A	p.A107T
MPS VI(MIM 253200)	Delayed diagnosis, on ERT, growth retardation, ventilator-dependent	*ARSB*	c.979C>T[1]	p.R327Stop
Oligosaccharidoses				
α–Mannosidosis(MIM 248500)	Severe MR, hearing loss	*MAN2B1*	c.2368C>T[a,2]	p.Q790X
	Early diagnosis, BMT, mild MR		c.2119C>T[a,2]	p.Q707X
Sphingolipidoses				
GM1-gangliosidosis(MIM 230500)	Infantile onset	*GLB1*	**c.1336 C>T**[a,b]	p.K446F
			c.1768C>T	p.R590C
Niemann-Pick type B(MIM 257220)	Obstructive sleep apnea, HSM	*SMPD1*	c.1244C>T[2]	p.A415V
Farber Lipogranulomatosis(MIM 228000)	Infantile onset, failure-to-thrive, cherry-red spot, painful joints, hoarseness, no subcutaneous nodules	*ASAH1*	**c.533T>C**[a,3] **/ c.1144A>C**[a,3]	P.W185R[a,]/ P.K382P[a]
Fabry disease(MIM 301500)	Isolated renal phenotype, end-stage renal disease	*GLA*	c.1277_1278delAA[4]	p.K426RfsStop24
Mucolipidoses				
I-Cell Disease(MIM 252500)	Infantile onset	*GNPTAB*	c.3503_3504delTC[5]	p.L1168fs
Mucolipidosis type III(MIM 252600)	Normal development, mild skeletal involvement	*GNPTG*	c.499dupC[6]	P.L167PfsStop32
Other disorders				
Batten Disease(MIM 204200)	Juvenile onset	*CLN3*	Homozygous deletions[a,b,c]	
CystinosisMIM 219800	Infantile onsetTreated early, normal growth, learning difficulties	*CTNS*	681G>A[a,7]	Splice
Pompe Disease(MIM 232300)	Infantile cardiomyopathy	*GAA*	c.1327-2A>G[8]	Splice

MR mental retardation, *HSM* hepatosplenomegaly, *ERT* enzyme replacement therapy, *BMT* bone marrow transplantation

[a] Reported only in Emiratis

[b] Novel mutation

[c] Homozygous deletions of exon 3, introns 6 and 8, and exons 11, 14, and 15 in the *CLN3* gene

[1] Karageorgos et al. 2004

[2] Lan et al. 2009

[3] Al-Jasmi 2012

[4] Eng and Desnick 1994

[5] Kudo et al. 2006

[6] Raas-Rothschild et al. 2000

[7] Ben-Rebeh et al.

[8] Kroos et al. 2008

With respect to MPS IIIB, five blood-related patients had the homozygous (novel) variant c.1694G>T in exon 6 of the *NAGLU* gene. The amino acid substitution (p.R565L) involved a highly conserved residue, Table 2. Other mutations affecting the same amino acid were previously shown to cause MPS IIIB (Beesley et al. 1998; Bunge et al. 1999; Weber et al. 1999). The two siblings with Batten disease had homozygous (novel) deletions involving exon 3, introns 6 and 8, and exons 11, 14, and 15 of the CLN3 gene (Table 2).

MPS I and II were mainly noted in Palestinians and Pakistanis. They had severe phenotype with central nervous system involvement. They had two (novel) premature stop codons [c.784delC (p.H262TfsX55) in the *IDUA* gene and

Table 3 Genotypes and phenotypes of LSD in non-Emiratis

Disorder	Gene	Phenotype	Nucleotide change	Amino acid change	Ethnic origin
Mucopolysaccharidoses (MPS)					
MPS I MIM 607015	*IDUA*	Severe disease with CNS involvement	c.784delC[a]	p.H262TfsX55	Pakistan
			c.192C>A[1]	p.Y64Stop	Palestine
MPS II MIM 309900	*IDS*	Severe disease with CNS involvement	c.1418delC[a]	p.P473Lfsx10	Palestine
MPS VI MIM 253200	*ARSB*	Diagnosed early, on ERT, mild limitation of joint movements	c.944G>A	p.R315Q	Sudan
Sphingolipidoses					
GM1-Gangliosidosis MIM 230500	*GLB1*	Infantile onset	c.914+4A>G[2]	Splice	Palestine
			c.1465-1466del AT[3]	p.I489fs	India
Sandhoff GM2-Gangliosidosis MIM 268800	*HEXB*	Infantile onset	16 kb deletion[4]		Iran
			c.850C>T	p.R284X	Pakistan
			c.884 C>G[a] / c.1507 T>C[a]	p.T295R / p.W503R	Lebanon
Tay-Sachs disease (MIM 272800)	*HEXA*	Infantile onset	c.2T>C[5]	p.Met1?	Iran
Multiple sulfatase deficiency MIM 272200	*SUMF1*	Tetralogy of fallot, hypoplastic lungs, hydrops fetalis, short limbs, inguinal hernia	c.603-2A>G[6]	Splice	Iran
Gaucher disease MIM 230900	*GBA*	Normal growth, development, and neurological exam, icthyosis, hoarsness, splenomegaly	c.854 T>C[a]	p.F285S[a]	Palestine
Gaucher disease type II MIM 230800	*GBA*	Progressive neurological impairment, died at 1 year of age	c.160G>A[7]	p.V54M	Palestine
Mucolipidosis					
Mucolipidosis type III MIM 252600	*GNPTG*	Mild skeletal involvement, normal development	c.499insC[a]	p.V167fs[a]	Pakistan
Lipid storage					
Niemann-Pick C disease MIM 257220	*NPC 1*	Juvenile onset	c.1408 G>C / c.1408 G>C[a] c.2509A>G/ c.2509A>G[a]	p.A470P[a] p.I837V[a]	India
			c.2974 G>T	p.G992W	Palestine
Other disorders					
Pompe Disease (MIM 232300)	*GAA*	Infantile cardiomyopathy	c.340_341insT[3]	p.K114*fs*	Palestine

[a] Novel mutation

[1] Bach et al. 1993

[2] Georgiou et al. 2004

[3] Ali et al. 2011

[4] Zhang et al. 1994

[5] Harmon et al. 1993

[6] Ben-Rebeh et al.

[7] Alfonso et al. 2004

c.1418delC (p.P473Lfsx10) in the *IDS* gene], causing truncated protein or mRNA degradation by the nonsense-mediated decay mechanism (Table 3).

The patient with Gaucher disease had a novel variant (c.854 T>C), Table 3. He presented in infancy with severe thrombocytopenia, splenomegaly, icthyosis, and hoarseness. Patient improved significantly on high dose of enzyme replacement therapy. At 7 years of age, he still has icthyosis and hoarseness but his growth, development, and neurological examination are all normal.

The patient with juvenile Niemann-Pick C disease had two distinct (novel) inherited homozygous missense var-

Table 4 Distribution of LSD in 26 Emirati tribes

Disorders	No. of patients	Tribes																										
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	Others*
MPS I	1												1															
MPS IIIB	5	1[a]	3[a]											1	1													
MPS IIIC	1															1												
MPS IVA	2								2																			
MPS VI	2						2																					
α-Mannosidosis	5					3[b]				2[b]																		
Fucosidosis	2																1										1	
GM1-gangliosidosis	14	10[c]																1	1							2		
Tay-Sachs	3																											3
Sandhoff	4																			1	1							2
Metachromatic leukodystrophy	2										2																	
Niemann-Pick B	1																								1			
Gaucher	1																											1
Fabry	1				1																							
Farber	2				2																							
Niemann-Pick C	1																					1						
Mucolipidosis II/III	7			5[d]																			1					1
Cystinosis	1																							1				
Pompe disease	8		2[e]									1[e]																5
Batten disease	2							2																				
Total	**65**	**11**	**5**	**5**	**3**	**3**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**			**12**

Values are number of patients

*Belong to unidentified tribes

[a] The same mutation

[b] Two distinct mutations

[c] Two families with two distinct mutations

[d] One family with the same mutation

[e] Two families with the same mutation

Table 5 Distribution of LSD mutations in 13 Emirati tribes

Tribe	LSD	Gene	Mutation	No of patients
A	MPS IIIB	*NAGLU*	c.1694G>T	1
	GM1-gangliosidosis	*GLB1*	c.1336 C>T	3
			c.1768C>T	7
B	MPS IIIB	*NAGLU*	c.1694G>T	3
	Pompe	*GAA*	c.1327-2A>G	2
C	Mucolipidosis III	*GNPTG*	c.499dupC	5
D	Fabry	*GLA*	c.1277_1278delAA	1
	Farber	*ASAH1*	c.533T>C c.1144A>C	2
E	α-Mannosidosis	*MAN2B1*	c.2119C>T	3
F	MPS VI	*ARSB*	c.979C>T	2
G	Batten	*CLN3*	Homozygous deletions	2
H	MPS IVA	*GALNS*	c.319G>A	2
I	α-Mannosidosis	*MAN2B1*	c.2368C>T	2
K	Pompe	*GAA*	c.1327-2A>G	1
V	Mucolipidosis II	*GNPTAB*	c.3503_3504delTC	1
W	Cystinosis	*CTNS*	681G>A	1
X	Niemann-Pick B	*SMPD1*	c.1244C>T	1

iants in the *NPC1* gene. Both were predicted to be damaging, with the c.1408 G>C variant being more so than the c.2509A>G variant (Table 3). The parents were carriers and a healthy sibling had normal alleles.

Table 4 shows distribution of the 20 distinct LSD in Emirati tribes. Among the 65 patients with LSD, 53 (82 %) were linked to 26 UAE tribes based on the last name. Twenty-seven (44 %) patients were clustered in five tribes (labeled A–E). Three tribes (A, B, and D) had two distinct LSD. MPS IIIB was found in four tribes, GM1-gangliosidosis in four tribes, α-mannosidosis in two tribes, Sandhoff in two tribes, and Pompe in two tribes. This tribal information is important for genetic counseling and disease prevention.

Table 5 shows distribution of the 16 distinct LSD mutations in Emirati tribes. The identified mutations were clustered in 13 tribes. The two distinct mutations of *GLB1* gene were found only in tribe A, affecting 10 patients with GM1-gangliosidosis from two families. For MPS IIIB, the founder mutation c.1694G>T in *NAGLU* was found only in tribes A and B, in whom cross tribal marriages were common. For Farber disease, two distinct mutations of *ASAH1* gene were identified in tribe D. For Pompe disease, the c.1327-2A>G mutation of *GAA* was present in tribes B and K. The remaining tribes had one mutation per disease.

The 10 patients with GM1-gangliosidosis in tribe A belong to two families; one family had the c.1336 C>T mutation and the other had c.1768C>T. The remaining patients with specific LSD disease within the tribe are siblings (Table 5).

Discussion

The overall prevalence of LSD in Emiratis is 1 in 3,717 live births (26.9 per 100,000 live birth), which is similar to that in Portugal (25 per 100,000) (Pinto et al. 2004). The prevalences of LSD in The Netherlands, Czech Republic, and Italy, on the other hand, are ~50 % less than UAE (14, 12, and 12 per 100,000, respectively), Table 1 (Poorthuis et al. 1999; Dionisi-Vici et al. 2002; Poupetova et al. 2010). Consistently, 37 of the 39 (95 %) Emiratis who had genotyping were homozygous and other 2 were compound heterozygous (Table 2). This finding reflects the high rate of consanguinity in UAE.

In Emiratis, GM1-gangliosidosis is the most common LSD (14 of 65 patients, or ~21 %), with a prevalence of ~1 in 21,000 live births. GM1-gangliosidosis is also the most common sphingolipidoses (14 of 28 patients, or ~50 %). GM2 Tay-Sachs, on the other hand, is the most prevalent sphingolipidoses in Portugal (Pinto et al. 2004), while Gaucher type 1 is the most prevalent sphingolipidoses worldwide(Poupetova et al. 2010). The treatable Gaucher type 1 and adult form of Pompe were not found in Emiratis (Table 1). The lethal Gaucher type 2 was found in one Emirati family.

In this series, most of the recognized cases of LSD are of the severe infantile form, while the treatable adult forms are less frequent. The severe forms of LSD are easily recognized by their striking features, while the milder forms are easily missed. Therefore, it is likely that the milder forms of LSD are under diagnosed in UAE. Since

data on disease prevalence were mainly based on clinical diagnosis, the reported prevalences should be considered as minimum estimates. A high risk group screening or newborn screening may thus reveal higher prevalences, especially in entities with a wide clinical spectrum such as late-onset Pompe disease.

In this study, mutation analyses were performed in 59 patients with LSD (39 Emiratis and 20 non-Emiratis). Thirty-five different mutations are described, 16 in Emiratis and 19 in non-Emiratis. Eleven mutations are novel and 24 are reported in the literature (Tables 2–3). Identification of these mutations will certainly aid the diagnosis and prevention of LSD.

The national inhabitants of UAE are ethnically diverse, with ancestries from north and south of Arabian Peninsula, Persia, Baluchistan, and East Africa. The majority of the current eight million inhabitants in UAE are expatriates. In spite of this mixed population, intermarriages are rare and consanguineous marriages within local tribes are the norm. Consanguinity leads to a higher birth prevalence of autosomal recessive diseases if a "founder" mutation is present in the tribe. Otherwise, it actually protects against the occurrence of recessive diseases. As shown in Tables 4–5, diseases with low birth prevalence (e.g., MPS VI and fucosidosis, with mutations are present in homozygous form) have an unusually high prevalence in our tribes with high degree of consanguinity. Other more frequently encountered diseases (e.g., MPS IIIA) are absent in the community.

Most of the families had more than one affected child (Tables 4–5), highlighting the need for genetic counseling, pre-implantation genetic diagnosis, and prenatal diagnosis. The data also strongly point to the importance of premarital testing and counseling, which could be tribal based. Early detection and treatment of LSD (with enzyme replacement or stem cell transplantation) prevents irreversible organ damages. Nevertheless, disease prevention through tribal-based premarital testing is the ultimate goal.

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Disclosure Statement

No competing financial interests exist.

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RESEARCH REPORT

Chitotriosidase Deficiency: A Mutation Update in an African Population

Silke Arndt · Angela Hobbs · Iain Sinclaire · Anthony B. Lane

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Abstract

Human plasma chitotriosidase activity is a commonly used diagnostic and therapeutic biomarker for non-neuronopathic Gaucher disease. Chitotriosidase deficiency is common in non-African populations and is primarily caused by a 24 bp duplication in the encoding gene (*CHIT1*). Allele frequencies for the 24 bp duplication range from 20–50 % outside Africa. The present study found chitotriosidase deficiency to be rare in the South African Black population (1.6 %) and the otherwise common 24 bp duplication is absent in this African population. Instead, chitotriosidase deficiency is caused by a 4 bp deletion across the exon/intron 10 boundary (E/I-10_delGAgt) of the *CHIT1* gene. The exact position of this mutation was found to differ from the previously reported location. Allele frequencies for six coding variants of *CHIT1* (p.G102S, p.G354R, 24 bp duplication, E/I-10_delGAgt, p.A442V/G) were determined and the 4 bp deletion was found to be in complete linkage disequilibrium (LD) with two of the coding variants (p.G354R and p.A442V). The in silico assessments of the two missense mutations in LD predict a protein-damaging nature and functional studies are needed to clarify if one or both abolish the enzyme's activity. Overall, the low frequency of chitotriosidase deficiency in South African Blacks makes chitotriosidase activity an excellent biomarker of choice in this population.

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S. Arndt (✉) · A. Hobbs · I. Sinclaire · A. Lane
Division of Human Genetics, National Health Laboratory Service and School of Pathology, University of the Witwatersrand, Cnr. DeKorte/Hospital streets, P.O. Box 1038, Johannesburg 2000, South Africa
e-mail: silke.arndt1@gmail.com

Introduction

Chitotriosidase (*CHIT1*) (EC 3.2.1.14) is a chitinase that hydrolyses chitin, a structural component found in fungi, nematodes, insects and shellfish. This enzyme is primarily secreted from human macrophages and is thought to play a role in the body's defence against chitin-containing pathogens. Elevated plasma chitotriosidase levels are observed in several lysosomal storage disorders including Gaucher disease, as well as in β-thalassaemia and atherosclerosis (Barone et al. 2003a; Boot et al. 1999; Guo et al. 1995). This finding has led to the utilisation of chitotriosidase activity as a biomarker for conditions involving chitinase secretion from human macrophages (Renkema et al. 1998, 1995).

One limitation in the use of chitotriosidase activity as a biomarker is the presence of genetic variants in the chitotriosidase encoding gene *CHIT1* which result in lack or reduced activity of this enzyme in some individuals. The most striking observation about this recessively inherited enzyme deficiency is its distinct difference in prevalence rates in various populations, as well as the dissimilar distribution of causative mutations. To date, three mutations that lead to deficient chitotriosidase expression (Boot et al. 1998; Grace et al. 2007) (Table 2) and three mutations that result in strongly reduced chitotriosidase expression in humans (Lee et al. 2007; Webb et al. 2006) (Table 2) are recorded in the Human Genome Mutation Database (www.hgmd.cf.ac.uk) (Stenson et al. 2003). The most common

mutation reported in the literature is a 24bp duplication in exon 10 of the *CHIT1* gene (OMIM 600031.0001) that results in aberrant splicing of the mRNA and subsequently leads to the diminished expression of an enzymatically inactive protein (Boot et al. 1998). Notably, chitotriosidase (chito) deficiency resulting from this 24bp duplication has been shown to be highly prevalent in Europeans and Asians, with allele frequencies reported to be ~ > 0.20 and > 0.50, respectively (Lee et al. 2007). A very different picture is observed in populations on the African continent, where chito deficiency is rare (0–2%) and the 24bp duplication is absent (Malaguarnera et al. 2003). Grace et al. (2007) screened 396 individuals from various ethnic groups with an unknown chito activity status for the presence of the 24bp duplication in the *CHIT1* gene and found that 3/70 (4.3%) Black Americans and 3/64 (4.7%) Caribbean Blacks carry the 24bp duplication allele compared to carrier rates of 7/88 (8%) and 33/244 (13.5%) in the Dominican and Puerto Rican population, respectively.

The study presented here was aimed at elucidating the overall prevalence of chito deficiency in the South African Black population and to investigate the underlying molecular mechanism. Those 10 individuals that showed either complete chito deficiency or low chito activity (≤10 nmol/h/ml) were earmarked for the analysis of six mutations: p.G102S (rs2297950) in exon 4, p.G354R (rs9943208) in exon 10, the 24bp duplication in exon 10, a 4bp deletion across the exon/intron-10 boundary (complex E/I-10) and a tri-allelic SNP p.A442V/G (rs1065761) in exon 12. In addition, estimates of the frequencies for these six *CHIT1* variants were also ascertained in the general South African Black population.

Materials and Methods

Subjects

Peripheral blood samples from 492 unrelated Black South African Bantu of unknown disease status who presented for paternity testing at the National Health Laboratory Service (NHLS) were used in the present project. The study was conducted in two parts with two groups of subjects. In part one, we assessed chito activity in 280 participants (group 1) and tested for the presence of the 24bp duplication in all chito-deficient individuals (0 nmol/h/ml) that were found in this group.

In part two, we assessed chito activity in 212 participants (group 2). Genotype information was generated for all subjects with reduced or absent chito activity. A subset (70/212) of participants with normal chito activity was further investigated in order to obtain background *CHIT1* allele frequency data for the Black South African Bantu population. Three out of six investigated loci harbour an allele that impacts on chito activity (Tables 1 and 2). Ethics clearance to undertake the study was obtained from the University of the Witwatersrand's Human Research Ethics Committee (Medical) (protocol number M050706).

Methods

Plasma Chitotriosidase Assay

Plasma chitotriosidase activity was measured in 492 participants (group 1 and 2), using an adapted biochemical assay (Hollak et al. 1994).

CHIT1 Mutation Detection

Genomic DNA was extracted from peripheral blood samples, using the FlexiGene® DNA kit (Qiagen, USA) in accordance with the manufacturer's protocol. DNA concentrations were spectrophotometrically assessed. Each sample was standardised to a working concentration of 100 ng/μL for further use.

The frequency of the 24bp duplication was assessed by subjecting genomic DNA to a PCR-based method described elsewhere (Boot et al. 1998). For group 2, ten sets of primers were designed to amplify the *CHIT1* coding region, covering all 12 exons and the intron/exon boundaries. Genomic DNA in the 2 chito-deficient individuals was amplified and each amplicon was sequenced in both directions using BigDye™ Terminator Ready Reaction Mix and the ABI Prism™ 3130XL genetic analyser (Applied Biosystems, USA). The SeqMan module from the Lasergene® software suite Lasergene® (DNASTAR Inc. Software, USA) was utilised for the evaluation of sequence variants compared to the published *CHIT1* reference sequence (NM_003465).

Population Screening for CHIT1 Mutations

In order to obtain allele frequency data for the Black South African population, a subset of 70 (out of 212) group 2 participants was subjected to *CHIT1* locus investigations. Five of the following six variants have been associated with reduced or deficient chito activity in the literature: p.G102S (rs2297950) in exon 4, p.G354R (rs9943208) in exon 10, the 24bp duplication in exon 10, a 4bp deletion across the exon/intron-10 boundary (complex E/I-10) and the tri-allelic SNP p.A442V (rs1065761) in exon 12 of the *CHIT1* gene. The additional SNP allele p.A442G (rs1065761) has no adverse effect on chito activity (Table 2).

For p.G102S, a PCR assay followed by RFLP analysis was applied (Supplementary Table 1). The 259bp long PCR amplicon was subjected to restriction enzyme digestion with *Hpa*II (10 units/10 μL PCR reaction) and the fragments were separated on a 4% Seakem agarose gel

Table 1 Chitotriosidase activity and *CHIT1* genotype information for Chito-deficient and Chito-impaired individuals

Chitotriosidase impaired individuals	Chito-activity [nmol/h/ml]	p.G102S (G>A) Exon 4 (rs2297950)	p.G354R (G>A) Exon 10 (rs9943208)	c.1049_1072dup24 Exon 10 (24bp duplication)	E/ I-10_delGAgt* (4bp deletion)	p.A442V/G (C>T/G) Exon 12 (rs1065761)
Group 1						
01	0	ND	ND	WT / WT	ND	ND
02	0	ND	ND	WT / WT	ND	ND
03	0	ND	ND	WT / WT	ND	ND
04	0	ND	ND	WT / WT	ND	ND
05	0	ND	ND	WT / WT	ND	ND
06	0	ND	ND	WT / WT	ND	ND
Group 2						
07	0	G/G	A/A	WT / WT	Del / Del	T/T
08	0	G/G	A/A	WT / WT	Del / Del	T/T
09	8.5	G/G	G/A	WT / WT	WT / Del	C/T
10	9.4	G/G	G/G	WT / WT	WT / WT	C/C
11	0.5	G/G	G/G	WT / WT	WT / WT	C/G
12	6.0	G/G	G/G	WT / WT	WT / WT	C/C
13	10.0	G/G	G/G	WT / WT	WT / WT	C/C
14	8.4	G/G	G/G	WT / WT	WT / WT	C/C
15	9.4	G/G	G/G	WT / WT	WT / WT	C/C
16	8.8	G/G	G/G	WT / WT	WT / WT	C/C

ND - not determined; WT- wild type

* c.1155delGA; 1156+1_1156+2delGT (see Fig. 1)

Table 2 Allele frequencies for six *CHIT1* mutations in unrelated Black South Africans

	p.G102S (G>A) Exon 4 (rs2297950)	p.G354R (G>A) Exon 10 (rs9943208)	24bp duplication in Exon 10	E/ I-10_delGAgt 4bp deletion	p.A442V (C>T) Exon 12 (rs1065761)	p.A442G (C>G) Exon 12 (rs1065761)
Mutant allele frequency	0.257 (36/140)	0.250 (5/20)	0 (0/984)	0.038 (5/130)	0.049 (6/122)	0.148 (18/122)
homozygote	0.057 (4/70)	0.200 (2/10)	0	0.030 (2/65)	0.032 (2/61)	0.049 (3/61)
heterozygote	0.286 (20/70)	0.100 (1/10)	0	0.015 (1/65)	0.032 (2/61)	0.197 (12/61)
Chitotriosidase activity of mutant alleles (assessed and reported by)	Reduced activity Lee et al. (2007)	No stable protein Grace et al. (2007)	Deficient Boot et al. (1998)	Deficient Grace et al. (2007)	Reduced activity Lee et al. (2007)	Normal activity Lee et al. (2007)

(FMC Bioproducts, USA) at 4°C. This restriction enzyme digest resulted in the presence of two fragments (240bp and 19bp) for the wild-type allele, whereas the p.G102S transversion abolishes the *Hpa*II restriction site and the 259bp fragment remains uncleaved. In the heterozygote state, three fragments were visible on the gel.

PCR amplification, followed by direct DNA sequencing was used to examine exon 10 and 12 of the *CHIT1* gene for the presence of p.G354R, the 24bp duplication, the 4bp deletion and p.A442V/G. PCR products were purified using DyeEx Kit™ 2.0 kit (Qiagen, Hilden, Germany) and then sequenced in both directions using BigDye™ Terminator Ready Reaction Mix and the ABI Prism™ 3130XL genetic analyser (Applied Biosystems, USA).

Evaluation of Missense Mutations

Variants detected in the coding region of *CHIT1* were assessed using two web-based tools, PolyPhen (http://genetics.bwh.harvard.edu/pph) and SNPs3D (http://www.snps3d.org/). Briefly, PolyPhen computes the impact of an amino acid substitution on a human protein by analysing its structural and functional characteristics. The program generates a position-specific independent counts (PSIC) score for the two amino acid variants. If the difference between the calculated scores for these two amino acid variants (ΔPSIC) is ≤ 0.5, the impact of the amino acid substitution on the protein's function is likely to be benign (Ramensky et al. 2002).

Table 3 Computationally predicted functional impact of coding DNA variants on CHIT1 using two web-based tools

Variant	PolyPhen ΔPSIC (http://genetics.bwh.harvard.edu/pph)	SNP3D SVM score (http://www.snps3d.org/)	Predicted effect on Chitotrosidase
G354R	2.975	–4.25	Deleterious
A442V	0.948	–1.07	Damaging
A442G	0.867	1.55	Inconclusive
	ΔPSIC $\leq$ 0.5 = benign	SVM scores > +0.5= benign	

SNPs3D uses a machine learning technique for the in silico analysis of coding non-synonymous variants. The SNPs3D algorithm combines two support vector machine (SVM) methods and computes the impact of an amino acid change on the structure and stability of the protein. In addition, it incorporates evolutionary sequence information by comparing the mutated amino acid position to orthologue sequences. The output is an SVM score. A positive SVM score classifies the amino acid change as non-deleterious, while a negative score indicates a deleterious effect on the protein's function. It has been noted that accuracy is significantly higher for SVM scores > 0.5 and < -0.5 (Yue and Moult 2006).

Results

Plasma chito activities and CHIT1 genotyping

A mean chito activity of 68.77 nmol/h/ml (SD+/− 46.19) was observed in Black South African individuals ($N = 210$) with detectable enzyme activity (range 0.5–346.16 nmol/h/ml). In total, 8 of the 492 (1.6%) unrelated Black individuals that were tested biochemically showed no detectable chito activity (0 nmol/h/ml) and 8 out of 212 individuals (3.8%) showed low enzyme activity (<= 10 nmol/h/ml), indicative of bearing a mutant *CHIT1* allele. The absence of the 24bp-duplication was confirmed in all individuals ($N = 0/16$) (Table 1).

Genotype analysis of ten chito-compromised probands from group 2 (N=212) revealed a 4bp deletion across the exon / intron 10 boundary (E/I-10_delGAgt) in homozygous state in Probands 7 and 8 and in heterozygous state in Proband 9 (Table 1). The exact position of this 4bp splice site deletion (E/I-10_delGAgt) and its impact on the mRNA splicing are shown in Fig. 1. All three individuals with the E/I-10_delGAgt allele also carried the p.G354R and the p.A442V variant. Based on our data, all three mutations appear to be in complete linkage disequilibrium (LD) with each other (Table 1). Since the three potentially damaging variants were present on each of the homozygote chito-deficient loci, we used online bioinformatic tools in conjunction with published information to assess the impact of mutations p.G354R and p.A442V independently (Table 3) (Ramensky et al. 2002; Yue and Moult 2006).

Discussion and Conclusion

The 1.6% overall prevalence of enzyme assay-based chitotriosidase deficiency detected in the Black SA Bantu population corroborates previous findings of notably lower prevalence of chito deficiency in African populations (0–2%) (Malaguarnera et al. 2003) in comparison with populations elsewhere in the world (Lee et al. 2007; Piras et al. 2007). In addition, our study revealed complete absence of the 24bp duplication in exon 10 of the *CHIT1* gene as the causative mutation for chito deficiency in the Black Southern African population. This finding differs from investigations of chito-deficient alleles in subjects of African ancestry in previous studies (Grace et al. 2007; Lee et al. 2007) where the 24bp duplication was detected at appreciable frequencies (4.3% and 7%, respectively). However, it is likely that the 24bp duplication allele in African Americans, Caribbean Hispanics and Black individuals of self-reported ethnicity, all living outside of Africa, is a consequence of gene flow, if one considers the high frequency of the 24bp duplication in their respective surrounding populations. The observation of the 4bp splice site deletion (E/I-10_delGAgt) in homozygous state in our study allowed the precise localisation of the deletion, which is shifted by 2bp from the published position (Grace et al. 2007). The postulated functional impact of this 4bp deletion as described in the current study (Fig. 1) is strongly supported by previous CHIT1 mutation expression studies (Grace et al. 2007).

Mutation analysis in Black South African chito-deficient individuals shows that the 4bp deletion is in complete linkage disequilibrium (LD) with two additional coding variants (p.G354R and p.A442V) within the *CHIT1* gene. It is not possible to accurately pinpoint the causal mutation that leads to the loss of chito activity in Black South Africans, since two of the three detected mutations (p.G354R, E/I-10_delGAgt) result in chito deficiency and the remaining one (p.A442V) results in reduced activity (Lee et al. 2007). It would be interesting to investigate

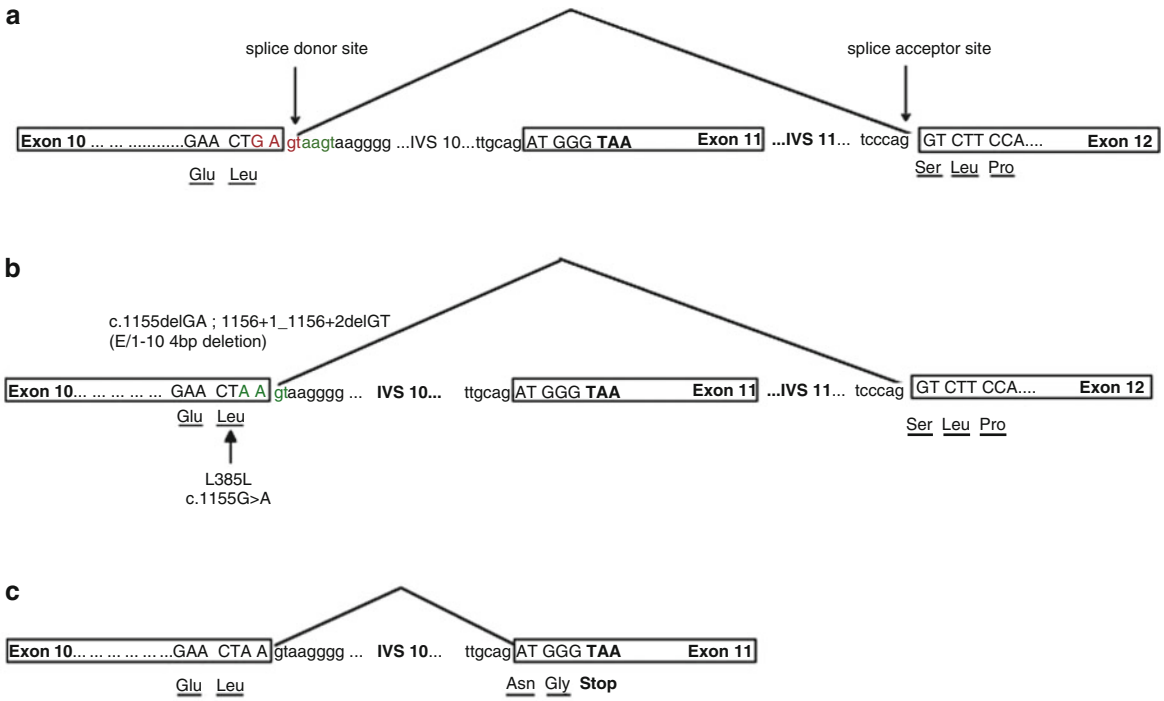

Fig. 1 (**a**) The normal sequence and exon splicing of *CHIT1* pre-mRNA, (exon 1-10 + exon 12) (Boot et al. 1998), with the 4bp deletion indicated in *red*. Flagged in *green* are the neighbouring nucleotides of the 4bp deletion. (**b**) As a result of the E/I-10 4bp deletion the pre-mRNA sequence of *CHIT1* is altered. The occurring frame shift leads to silent mutation p.L385L as well as the creation of an alternative splice donor site. In the majority of splicing events (4/6 times) the splicosome recognises the alternative splice site and normal splicing occurs (exon 1-10 + exon 12) (Grace et al. 2007). (**c**) In 2/6 splicing events, the use of the alternative splice site leads to aberrant mRNA splicing. The inclusion of exon 11 in the mRNA leads to the introduction of a premature stop codon and no translation into protein occurs (Grace et al. 2007)

allelic variants that occur at the *CHIT1* locus in other African populations and establish if these three mutations are, firstly, present and, secondly, in LD with each other.

To date, the answers as to why the global population differences in chitotriosidase deficiency emerged and what the exact biological function of this enzyme is remain controversial (Barone et al. 2003b; Chien et al. 2005; Choi et al. 2001; Gordon-Thomson et al. 2009; Hall et al. 2007; Hise et al. 2003). In populations living outside Africa, chitotriosidase deficiency might confer a selective advantage against atopy by modulating the immune response (van Eijk et al. 2005) and, more recently, it was reported that heterozygosity for the 24bp duplication is associated with longevity in Mediterranean populations (Malaguarnera et al. 2010).

In conclusion, regardless of the driving force behind the emergence of chitotriosidase deficiency, we have shown that the prevalence of chitotriosidase deficiency in Black South Africans is low and it is therefore a conducive biomarker.

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CASE REPORT

NDUFS8-related Complex I Deficiency Extends Phenotype from "PEO Plus" to Leigh Syndrome

Adela Della Marina · Ulrike Schara · Angela Pyle · Claudia Möller-Hartmann · Elke Holinski-Feder · Angela Abicht · Birgit Czermin · Hanns Lochmüller · Helen Griffin · Mauro Santibanez-Koref · Patrick F. Chinnery · Rita Horvath

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Abstract With over 1,000 nuclear genes that could potentially cause a mitochondrial disorder, the current diagnostic approach requires targeted molecular analysis, guided by a combination of clinical and biochemical features. However, the expanding molecular and clinical spectrum means that this approach does not always yield a result. Here we report the unusual clinical presentation of "Progressive External Ophthalmoplegia (PEO) plus" Leigh syndrome in three children from a consanguineous family where exome sequencing identified mutations in *NDUFS8*. *NDUFS8* is a nuclear-encoded structural core protein of complex I, and mutations are expected to cause infantile onset and severe disease. Our patients had a later onset, milder and a clinically distinct phenotype, and this gene would not normally be considered in this context. Being untargeted to specific genes, whole exome analysis has the potential to re-write the phenotype and reveal an unexpected molecular aetiology, as illustrated by this family.

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ADM, US and AP as first authors and RH and PFC as last authors contributed equally to this study

A. Della Marina · U. Schara
Pediatric Neurology, University of Essen, Essen, Germany

A. Pyle · H. Lochmüller · H. Griffin · M. Santibanez-Koref · P.F. Chinnery · R. Horvath (✉)
Institute of Genetic Medicine, Newcastle University, NE1 3BZ Newcastle upon Tyne, UK
e-mail: Rita.Horvath@ncl.ac.uk

C. Möller-Hartmann
Institute of Diagnostic and Interventional Radiology and Neuroradiology, University of Essen, Essen, Germany

E. Holinski-Feder · A. Abicht · B. Czermin
Medical Genetic Center, Munich, Germany

Introduction

Mitochondrial complex I (NADH ubiquinone oxidoreductase) is the largest enzyme of the mitochondrial respiratory chain and catalyzes the transfer of electrons from NADH through the respiratory chain (Distelmaier et al. 2009; Pagniez-Mammeri et al. 2012). It is composed of 37 nuclear-encoded and 7 mitochondrial-encoded subunits, based on a recent study, which showed that the NDUFA4 subunit is actually a subunit of complex IV (Balsa et al. 2012), and requires several additional proteins for its assembly. Mutations in many of these genes were reported in patients with different clinical presentations associated with isolated complex I deficiency, which is the most frequently encountered defect, causing ~25–35 % of all disorders of the mitochondrial energy metabolism (Distelmaier et al. 2009; Pagniez-Mammeri et al. 2012; Nouws et al. 2012).

Mutations in the mitochondrial subunit genes (*MTND1-MTND6*) of complex I cause maternally inherited disease with variable clinical presentations such as Leber's hereditary optic neuropathy, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes or Leigh syndrome. More frequent clinical presentations of nuclear gene defects are Leigh syndrome (25 %), neonatal cardiomyopathy with lactic acidosis, leukoencephalopathies or other undefined encephalomyopathies, which follow Mendelian inheritance. In general, mutations in assembly factor genes result in later onset and milder phenotypes compared to deficiencies of nuclear-encoded structural complex I subunit genes (Nouws et al. 2012). The number of nuclear mutations responsible for isolated complex I deficiency is increasing rapidly, which was recently facilitated by the new high-throughput technologies. However, thorough clinical reports on patients are still lacking (Haack et al. 2012; Calvo et al. 2010).

Here we report three children with a mild and variable clinical presentation due to a homozygous mutation in a nuclear complex I structural subunit gene, a totally unexpected finding detected through non-targeted whole exome sequencing.

Case Reports

Patient 1

The now 13-year-old index patient (Fig. 1a) is the first of three children of consanguineous Afghan parents (first cousins). Our index patient was born at term after uneventful pregnancy. He exhibited generalized muscular hypotonia from infancy with delayed motor milestones (sitting at 16 months, walking at 24 months, frequent falls). His speech development was delayed (first words at age 24 months). Facial hypotonia and mild ptosis were also noted.

From 5 years of age he suffered from a slowly progressive muscle weakness, and developed dysarthria and an ataxic gait. There was a marked worsening of his symptoms, especially of muscle strength and respiratory function, frequently caused by infections. Currently, at the age of 13 years he is able to walk only a few steps independently. He complains of pain after physical exercise and for longer distances he uses a wheelchair.

On clinical examination his height was on the 3rd percentile (144 cm) and weight 2 kg below the 3rd percentile (31.2 kg). He showed facial hypotonia, mild ptosis, external ophthalmoplegia (Fig. 1a) and had intermittent hypersalivation and difficulties in swallowing. He had atrophic muscles with thoracolumbal scoliosis and contractures; deep tendon reflexes were reduced. Further symptoms were dystonic posturing, decelerated movements and an ataxic gait. He had a reduced intelligence quotient (IQ) 61 (Snijders Oomen nonverbal Test for Intelligence), was not able to read or write apart from his own name and attends special schooling.

Laboratory measurements including creatine kinase (CK) were normal, but serum lactate was mildly elevated (23.5 mg/dl, normal<19.8 mg/dl). Echocardiography detected a borderline left ventricular dysfunction (fractional shortening 25.6 %); electrocardiogram was normal.

Cranial magnetic resonance imaging (MRI) at 5 years of age showed bilateral increased signals in the putamen. MRI at age 9 years showed increased signals in the bilateral putamen, nucleus caudatus and frontal subcortical region (Fig. 1b), consistent with mitochondrial encephalopathy (Leigh syndrome).

Substitution with creatine and riboflavin was initiated at 10 years of age, with no clearly demonstrable effect.

Patient 2

The younger brother of the index patient had a normal motor and mental development until 5 years of age, when he developed dysarthria. Severe myopia was diagnosed at 6 years of age, followed by slowly progressive muscle weakness leading to frequent falls. Currently, at 12 years of age (Fig. 1a), his weight is on 25th percentile (35 kg) and his height on 75th percentile (154 cm). He showed mild proximal muscular hypotonia with difficulties in uprising from a squatting position. His exercise tolerance was reduced, and he climbed stairs by holding on to the hand rail. He also complained of pain after exercise. His symptoms worsen during febrile infections.

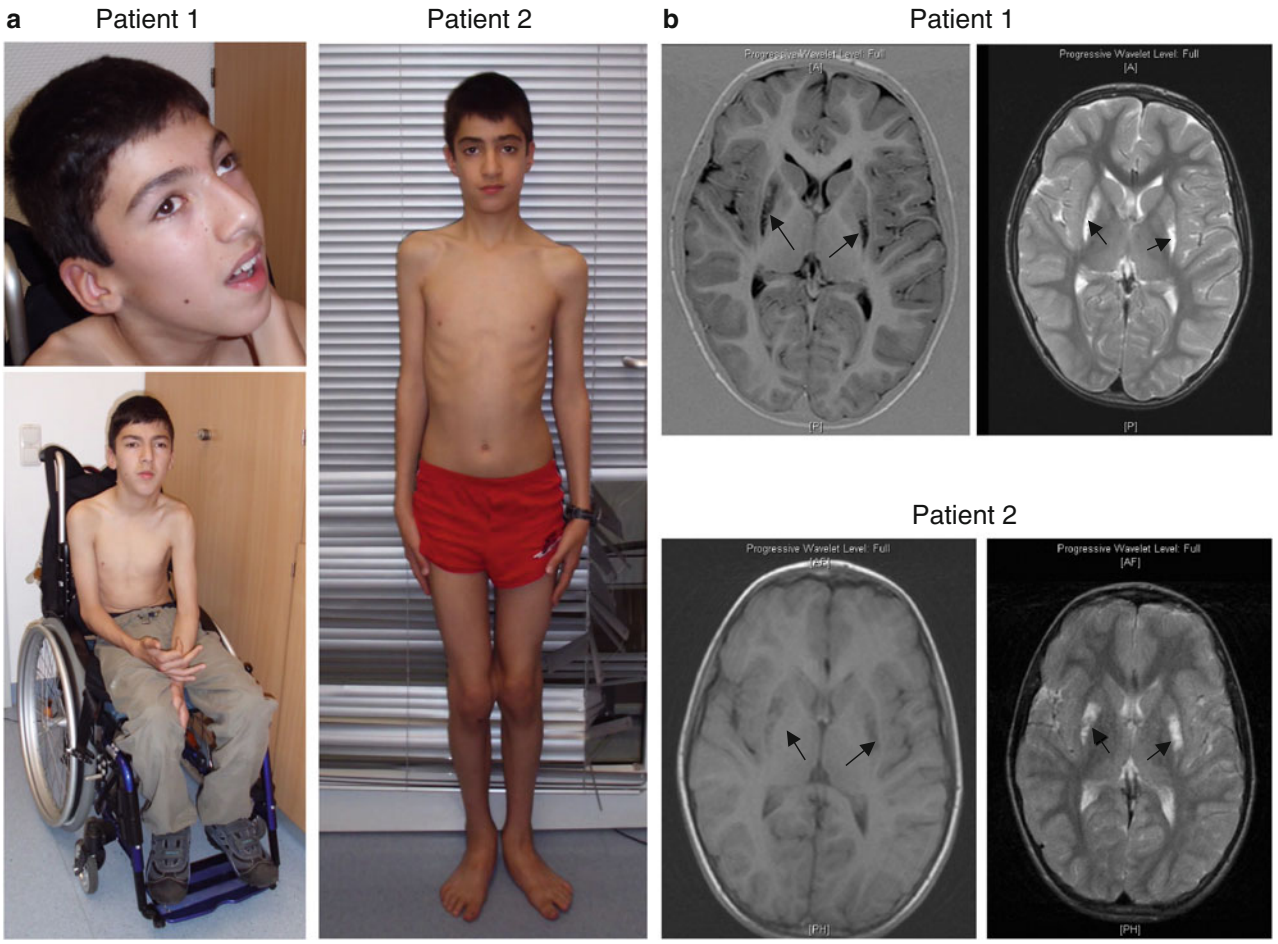
Clinical examination revealed mild ptosis and ophthalmoplegia with intermittent horizontal nystagmus and dysarthria, but normal deep tendon reflexes. He had mild cognitive impairment (Kaufman-Assessment Battery for Children: IQ 73); he attends a school for handicapped children. Cranial MRI at 8 years of age showed abnormal signal in the bilateral putamen (Fig. 1b). Echocardiography detected borderline left ventricular dysfunction (fractional shortening 25.6 %); electrocardiogram was normal, as well as his laboratory findings including CK and lactate.

Patient 3

The third child of the family is a girl, currently 9 years old. She reached her motor and mental milestones age appropriately. At 7 years of age she had mild generalized muscular hypotonia, scapular winging and mild knee contractures. Deep tendon reflexes were normal. She had difficulties in rising from a squatting position, but her walking distance was unlimited. She had myopia, intermittent nystagmus and dysmetria, but no gait ataxia. She had normal cognitive development until 9 years of age (Kaufman-Assessment Battery for Children: IQ 84), and she had been visiting a mainstream primary school from age 7. Because of mild symptoms, no laboratory findings or MRI were performed. Echocardiography and electrocardiogram were normal.

Results

Muscle biopsy of patient 1 at 5 years of age detected non-specific variation of fibre size, no ragged-fed fibres in the Gomori-Trichrome stain and enzyme histochemistry for NADH-CoQ-Oxidoreductase (NADH), cytochrome c



c

Patient	Variant Type	Total predicted (*)	Homozygous on Target (a)	1000 Genomes & dbSNP135 (b)	Homozygous rare/novel Variants (c)	Number of homozygous possibly pathogenic mutations			
						Homozy-gous	Homozygous protein altering (d)	Homozygous predicted disease causing (e)	Homozygous mtochondrial
Patient 1	SBV	362,804	23,077	22,598	165	62	30	15	1
	Indel	29,731	1,351	986	43				
Patient 2	SBV	333,390	22,324	21,926	140				
	Indel	28,893	1,221	902	26				

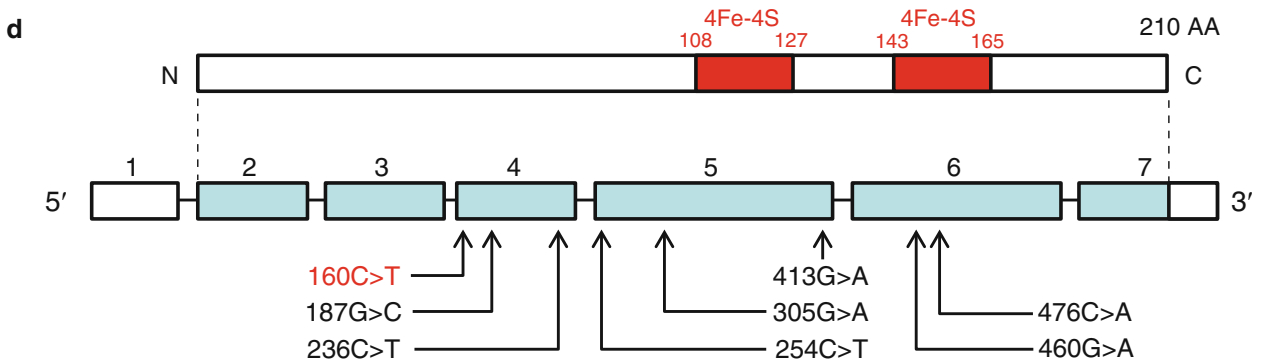


Fig. 1 (**a**) The picture shows patient 1 (13 years) and patient 2 (12 years). Note external ophthalmoplegia on upgaze in patient 1. (**b**) Brain MRI of patient 1 at 9 years of age showed hyperintense lesions in the bilateral putamen and nucleus caudatus as well as in the bilateral frontal subcortical region on T2w sequences without enhancement after gadolinium. The lesions in the bilateral putamen and nucleus caudatus were hypointense on T1 images. There was a slight ventricular asymmetry, but the configuration of the

oxidase (COX) and succinate dehydrogenase (SDH) revealed no abnormalities (data not shown).

Biochemical analysis of the respiratory chain enzymes in muscle was done by standard methods and showed a reduction of complex I activity, NADH-CoQ-Oxidoreductase (0.05U/U Citrate Synthase, normal range: 0.17–0.56). The activities of complex II, III and IV were normal.

Genetic analyses: mitochondrial DNA deletions, mtDNA depletion and point mutations were excluded by standard methods. Genetic analysis of *POLG* was negative.

Whole exome sequencing was performed as described previously in patient 1 and 2 (Horvath et al. 2012). We detected 62 novel homozygous changes shared in both patients, 30 were protein altering and 15 of these were predicted to be disease causing. Only one of these homozygous changes was in a mitochondrial complex I related gene, in *NDUFS8* (Fig. 1c). The mutation c.160C>T, p.Arg54Trp (Fig. 1d) is predicted to be disease causing (Mutation Taster) (De Sury et al. 1998) and was absent in 222 control chromosomes. This mutation co-segregated within the family as determined by Sanger sequencing, it was found heterozygous in the healthy parents and homozygous in all three affected siblings.

Discussion

Although mutations in several nuclear and mitochondrial genes have been reported in association with complex I deficiencies, there are few reports describing clear genotype-phenotype correlations to direct diagnostic genetic testing (Tuppen et al. 2010). The patients reported here have – unlike deficiencies of structural nuclear complex I subunit genes – a subtle onset, slowly progressive neuromuscular condition, with ptosis, eye movement abnormalities, exercise intolerance, muscle weakness and pain, dysarthria and ataxia, resembling "progressive external ophthalmoplegia (PEO) plus". Cognitive development was variable, although differences in the younger siblings could be explained by an earlier phase of the disease. Brain MRI showed Leigh syndrome and isolated complex I deficiency was detected in skeletal muscle of patient 1, pointing to a mitochondrial disease. The high number of nuclear genes involved in the structure and assembly of complex I and the lack of genotype/phenotype data prompted us to perform whole exome sequencing. We detected 15 possible disease causing homozygous changes in the two affected patients, but only c.160C>T, p.Arg54Trp altered a complex I related gene, *NDUFS8*. This mutation is most likely causative for the disease in our patients, since it co-segregates with the symptoms, and alters a highly conserved amino acid, but does not disrupt the [4Fe-4S] cluster, and thus may result in some residual protein function.

By searching the literature we identified eight patients carrying *NDUFS8* mutations to date (Table 1); however, the published clinical information was very brief in five cases (Haack et al. 2012; Calvo et al. 2010; Tuppen et al. 2010; Procaccio et al. 2004; Loeffen et al. 1998). Some patients had a severe disease with feeding difficulties, respiratory problems, epilepsy and hypertrophic cardiomyopathy and died within the first months of life (Tuppen et al. 2010; Loeffen et al. 1998). Others, including our patients show normal or mildly impaired motor development in the first years of life and develop a slowly progressive neurological disease at the end of the first decade (Procaccio and Wallace 2004, this paper). All reported mutations are missense mutations (Table 1, Fig. 1d), and no patients are reported to date with nonsense mutations in *NDUFS8*, suggesting that a complete absence of this protein may have deleterious effect leading to intrauterine lethality. Relatively mild phenotypes associated with Leigh syndrome are not so uncommon in mitochondrial disorders due to mutations in some nuclear assembly genes, as reported in patients with mutations in *SURF1* (Piekutowska-Abramczuk et al. 2009).

Defining the molecular basis of human diseases is important for genetic counselling and for prenatal genetic testing. However, limited clinical data on new and existing disease genes makes it difficult to know which genes to target. More detailed clinical descriptions of patients carrying mutations in different genes would be highly valuable for clinicians, who need to diagnose and counsel patients with mitochondrial disease. This will be facilitated by next generation sequencing of either large panels of relevant genes (Calvo et al. 2010), whole exome (Haack et al. 2012) and ultimately whole genome analysis. Our chapter shows the success of this approach.

Fig. 1 (continued) cerebellum and brainstem were normal. Brain MRI of patient 2 at 8 years of age showed an abnormal symmetrical signal in the bilateral putamen (hypointense on T1 images, hyperintense on T2 images) but no other malformations of the brain structures. (**c**) Exome sequencing detected 62 novel homozygous changes shared in both patients; 30 were protein altering and 15 of these were predicted to be disease causing. Only one of these homozygous changes was in a gene encoding a known mitochondrial protein, NDUFS8, a complex I related gene. (**d**) Localization of the c.160C>T, pArg54Trp mutation in the *NDUFS8* gene. 4Fe-4S refers to the iron-sulphur binding domains

Table 1 Summary of all previously reported patients compared to our patients with *NDUFS8* mutations

Reference	Onset	Age/death	Clinical presentation	Brain MRI	Muscle biopsy	RC in cell lines	Mutation
Previously described patients							
Loeffen et al. 1998	5 weeks	Died at 11 weeks	Poor feeding, apnea, cyanosis, spasticity, seizures, hypertrophic cardiomyopathy	LS	Histology n.d. complex I↓ (39% of normal)	Fibroblasts complex I↓ (69% of normal)	c.236C>T/p.Pro79Leu c.305G>A/p.Arg102His compound heterozygous
Procaccio and Wallace 2004	7 years	Alive at 9 years	Toe walking, nystagmus, dysarthria, dystonia, ataxic gait	LS	Histology normcomplex I↓ (31% of normal)	Lymphoblast complex I↓ (43% of normal)	c.254C>T/p.Pro85Leu c.413G>A/p.Arg138His compound heterozygous
Haack et al. 2012	n.d.	n.d.	Mitochondrial encephalopathy, cardiomyopathy, muscular hypotonia, respiratory insufficiency	n.d.	Histology n.d. complex I↓ (38% of normal)	Fibroblastscomplex I↓ (52% of normal)	c.279C>T/p.Arg79Trp c.476C>A/p.Ala159Asp compound heterozygous
	n.d.	n.d.	Muscular hypotonia, dyskinesia, epilepsy, lactic acidosis	LS	Histology n.d. complex I↓ (8% of normal)	Fibroblastscomplex I↓ (54% of normal)	c.187G>C/p.Glu63Gln homozygous
	n.d.	n.d.	Muscular hypotonia, hypertrophic cardiomyopathy, lactic acidosis	LS	n.d.	n.d.	
Tuppen et al. 2010	4 weeks	Died at 12 weeks	Poor feeding, apnea, lactic acidosis, seizures, failure to thrive, respiratory insufficiency	n.d.	Histology n.d. complex I↓ (30% of normal)	n.d.	c.236C>T, p.Pro79Leu homozygous
Calvo et al. 2010sibs	n.d.	n.d.	Mitochondrial encephalomyopathy	Leuko-encepha-lopathy	Complex I↓	n.d.	c.460G>A, p.Gly154Ser homozygous
Patients described in this paper							
P1	Birth	Alive at 13 years	Developmental delay, PEO, muscular hypotonia, ataxic gait, dysarthria, dystonia, respiratory problems	LS	Histology normcomplex I↓ (25% of normal)	n.d.	c.160C>T, p.Arg54Trp homozygous
P2	5 years	Alive at 11 years	Dysarthria, muscular hypotonia, myopia, ptosis, PEO, swallowing difficulties	LS	n.d.	n.d.	
P3	9 years	Alive at 9 years	Muscular hypotonia, scapular winging, dysmetria, nystagmus, myopia	n.d.	n.d.	n.d.	

Abbreviations: + present, *n.d.* not determined; *PEO* progressive external ophthalmoparesis, *LS* Leigh syndrome, *RC* respiratory chain

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Synopsis

Here we report three children with a mild and unusual clinical presentation of Leigh syndrome with progressive external ophthalmoparesis due to a homozygous mutation in a nuclear complex I structural subunit gene detected through non-targeted whole exome sequencing.

Author Contribution

Adela Della Marina and Ulrike Schara participated in the clinical follow-up of the patients and in the drafting the manuscript.

Angela Pyle performed whole exome sequencing and participated in the drafting of the manuscript.

Claudia Möller-Hartmann analyzed and interpreted the MRI images.

Elke Holinski-Feder, Angela Abicht and Birgit Czermin did the diagnostic work-up.

Hanns Lochmüller was involved in the biochemical and histological analyses.

Helen Griffin and Mauro Santibanez-Koref did the bioinformatic analysis of the exome data.

Patrick F. Chinnery and Rita Horvath are responsible for the clinical and diagnostic work-up, the study designs and for drafting of the manuscript.

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Ethical Issues

The study was approved by the County Durham & Tees Valley 1 Research Ethics Committee (08/H0905/106 "Nuclear genes in mitochondrial disease").

The parents gave written consent for the study and for presenting the data and photographs of the patients.

Competing Interest Statement

The authors have no competing interests.

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RESEARCH REPORT

Accuracy of Six Anthropometric Skinfold Formulas Versus Air Displacement Plethysmography for Estimating Percent Body Fat in Female Adolescents with Phenylketonuria

Teresa D. Douglas · Mary J. Kennedy · Meghan E. Quirk · Sarah H. Yi · Rani H. Singh

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Abstract *Background:* The reliability of studies investigating biological and therapeutic factors that influence body composition in PKU patients depends on accurate anthropometric measurements.

Objective: To determine the precision of six anthropometric skinfold equations versus air displacement plethysmography (ADP) for predicting body fat (BF) percentage in female adolescents with PKU.

Design: Skinfold and ADP measurements were recorded from a cross section of 59 female patients with PKU, ages 10–19 years. Anthropometric measures were used to calculate percent BF using equations published by Peterson et al., Loftin et al. (TAAG), Slaughter et al., Wilmore and Behnke, Durnin and Womersley, and Jackson et al. Bland-Altman agreement analysis and Lin's concordance correlation coefficient (ρ_c) were used to determine the precision of each equation compared with percent BF determined by ADP.

Results: Adolescent females with PKU had a mean BF content of 33% measured by ADP, with an inverse association to birth cohort ($r = -0.3$, $P = 0.016$). Based on the Bland-Altman method for evaluating agreement, only Peterson's equation did not differ significantly from ADP percent BF results ($P = 0.23$). Peterson's skinfold equation yielded percent BF estimates with the smallest mean difference from ADP and the smallest standard deviation (0.76 ± 4.8), whereas Slaughter's equation had the largest (-7.7 ± 7.4). Loftin's TAAG equation had the least mean percent error (2.2%), while Slaughter's equation had the highest (19%). Both TAAG and Peterson's equations had the highest concordance correlation coefficients ($\rho_c = 0.8$, $\rho_c = 0.8$), while Slaughter's equation had the lowest ($\rho_c = 0.3$).

Conclusions: Peterson's equation is a precise surrogate for ADP when estimating percent BF in female adolescents with PKU, though Loftin's TAAG equation is also effective. Observed decreases in adiposity correlating with birth cohort could reflect steady improvements in patient nutrition care.

Abbreviations

ADP	Air displacement plethysmography
BF	Body fat
DXA	Dual-energy x-ray absorptiometry
PKU	Phenylketonuria
UWW	Underwater weighing

Introduction

Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism characterized by the inability of phenylalanine hydroxylase to efficiently convert phenylalanine (Phe) into tyrosine. To prevent permanent neurological damage from high blood Phe concentrations, patients with PKU are instructed to follow a lifelong dietary regimen of

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M.J. Kennedy · R.H. Singh (✉)
Department of Human Genetics, Division of Medical Genetics, Nutrition Metabolic Program, Emory University School of Medicine, 2165 North Decatur Road, Decatur 30033, Georgia
e-mail: rsingh@emory.edu

T.D. Douglas · M.E. Quirk · R.H. Singh
Graduate Division of Biological and Biomedical Sciences, Nutrition and Health Sciences Program, Emory University School of Arts and Sciences, Decatur, Georgia

S.H. Yi
Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA

low-Phe foods along with amino acid-rich medical food as a primary source of protein nutrition (Santos et al. 2006).

The effects that PKU, and the corresponding dietary regimen, may have on weight gain have been the subject of prior studies, including some that indicate a higher risk of overweight in those diagnosed with PKU (White et al. 1982; McBurnie et al. 1991; Acosta et al. 2003). This trend has been seen for inborn errors of protein metabolism in general (Wilcox et al. 2005). Other research has specifically investigated body composition in early-treated patients with PKU. A study using total body electrical conductivity found a positive association between grams per kilogram of natural protein intake and fat-free mass, even when no mean difference in body composition could be found between subjects with PKU and controls (Huemer et al. 2007). However, other studies have reported significantly less lean body mass (Allen et al. 1995) and greater percent body fat (BF) (Albersen et al. 2010) in PKU pediatric and adolescent females versus controls. The lack of significant body composition difference between patients and controls in the Huemer study, while relevant findings occurred for Albersen and Allen, may be attributed to the different measurement approaches used: skinfold measures for the Allen study and air displacement plethysmography (ADP) for the Albersen comparison, as opposed to Huemer's use of body electrical conductivity; although other cohort variables may also have been contributory. The significant differences the Albersen and Allen studies found within their female child and adolescent subgroups led us toward selecting this demographic for our analysis.

Precise analytical methods and measurement tools are essential when conducting body composition research. ADP is a widely accepted approach for measuring body composition (McCrory et al. 1995; Nunez et al. 1999; Ginde et al. 2005), but is not always feasible due to space, technician, and monetary requirements. In research designs and clinical settings where technologies such as DXA, UWW, or ADP are impractical, it is important to have an economical yet comparably accurate alternative for determining body composition. Alternatively, interpretive equations that calculate percent BF or fat-free mass from skinfold measurements are easily performed, low in cost, and have been used for decades (Sloan et al. 1962; Brook 1971; Katch and McArdle 1973; Lohman 1981). The reliability of such equations, however, is often limited to the populations from which they originate (Brandon 1998; Michener et al. 2000; Kohli et al. 2009). Research has shown that unique medical populations, such as patients with congenital adrenal hyperplasia, cystic fibrosis, Crohn's disease, or who require antipsychotic medications, all conditions affecting nutrient utilization, may require customized interpretive measurement protocols or equations to obtain accurate body composition information (Dung et al. 2007; Sharpe et al. 2008; Wells et al. 2008; Gonçalves et al. 2012). Though research validating skinfold techniques to ADP has been reported for select groups (Gonzalez-Aguero et al. 2011; Gomez-Ambrosi et al. 2012; Lingwood et al. 2012), the usability of such equations has yet to be evaluated for those affected by inborn errors of metabolism, including the PKU population. Thus, the aim of this comparative analysis was to determine the precision and accuracy of six anthropometric skinfold equations versus ADP in a group of female adolescents with PKU.

Subjects and Methods

Subjects

Subjects were a cross section of adolescent females with PKU who attended Emory University's Annual Metabolic Camp at least once from 1999 to 2006. All subjects were early diagnosed through newborn screening with access to traditional diet treatment from infancy. PKU phenotypes ranged from classic to mild. Each camper represents only one data point, since those returning to camp in consecutive years were not remeasured to avoid repeats in the data sample. Of the 69 study volunteers who met the age criteria of 10–19 years (mean $\pm$ SD $=$ 14.4 $\pm$ 2.5), 59 (85%) agreed to measurement by both ADP and skinfold calipers. Data from the other ten subjects were excluded from comparative analysis due to a lack of either measure. Legal guardians and participants provided consent and assent, respectively, prior to participation in the research protocol. This protocol was approved by Emory University's Institutional Review Board.

Data Collection

ADP, a well-accepted and prevalent method for evaluating body composition (Fields et al. 2005), using the BOD POD® apparatus (Life Measurement Inc, Concord, CA), was selected as the reference standard because of convenience, availability, and comparability with the gold-standard methods of underwater weighing (UWW) (Nunez et al. 1999; Ginde et al. 2005) and dual-energy x-ray absorptiometry (DXA) (Nicholson et al. 2001; Ballard et al. 2004).

The Emory University Hospital Clinical Interaction Site (EUH CIS, formerly the EUH General Clinical Research Center) is a National Institutes of Health sponsored research site that provides trained professional research staff and quality standardized calibrated equipment for accurate measurement of anthropometric parameters. Licensed registered dietitians at the EUH CIS performed ADP body

Table 1 Female-specific body composition equations compared with ADP when calculating percent BF. Skinfold measurements in millimeters

Source	Formula	Age specificity
Peterson	%BF = 22.189 + 0.064 (age) + 0.604 (BMI) − 0.145 (height) + 0.309 (subscapular + triceps + thigh + suprailliac) − 0.001 (subscapular + triceps + thigh + suprailliac)^2	18–54 years
TAAG	%BF = −23.39 + 2.27 (BMI) + 1.94 (triceps) − 2.95 (race*) − 0.52 (age) − 0.06 (BMI × triceps)	10–15 years
Slaughter	%BF = 1.33 (subscapular + triceps) − 0.013 (subscapular + triceps)^2 − 2.5	8–18 years
Wilmore/Behnke	BD = 1.062 − .001[0.68 (subscapular) − 0.39 (triceps) − 0.25 (thigh)]	17–47 years
Durnin/Womersley	BD = 1.152 − 0.069 × log(subscapular + triceps + suprailliac)	16–19 years
Jackson	BD = 1.096 − .001[0.695 (triceps + abdominal + thigh + suprailliac) + 0.001 (triceps + abdominal + thigh + suprailliac)^2 − 0.071 (age)]	18–55 years

*Race is designated as 1 for African American and 0 for non-African American

density and skinfold caliper measurements. Height (cm) and weight (kg) were measured and recorded by a research nurse during the visit. Dietetic and nursing personnel all had prior experience and training in collection of anthropometric measures in accordance with well-established published standards (ACSM 2000, CDC 2000–2005). Anthropometric data collection occurred between 8am and 1pm each year on the day of arrival to camp, dependent upon the subject's time of arrival. All subjects were fasting for at least 8 hours at the time of data collection. Information about date of birth and demographics were obtained with a questionnaire. Birth cohort was determined by year born such that subjects born within the same year were considered to be of the same birth cohort. Plasma amino acids were analyzed by HPLC (Biochrom 30 Amino Acid Analyzer) at Emory Genetics lab.

Anthropometric Calculations

ADP uses air displacement to determine body volume. The subject's weight is divided by the measured volume to yield whole body density (BD) in grams per cubic centimeter (McCrory et al. 1998; Higgins et al. 2001).

The six equations selected for this comparative analysis (Table 1) incorporate anthropometric and demographic predictors that can be observed easily in a clinical environment and had to be gender specific. Slaughter's (Slaughter et al. 1988), TAAG (Trial of Activity for Adolescent Girls) (Loftin et al. 2007), Durnin/Womersley (Durnin and Womersley 1974), Peterson's (Peterson et al. 2003), Wilmore/Behnke (Wilmore and Behnke 1970), and Jackson's (Jackson et al. 1980) skinfold equations met this criterion. Since adolescent-specific equations were sparse in the literature, and nonexistent for the full age spectrum of our study sample, equations for age groups below and above 18 years were included in the analysis.

Triplicate caliper measurements for each skinfold site were averaged for each person. Averaged values were then used for the six selected equations. Peterson's, TAAG, and Slaughter's equations calculate percent BF directly, whereas the Wilmore/Behnke, Durnin/Womersley, and Jackson equations calculate BD.

BD determined either by ADP or skinfold equations was converted to percent BF values using the Siri formula (Siri 1993), and body mass index (BMI) was calculated from height and weight information. Sex- and age-specific BMI percentiles, and height-for-age percentiles, were determined from the Centers for Disease Control (CDC) 2000 growth charts (Kuczmarski et al. 2000). Percentile ranks for percent BF results in study participants were determined from published age-adjusted gender-specific US pediatric reference curves (Kelly et al. 2009). "Underfat" was identified at the 2nd percentile or less as set forth in the *International Journal of Obesity* (McCarthy et al. 2006). "Overfat" was identified when percent BF was at or above the 80th percentile per Flegal's NHANES-based recommendation (Flegal et al. 2010). Percentile values between these two adiposity classes were identified as being within the "healthy fat" range, respectively.

Statistical Analysis

The Bland-Altman method for assessing agreement (Bland and Altman 1986, 2003) was used to compare calculated percent BF from each skinfold equation against ADP. Statistical significance was set at $P = 0.05$. Further confirmation of agreement was performed using both Pearson correlation (r) and Lin's concordance correlation coefficient (ρ_c) (Lin 1989; Lin 2000).

The percent error of each skinfold equation for each individual was calculated with the following equation:

$$\frac{(\text{Equation percent BF result} - \text{ADP percent BF result}) \times 100}{\text{ADP percent BF result}}$$

Percent errors for all participants were then averaged to determine the mean percent error of each equation compared with ADP.

Linear regression in SPSS 17.0 was used to investigate covariate relationships with percent BF or with the precision error for each anthropometric formula.

Results

Demographics, BMI, and ADP Body Composition

Table 2 presents demographics and anthropometrics of the final study sample ($n = 59$). Table 3 provides the percentile ranks of the study sample for percent BF, BMI, and height-for-age. Mean height-for-age fell below the 50th percentile, but was still within the normal range. Seven (12%) study participants had a height-for-age below the 5th percentile. BMI percentiles ranged from 5th to 99th and averaged within the 71st (±25 SD). Forty-four percent of the study group exceeded the CDC BMI-for-age healthy weight criterion (5th–84th percentiles). Percent BF, as measured by ADP, averaged 32.5% (±9.5 SD) and ranged from 10.5% to 58.2%. Percentile values for ADP percent BF indicated 42% of study participants had body fat amounts above healthy fat criterion (3rd–79th percentiles). Neither BMI percentiles nor percent BF were associated with age or concurrent plasma Phe concentration; however, an inverse association between birth cohort (year of birth) and percent BF was observed ($P = 0.016$). This association remained significant ($P = .049$) even after controlling for periodic changes to dietetic staff. BMI percentiles were not associated with birth cohort.

Mean Values and Correlations

Table 4 provides mean BF results for each equation, as well as correlations with the ADP values. Percent BF calculated from Peterson's equation had the highest correlation to ADP ($r = 0.88$, $\rho_c = 0.80$), whereas Slaughter's equation had the lowest ($r = 0.67$, $\rho_c = 0.31$).

Bland-Altman Agreement

When calculated differences were plotted against individual averages for ADP and the specified equation to determine bias, Peterson's equation had an intercept closest to zero, with data points evenly distributed, while Slaughter's equation had an intercept far below zero with a steep inverse bias (Fig. 1).

Bland-Altman statistics for all six anthropometric equations compared with ADP are provided in Table 5. Peterson's equation had both the smallest mean difference

Table 2 Demographic and anthropometric distributions for 59 female patients with PKU

	N	%	Mean	±SD	Range
Age (years)			14.2	2.3	10.6–19.8
10–14.9	37	63			
15–19.9	22	37			
Ethnicity					
African American	4	7			
Caucasian	53	90			
Other/unclassified	2	3			
Height (cm)			155.0	6.4	134.4–166.9
Weight (kg)			56.4	15.0	30.5–115.6
BMI			23.3	5.0	15.6–41.5
Percent BF by ADP measurement			32.5	9.5	10.5–58.2

Table 3 Growth percentile values for 59 female patients with PKU

	N	%	Mean	±SD	Range
Height-for-age percentiles			38	25	1–84
<5th	7	12			
≥5th	52	88			
BMI-for-age percentiles			71	25	5–99
<5th	0	0			
5th to <85th	33	56			
85th to <95th	16	27			
≥ 95th	10	17			
Percent BF percentiles			63	31	0–99
<2nd	2	3			
3rd to <80th	32	55			
≥ 80th	25	42			

Table 4 Percent BF central statistics for each anthropometric equation and correlations with ADP values. Pearson correlation is r; ρ_c is Lin's concordance correlation coefficient

Method	Mean % BF ± SD	Range in % BF	r with ADP	ρ_c with ADP
Peterson	33 ± 6.3	20–48	0.88	0.80
TAAG	31 ± 7.9	15–45	0.84	0.80
Slaughter	25 ± 4.2	14–29	0.67	0.31
Wilmore/ Behnke	27 ± 4.2	20–38	0.84	0.50
Durnin/ Womersley	30 ± 5.6	17–39	0.84	0.69
Jackson	26 ± 6.6	11–37	0.86	0.61

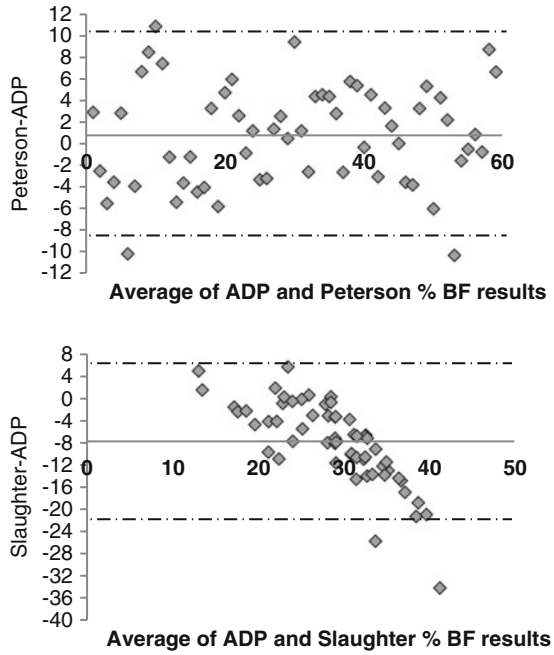

Fig. 1 Diagram of bias for the Peterson and Slaughter skinfold equations compared with reference ADP. Dashed lines indicate upper and lower limits of agreement (mean ± 2SD). Centerline is mean difference of all points from ADP

from ADP and smallest standard deviation; it was the only equation with a nonsignificant *P* value, as well, revealing good agreement with ADP. All other equations had significant *P* values, indicating poor agreement with the reference ADP BF results. When percent error values for all participants were averaged, TAAG had the smallest mean percent error, followed by Durnin/Womersley and Peterson's equations. The Slaughter equation, along with the Wilmore/Behnke and Jackson equations, had the largest mean percent errors.

All six equations fulfilled the Bland-Altman criterion that at least 95% of the difference values fall within the limits of agreement (mean bias ± 2SD), with values outside these limits classified as outliers.

Variation in Percent Error

All equations showed variability in the percent error from ADP for individual body composition results (Fig. 2), with the Slaughter equation underestimating percent BF by as much as 58% and Peterson's equation overestimating percent BF by as much as 104%. All skinfold equations revealed a significant inverse association ($P < .0001$) between percent BF and percent error bias from ADP, indicating that individuals with lower BF were more likely to receive inflated percent BF results from the equations, whereas individuals with higher BF were more likely to receive underestimates. Changes in clinical staff who collected skinfold measurements from 1999 to 2006 were not associated with the percent error from reference ADP.

Table 5 Results from Bland-Altman analysis, evaluating difference of each body composition equation from ADP reference. Limits of agreement are mean difference ± 2 SD

Formula	Mean % error	Mean difference (±SD)	*P* value	Limits of agreement
Peterson	+7.0 %	0.76 ± 4.8	$P = 0.230$	−8.8, 10.3
TAAG	−2.2 %	−1.58 ± 5.2	$P = 0.024$	−12.0, 8.9
Slaughter	−19.3 %	−7.70 ± 7.4	$P < 0.0001$	−22.5, 7.1
Wilmore/ Behnke	−10.4 %	−5.05 ± 6.2	$P < 0.0001$	−17.5, 7.4
Durnin/ Womersley	−3.0 %	−2.41 ± 5.7	$P = 0.002$	−13.8, 9.0
Jackson	−17.8 %	−6.32 ± 5.0	$P < 0.0001$	−16.4, 3.7

Discussion

Agreement of Tested Equations with ADP Results

Though none of the equations tested is perfect for determining individual body composition, Peterson's skinfold equation proved superior at determining percent BF from anthropometric measurements in a female adolescent population with PKU in the clinical setting or when more sophisticated methods of determining body composition (i.e., DXA or ADP) may not be available.

Peterson's equation, introduced in 2003 and originally designed for women over 18 years of age, has not been used before to determine percent BF in adolescents. It was therefore surprising that Peterson's equation proved to be the most precise estimator of percent BF in this analysis; indeed, it was superior even to three other equations designed specifically for adolescent females. Whether the precision of Peterson's equation in this comparative analysis was due to the choice of reference standard (ADP rather than DXA or UWW), our study's particular patient population, or the quality of the equation itself remains to be seen in future investigations.

In this study of female adolescents with PKU, Slaughter's equation arguably showed the poorest precision in determining percent BF, as it had the greatest mean percent error, the largest mean difference and standard deviation from the ADP values, and the weakest correlation to ADP results. Other studies have reported similar results for Slaughter's equation (Rodriguez et al. 2005; Loftin et al. 2007), though not consistently (Wong et al. 2000; Wells et al. 2008; Gonzalez-Aguero et al. 2011), and none others

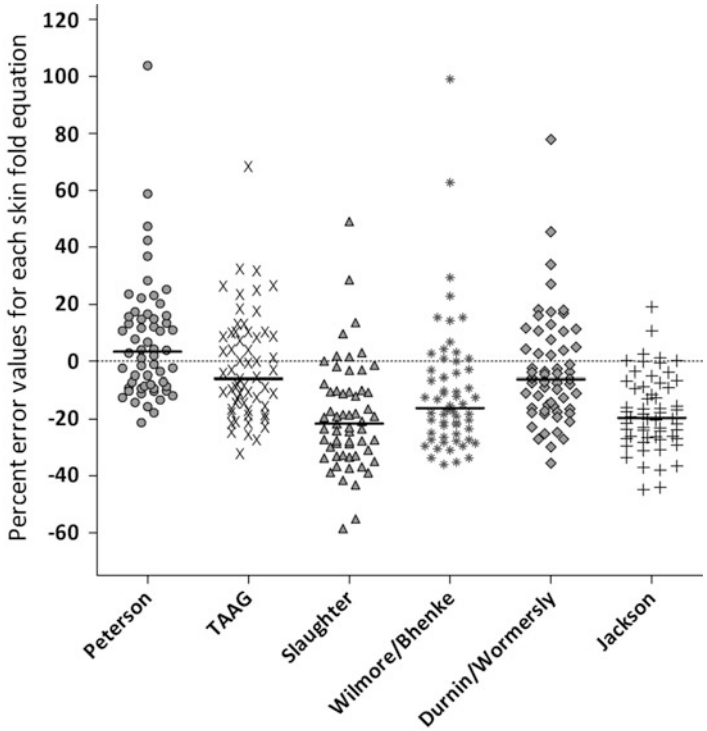

Fig. 2 Percent error between skinfold equation percent BF results and ADP for all study participants

were PKU specific. The discrepancy between these studies and our own may be due to differences in the equations selected for comparison, as well as measuring techniques, reference standard (DXA or UWW rather than ADP), or patient population medical status and demographics.

The TAAG equation, despite showing poor agreement via the Bland-Altman analysis, does have a smaller mean percent error than either the Peterson or Durnin/Womersley equations, and a concordance correlation coefficient that matches Peterson's formula. In addition, the TAAG equation requires fewer anthropometric measures than Peterson's equation, making it an appropriate alternative to Peterson's equation, when measuring multiple skinfold sites would be impractical.

When using any skinfold equation, however, investigators and clinicians must consider the fact that the degree of error correlates strongly with body fat content and is greatest when body fat content is in the upper or lower extremes, as observed in both our study and others (Swan and McConnell 1999; Daniel et al. 2005).

Adiposity in PKU and Clinical Relevance

The mean percent BF of study participants with PKU was similar to the 33% BF mean value reported from NHANES 1999 to 2004 DXA results in females ages 8–19 years (Kelly et al. 2009; Flegal et al. 2010). Therefore, based on the cross-sectional sampling for this study, adolescent females with PKU may not have higher body fat percentages than adolescent girls within the US background population. Whether this will remain the case in the future, given how the prevalence of overweight has been increasing steadily in US children, remains to be seen.

This does not mean that weight gain and body composition of patients with PKU is not worthy of attention. A 2010 article comparing twenty 6–16-year-old PKU children with age- and gender-matched healthy Dutch controls discovered that even though BMI was similar, there was a significantly higher BF percentage as measured by ADP in the children with PKU. Particularly in the subgroup of females ages 11–16 ($n = 8$), percent BF was 30% for females with PKU, but only 21.5% for age-matched healthy controls (Albersen et al. 2010). This, however, may not be contradictory to our result that percent BF is no higher in patients with PKU, since we are comparing our results to NHANES data gathered from over 4,300 US pediatric females from the general population (Kelly et al. 2009; Flegal et al. 2010), rather than against a healthy matched European control group. Also, when standardized by age, a higher proportion of PKU adolescent females within our study had a BMI-for-age status above the 85th percentile (44% of study subjects versus 33% of NHANES subjects) and percent BF values exceeding the 80th percentile (42% study subjects versus 20% NHANES) (Flegal et al. 2010). A study from Poland also showed that male and female adolescents with PKU whose serum Phe

levels were within recommended levels had greater lean body mass and bone mineral density than adolescents with serum Phe above recommended levels (Adamczyk et al. 2011). Therefore, the physiological impact of hyperphenylalaninemia, as well as the impact of dietary restriction, on weight gain, muscle mass, fat mass, and bone density are still research areas of clinical importance for disease management and patient care.

Of additional interest is the inverse trend we uncovered between birth cohort and percent BF in the adolescent females with PKU, which indicated that a girl with PKU who reached the age of 15 years in 2006 would likely have lower body fat than one who reached the same age in 1999. We speculate that continuing improvements in medical and nutrition management of patients with PKU have led to the overall decreases in percent BF. It will be interesting to observe if this trend continues given the emerging treatment options for patients with PKU, such as sapropterin dihydrochloride (BH4) and pegylated phenylalanine lyase (PEG PAL) therapies, which may affect body composition or weight status if they result in more liberalized diets. Accurate investigation of these changing factors on body composition, as well as routine clinical monitoring, requires anthropometric measurement techniques that are reliable and precise.

Conclusion

The comparative investigation of six skinfold equations to ADP indicated that Peterson's skinfold equation is an adequate substitute for ADP when determining body composition in female adolescents with PKU; therefore, the development of a new modified body composition formula was not necessary. Also shown was the superiority of Peterson's equation over five other skinfold equations tested, including three designed specifically for adolescent females, though the TAAG equation may be appropriate in circumstances when the measurement of more than one skinfold site is impractical.

In addition, mean percent body fat composition of female adolescents with PKU did not differ from an NHANES sampling representative of the US background female adolescent population. Even so, a larger proportion of the study group demonstrated adiposity beyond the healthy BMI and percent BF percentile criterion, though we did discover a trend toward decreasing percent BF associated with birth cohort.

This is the first study focused on determining the most accurate method of estimating body composition of individuals with PKU when using skinfold equations. Future studies should investigate the validity of these methods in other sizable demographic groups within the PKU population, as well as longitudinal changes affected by various emerging therapies.

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Synopsis

When determining percent body fat in adolescent females with PKU, Peterson's anthropometric equation is closest to ADP in accuracy and reliability.

Contributions of Individual Authors

Teresa D. Douglas: Performed data cleaning, analysis of data, and writing all parts of manuscript.

Mary J. Kennedy: Project coordinator, writing and submission of IRB documents, obtained informed consent, organizing and coordinating of patients on the day of measurement, coordinating with staff and faculty at the Emory University Hospital Clinical Interaction Site.

Meghan E. Quirk: Made significant contributions in reviewing the article and in recommending large-scale revisions, additional analysis, and statistical approaches

Sarah H. Yi: Assisted with project coordination, obtaining informed consent, measuring of patients, and data entry and organization.

Rani H. Singh: Principal Investigator, developed initial project protocol, obtained essential funding every year; supervising of staff, volunteers, and graduate students involved in project coordination, data management, and data reporting.

Author Serving as Guarantor

Rani H. Singh, PhD. RD

Competing Interest Statement

All the authors of this article have no competing interests to declare.

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Details of Ethics Approval

Research protocol, informed consent documents, and all procedures were submitted to Emory University Institutional Review Board (IRB), receiving approval. Project review and approval by IRB was conducted annually.

Patient Consent Statement

Written informed consent was received from participants 18+ years of age. For study participants who were 10–17 years old, written consent was received from the parents, and written or verbal assent (as age appropriate) from the participating minor. Consent/assent procedures were completed prior to any study measurements being performed. Consenting patients were at liberty to refuse anthropometric measures per their own discretion. Patients not providing informed consent were not involved in the research protocol.

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CASE REPORT

Noncompaction of the Ventricular Myocardium and Hydrops Fetalis in Cobalamin C Disease

Pranoot Tanpaiboon · Jennifer L. Sloan · Patrick F. Callahan · Dorothea McAreavey · P. Suzanne Hart · Uta Lichter-Konecki · Dina Zand · Charles P. Venditti

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Abstract Cobalamin C disease (*cbl*C), a form of combined methylmalonic acidemia and hyperhomocysteinemia caused by mutations in the *MMACHC* gene, may be the most common inborn error of intracellular cobalamin metabolism. The clinical manifestations of *cblC* disease are diverse and range from intrauterine growth retardation to adult onset neurological disease. The occurrence of structural heart defects appears to be increased in *cblC* patients and may be related to the function of the MMACHC enzyme during cardiac embryogenesis, a concept supported by the observation that *Mmachc* is expressed in the bulbis cordis of the developing mouse heart. Here we report an infant who presented with hydrops fetalis, ventricular dysfunction, and echocardiographic evidence of LVNC, a rare congenital cardiomyopathy. Metabolic evaluations, complementation studies, and mutation analysis confirmed the diagnosis of *cblC* disease. These findings highlight an intrauterine cardiac phenotype that can be displayed in *cbl*C disease in association with nonimmune hydrops.

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P. Tanpaiboon · U. Lichter-Konecki · D. Zand
Division of Genetics and Metabolism, Children's National Medical Center, Washington, DC 0010, USA

J.L. Sloan · C.P. Venditti (✉)
Organic Acid Research Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Building 49, Room 4A18, Bethesda, MD 20892, USA
e-mail: venditti@mail.nih.gov

P.F. Callahan
Child Cardiology Associates, Fairfax, VA 22031, USA

D. McAreavey
Critical Care Medicine Department, National Institutes of Health (NIH), Bethesda, MD 20892, USA

P.S. Hart
Office of the Clinical Director, Medical Genetics Branch, National Human Genome Research Institute (NHGRI), National Institutes of Health (NIH), Bethesda, MD 20892, USA

Introduction

Cobalamin C disease (*cbl*C), a form of combined methylmalonic acidemia and hyperhomocysteinemia, may be the most common inborn error of intracellular cobalamin metabolism and is caused by mutations in *MMACHC* gene located on chromosome 1p34 (Lerner-Ellis et al. 2006). In the "early onset" form of *cbl*C, infants present with failure to thrive, developmental delay, visual impairment, and hematologic problems (Carrillo-Carrasco et al. 2011; Rosenblatt et al. 1997). Malformations including congenital microcephaly and congenital heart diseases are frequently present (Andersson et al. 1999). While the phenotypic spectrum of *cblC* disease is broad and age of onset is variable, encompassing prenatal and adult presentations, a prenatal cardiac presentation of right ventricular dilated cardiomyopathy in the third trimester has been documented in one infant with *cblC* disease (De Bie et al. 2009). Here, we describe a patient with *cblC* disease who presented with the combination of hydrops fetalis and left ventricular noncompaction (LVNC), review the previous reports of cardiomyopathy in *cbl*C disease, and highlight the need to consider the diagnosis of *cblC* disease in the setting of nonimmune hydrops fetalis, especially when cardiac disease is present in the fetus.

Case Report

Clinical investigations were conducted through NIH study 04-HG-0127 "Clinical and Basic Investigations of Methylmalonic Acidemia and Related Disorders" (clinicaltrials.gov identifier: NCT00078078) in compliance with the Helsinki Declaration. The patient was born to a healthy 35-year-old primigravida. A routine prenatal ultrasound at 12 weeks showed an increased nuchal translucency and subsequently chorionic villus sampling demonstrated a normal female karyotype, 46,XX. At 20 weeks, a prenatal ultrasound showed a thickened nuchal fold. Fetal echocardiography suggested possible tricuspid atresia and insufficiency with poor right ventricular function. Repeat evaluation at 24 weeks demonstrated questionable pulmonic stenosis and impaired biventricular function. Significant hydrops fetalis was also noted and the fetus was not expected to survive. At 30 weeks, a fetal echocardiogram revealed poor right ventricular function with preserved left ventricular systolic function. Deep trabeculations of both ventricles with a thickened septum were clearly visualized at the apex (Fig. 1a). Surprisingly, the hydrops nearly resolved by 35 weeks.

This infant was delivered at 38 weeks by scheduled Cesarean section. The birth weight was 2.72 kg (10th percentile), birth length was 49.3 cm (50th percentile), and head circumference was 34 cm (50th percentile). Clinical heart failure manifested shortly after birth and required inotropic support including dopamine, dobutamine, and digoxin. An echocardiogram on the first day of life demonstrated bilateral poor ventricular function, hypertrabeculation of the left ventricle, perfusion of trabecular recesses by Doppler color flow, and > 2:1 ratio of compacted to noncompacted myocardium, consistent with left ventricular noncompaction (LVNC). A screen for metabolic disorders was conducted and revealed methylmalonic aciduria (urine methylmalonic acid (MMA) 330 μmol/mmol creatinine; normal< 5.1), hyperhomocysteinemia (total homocysteine (tHcy) 180 μmol/L; normal 5–13), and hypomethioninemia (methionine 6 μmol/L; normal 8–51) consistent with a diagnosis of an intracellular vitamin B12 disorder. Hydroxycobalamin, betaine, carnitine, folate, and aspirin were administered and a reduced protein diet was prescribed. After treatment, the levels of urine MMA and plasma tHcy decreased significantly (urine MMA 165 μmol/mmol; creatinine and tHcy 59.2 μmol/L), and plasma methionine increased to the normal range (22 μmol/L). The diagnosis of *cbl*C was established by cellular biochemical studies in the laboratory of Dr. David Rosenblatt (McGill University, Montreal, Canada). Her fibroblasts demonstrated diminished incorporation of propionate and methyl-THF, diminished synthesis of adenosyl- and methylcobalamin, and failed to complement cells of the *cbl*C class.

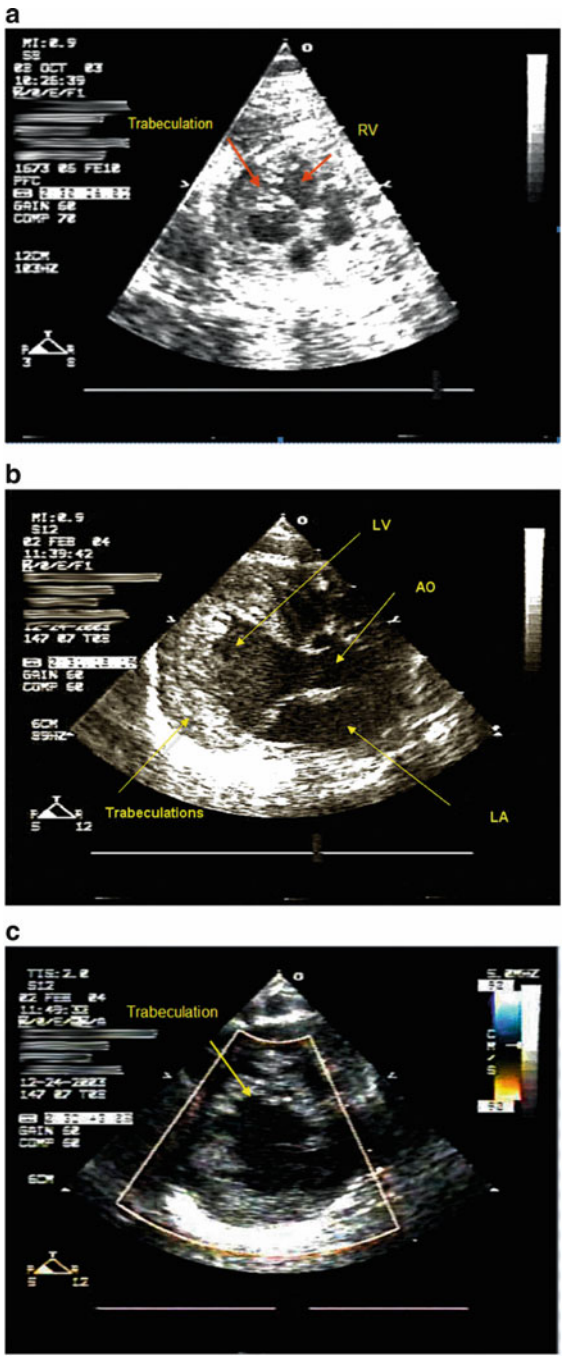

Fig. 1 Selected echocardiographic images. (**a**) Fetal echocardiography at 30 weeks (parasternal short axis view) showed hypertrabeculation (*arrows*) of both ventricles. (**b**) Echocardiography at 1 month (parasternal long axis view) revealed a globular apex. (**c**) Echocardiography at 1 month (parasternal short axis view) shows multiple deep trabeculations at the apex of the left ventricle

Sequence analysis of the *MMACHC* gene detected a homozygous mutation, c.271dupA. To rule out the possibility that uniparental disomy (UPD) at the *MMACHC* locus was present in the patient, 21 markers spaced along chromosome 1 that mapped 8.3 Mb telomeric and

1.0 Mb centromeric to the *MMACHC* gene were analyzed. The patient was heterozygous at 19 informative markers indicating there was no evidence of UPD or microdeletion around the *MMACHC* locus (data not presented). Other postnatal genetic investigations included a high-resolution karyotype and subtelomere FISH studies, both of which were normal.

At 1 month of age, an echocardiogram demonstrated poor left ventricular (LV) function, with estimated ejection fraction (LVEF; 43%) and decreased LV fractional shortening (LVFS; 20%). Noncompaction was noted at the apex of the LV (Fig.1b, c). At 8 months, LV function had improved (70% EF and 40% LVFS), but noncompaction was still evident at the apex of the LV. At 30 months of age, ventricular function had stabilized and the cardiac morphology, except for a mild globular appearance of the left ventricle, appeared normal. Digoxin therapy had been initiated in early life and was continued.

Other clinical findings consistent with *cbl*C deficiency, such as feeding difficulties, macular changes, and developmental delay were present. Both parents had normal echocardiograms.

Discussion

Cardiac manifestations of *cbl*C disease have been described and include several congenital abnormalities (Andersson et al. 1999; Martinelli et al. 2010; Profitlich et al. 2009), cardiomyopathy (Martinelli et al. 2010; Profitlich et al. 2009), and endocardial fibrosis (Baumgartner et al. 1979; Geraghty et al. 1992; McCully 1969). At least 15 patients with *cbl*C have been reported with cardiomyopathy (Table 1; Baumgartner et al. 1979; Brandstetter et al. 1990; Carmel et al. 1980; Chenel et al. 1993; De Bie et al. 2009; Longo et al. 2005; Geraghty et al. 1992; Ogier de Baulny et al. 1998; Profitlich et al. 2009). However, the specifics regarding the type and echocardiographic manifestations of the cardiac lesions have not been fully delineated. Only three out of fifteen previously reported patients were diagnosed with LVNC (Profitlich et al. 2009); one additional patient displayed prominent left ventricular apical trabeculation but did not meet the criteria of noncompaction cardiomyopathy (Profitlich et al. 2009). Although an association with the underlying disorder has been assumed, none of the previously reported patients were evaluated for familial and genetic causes of noncompaction and, except for this report, parental echocardiograms were not performed. Biochemical and molecular genetic data (Table 2) have not uniformly been documented, although in several instances, the patients have had mutations and a clinical course consistent with "early onset" disease (Rosenblatt et al. 1997). Cobalamin C disease has also been documented in a single patient who presented with dilated cardiomyopathy, diminished systolic function, hypocontractility of the left ventricle, and intrauterine growth retardation (De Bie et al. 2009). Left ventricular noncompaction (LVNC) was not described in this infant. It therefore remains to be determined whether LVNC is present in all *cblC* patients, if there is a correlation with *MMACHC* genotype(s), an association with the magnitude of metabolite elevations and/or the age of diagnosis or treatment.

Left ventricular noncompaction is a genetically heterogeneous condition that occurs because of failure of normal myocardial maturation during cardiac embryogenesis (Chin et al. 1990; Ichida 2009) and results in prominent trabeculations, particularly in the left ventricle (Chin et al. 1990; Ichida. 2009). LVNC can occur in patients with malformation syndromes, including velocardiofacial syndrome (Madan et al. 2010), Sotos syndrome (Martinez et al. 2011), as well as states of aneuploidy and mosaicism (Beken et al. 2011; McMahon et al. 2005; Sellars et al. 2011; Wang et al. 2007). Furthermore, LVNC has been reported in association with several distinct inborn errors of metabolism (IEM) including Barth syndrome (Bleyl et al. 1997); Pompe disease (Finsterer et al. 2006); Fabry disease (Stöllberger et al. 2003); cobalamin C disease (Profitlich et al. 2009); numerous mitochondrial disorders including Leber's hereditary optic neuropathy (LHON) (Finsterer et al. 2002); mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke (MELAS) syndrome as well as less well-defined mitochondriopathies (Finsterer et al. 2006; Stöllberger et al. 1999). In at least one case, isolated LVNC has manifested as fetal hydrops (Richards et al. 2009).

There are several genes associated with isolated LVNC including G4.5 (*TAZ*), dystrobrevin (*DTNA*), LIM domain-binding protein 3 (*Cypher/ZASP: LDB3*), Lamin A/C (*LMNA*), *SCN5A*, and sarcomere proteins (β-myosin heavy chain, α-cardiac actin (*ACTC*), cardiac troponin T [*TNNT2*], tropomyosin 1 [*TPM1*]) (Xing et al. 2006; Ichida 2009; Chang et al. 2011). These gene products have functions related to cytoskeletal and/or mitochondrial function (Tang et al. 2010). Another six loci located on chromosomes 1p36 (Thienpont et al. 2007), 1q43 (Kanemoto et al. 2006), 5q35 (Pauli et al. 1999), 8p23 (Blinder et al. 2011), and 11p15 (Sasse-Klaassen et al. 2004) also have been linked to the phenotype of nonsyndromic LVNC. While a number of genes and candidate loci have been reported with LVNC, only a minority of affecteds have been found to harbor causative mutations (Finsterer et al. 2004; Ichida 2009; Tang et al. 2010; Xing et al. 2006). Although we and others have not able to rule out other genetic causes of LVNC in manifesting patients, that fact that other *cblC* patients have been diagnosed with LVNC (Profitlich et al. 2009) and that the parents were unaffected in the case reported here provides some support for an association between *cblC* disease and LVNC.

Table 1 Clinical features of congestive heart failure and cardiomyopathy in *cblC* disease

Reference	Number of patients	Cardiac manifestations	Echocardiography	Pathology
Baumgartner et al. 1979	1	Congestive heart failure	N/A	Endocardial fibrosis, intima, and inner elastic membrane destruction of the coronary arteries
Carmel el al. 1980	1	Congestive heart failure	Cardiomyopathy	N/A
Brandstetter et al. 1990	1	Congestive heart failure	Right ventricular dilated cardiomyopathy	N/A
Geraghty et al. 1992	1	Congestive heart failure	N/A	Patchy endocardial fibrosis, intimal proliferation in the coronary arteries
Chenel et al. 1993	1	Congestive heart failure	Cardiomegaly, hypertrophic ventricular septum	N/A
Ogier de Baulny et al. 1998	3	Cardiomyopathy	N/A	N/A
Longo et al. 2005	2	Cardiomyopathy	N/A	N/A
Profitlich et al. 2009	4	Asymptomatic with low LV shortening fraction	Left ventricular noncompaction ($n = 3$), prominent left ventricular trabeculation ($n = 1$)	N/A
De Bie et al. 2009	1	Symmetrical intrauterine growth retardation	Thickened right ventricle, dilated cardiomyopathy, and small perimembranous ventricular septal defect	N/A
This report	1	Hydrops fetalis	Left ventricular noncompaction	N/A

Table 2 Enzymatic, biochemical, and molecular aspects of *cblC* patients with congestive heart failure and cardiomyopathy

Reference	Number of patients	Cellular biochemistry/ molecular genetics	Plasma total homocysteine (tHcy)(μmol/L; normal = 3–9.8)	Homocysteine plasma (μmol/L; normal = 0) (P) or urine(U) (mmol/mol Cr; normal = 0)	Plasma methionine (μmol/L; normal = 8–51)	Urine MMA (mmol/mol Cr; normal < 10)
Baumgartner et al. 1979	1	–	N/A	P = present U = high	Low	High
Carmel et al. 1980	1	+	N/A	P = present U = 151.7	7	4,751
Brandstetter et al. 1990	1	+	N/A	P = present U = 3.1	3	1,705
Geraghty et al. 1992	1	-	N/A	P = 37 U = N/A	0	1,187
Chenel et al. 1993	1	+	N/A	P=25 U=1,800	8	2,000
Ogier de Baulny et al. 1998	3	–	N/A	P = N/A U = N/A	N/A	N/A
Longo et al. 2005	2	+	71	P = N/A U = N/A	N/A	285
		+	52	P = N/A U = N/A	N/A	170
Profitlich et al. 2009	4	271dupA/271dupA	95	P = N/A U = N/A	N/A	266
		271dupA/271dupA	107	P = N/A U = N/A	N/A	196
		547_8delGT/ 285dupA	64	P = N/A U = N/A	N/A	31
		608G>A/608G>A	42	P = N/A U = N/A	N/A	20
De Bie et al. 2009	1	271dupA/271dupA	236 or 45	P = 66 U = N/A	5	High
This report	1	271dupA/271dupA	55	P = 2 U = N/A	42	250

The presence of LVNC in utero with cardiac dysfunction in the propositus is consistent with a model in which the MMACHC gene product may play a role in cardiac development. In a recent study, *Mmachc* mRNA was present and highly expressed in the bulbus cordis and endocardial cushion at E11 in mice (Pupavac et al. 2011). The role of MMACHC in development is unknown but given that MMADHC, a related enzyme in the pathway and a MMACHC binding partner (Plesa et al. 2011), was not expressed in the same temporal/spatial pattern may indicate a unique role for MMACHC in cardiac physiology and/or development (Pupavac et al. 2011). Other factors related to the biology of MMACHC may be contributory: MMACHC can localize to mitochondria (Pagliarini et al. 2008) and *cblC* patients and their fibroblasts have increased markers of oxidative stress suggesting underlying mitochondrial dysfunction (Richard et al. 2009; Mc Guire et al. 2009). In addition, patient-derived cells display global dysregulation of cytoskeletal proteins, such as actin, lamin A/C, and collagen VI (Hannibal et al. 2011). Therefore, it seems reasonable that MMACHC deficiency could result in developmental cardiac defects, possibly by inducing secondary cellular ultrastructural changes and/or mitochondrial dysfunction. This hypothesis is consistent with the observation that genes responsible for LVNC have functions associated with cytoskeletal and/or mitochondrial metabolism, a finding that perhaps could be investigated in experimental animal models of *cblC* disease.

To our knowledge, this is the first case of LVNC presenting prenatally in a patient with *cblC* disease. The fact that a number of other *cblC* patients have also been reported to have cardiomyopathy (Table 1) extends the hypothesis of a disease association between *cblC* and cardiac pathology (McCully 1969; Profitlich et al. 2009) and highlights the importance of assessing all patients with *cblC* for cardiac disease. The findings in the patient reported here also emphasize that a metabolic evaluation for *cblC* should be considered in the setting of nonimmune hydrops fetalis, especially if fetal cardiomyopathy is present.

Synopsis

A case report of prenatal onset left ventricular noncompaction and hydrops in cobalamin C disease.

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CASE REPORT

Primary Carnitine (OCTN2) Deficiency Without Neonatal Carnitine Deficiency

L. de Boer · L.A.J. Kluijtmans · E. Morava

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Abstract Although the diagnosis of a primary carnitine deficiency is usually based on a very low level of free and total carnitine (free carnitine: 1–5 μM, normal 20–55 μM) (Longo et al. 2006), we detected a patient via newborn screening with a total carnitine level 67 % of the normal value. At the age of 1 year, after interruption of carnitine supplementation for a 4-week period the carnitine profile was assessed and the free carnitine level had dropped to 10.4 μmol/l (normal: 20–55 μM) and total carnitine level had dropped to 12.7 μmol/l (normal: 25–65 μM). Transient carnitine deficiency was not likely anymore and DNA mutation analysis of the OCTN2 (SLC22A5) gene showed a homozygous c.136C>T (p.P46S) mutation, confirming the diagnosis of primary carnitine deficiency. We would like to emphasize that neonates with a primary carnitine deficiency might present with relatively high levels of total carnitine due to placental carnitine transfer, and also draw the attention to the importance of regular follow-up and the significance of genetic diagnostics in patients with a nonclassical presentation.

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L. de Boer · E. Morava
Department of Pediatrics, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

L.A.J. Kluijtmans
Department of Laboratory Medicine, Laboratory of Genetic, Endocrine and Metabolic Diseases, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

L. de Boer (✉)
PO Box 9101, Nijmegen, The Netherlands
e-mail: ldeboer@kpnplanet.nl

Introduction

Although the diagnosis of a primary carnitine deficiency is usually based on a very low level of free and total carnitine (free carnitine: 1–5 μM, normal 20–55 μM) (Longo et al. 2006), we detected a patient via newborn screening with a total carnitine level 67 % of the normal value.

Primary carnitine deficiency is an autosomal recessive disease caused by a defect in OCTN2 (OMIM 212140), a carnitine transporter, due to various mutations of the *SLC22A5* gene. OCTN2 is expressed in muscle, heart, kidney, lymphoblasts, and fibroblasts. *SLC22A5* mutations result in impaired fatty acid oxidation in skeletal and heart muscle (Longo et al. 2006). In addition, renal wasting of carnitine results in low serum levels, usually with free carnitine levels between 1 and 5 μM. The disease can present in infancy with acute hypoglycemic episodes or in childhood with cardiomyopathy and muscle weakness. Carnitine supplementation can prevent these episodes. Usually total carnitine levels are < 2.5–10 % of the normal value and in urine elevated levels of free carnitine are detected (Longo et al. 2006).

A male neonate was referred to our hospital with a suspicion on primary carnitine deficiency because of a positive newborn screening (NBS). The free carnitine level (C0) in the NBS bloodspot was 3.9 μmol/l (age: 7 days). The boy, born at term with a birth weight 3,590 g, was in a good clinical condition and showed no signs of muscle weakness. Confirmatory testing at the age of 11 days in our laboratory showed a free carnitine of 12.4 μmol/l (normal 20–55 μmol/l) and a total carnitine concentration of 16.8 μmol/l (normal 25–65 μmol/l). Plasma carnitine levels in the mother were normal (free: 25.3 μM, total: 29.6 μM). Because of a relatively high carnitine level for the suspected diagnosis of primary carnitine deficiency, the

possibility of transient carnitine deficiency was also considered in the differential diagnosis, and a carnitine supplementation of 50 mg/kg was initiated. The dose was adjusted to 30 mg/kg upon controlling carnitine levels after 6 months of therapy. On supplementation, total carnitine values remained between 50.3 μmol/l and 63.3 in the first year of life and the child showed no clinical symptoms. At the age of 1 year, after interruption of carnitine supplementation for a 4-week period the carnitine profile was assessed again. Unexpectedly, the free carnitine level had dropped to 10.4 μmol/l (normal: 20–55 μM) and total carnitine level had dropped to 12.7 μmol/l (normal: 25–65 μM). A urine sample showed elevated excretion of free carnitine (50.0 μmol/mmol kreatinine; normal < 35 μmol/mmol). Supplementation of L-carnitine was resumed. A cardiac ultrasound showed no abnormalities. DNA mutation analysis of the *OCTN2* (*SLC22A5*) gene showed a homozygous c.136C>T (p.P46S) mutation in the patient and heterozygous state in both the parents, confirming the genetic diagnosis of a primary carnitine deficiency.

The male patient is now 4 years old and on continuous supplementation of L-carnitine, 60 mg/kg; the plasma carnitine levels, both free and total, remain in the normal range. Clinically, the patient showed a transient motor developmental delay with starting to walk at 20 months, but his development is now adequate for his age. He has a mildly decreased muscle strength and has a history of muscle pain, which has resolved spontaneously. Temporarily increasing the carnitine dose to 100 mg/kg had no effect on these complaints. On the contrary, he started to complain about side effects of the carnitine, including diarrhea and intestinal discomfort. At follow-up, his cardiac function has always been normal.

To our knowledge, the *SLC22A5* c. 136 C>T mutation has not been described before in homozygous state. It has been described as a heterozygous mutation in asymptomatic mothers of patients. Although these carrier individuals showed a decreased carnitine transport, it was higher than symptomatic patients (Rose et al. 2012). It has been also described in a patient with severe decreased carnitine transport in fibroblasts (Schimmenti et al. 2007), in combination with a null mutation, c. 844C>T, p.R282X, identified before as a pathogenic mutation (Wang et al. 1999). In a study on glycosylation of the OCTN2 carnitine transporter (Filippo et al. 2011), it is postulated that the p. P46S mutation retains residual transport activity and might be responsible for a milder or no overt phenotype. This could explain the relatively high carnitine level detected in our patient.

In summary, we would like to emphasize that neonates with a primary carnitine deficiency might present with relatively high levels of total carnitine due to placental carnitine transfer, and also draw the attention to the importance of regular follow-up and the significance of genetic diagnostics in patients with a nonclassical presentation.

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CASE REPORT

MNGIE Syndrome: Liver Cirrhosis Should Be Ruled Out Prior to Bone Marrow Transplantation

Armin Finkenstedt • Melanie Schranz • Sylvia Bösch • Daniela Karall • Sabine Scholl Bürgi • Christian Ensinger • Mathias Drach • Johannes A. Mayr • Andreas R Janecke • Wolfgang Vogel • David Nachbaur • Heinz Zoller

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Abstract Mitochondrial neurogastrointestinal encephalopathy (MNGIE) is an autosomal recessive mitochondriopathy caused by loss-of-function mutations in the thymidine phosphorylase gene. The disease leads to premature death and is characterized by gastrointestinal dysmotility and cachexia, external ophthalmoplegia, a sensorimotor neuropathy, and leukoencephalopathy. Bone marrow transplantation (BMT) is the only potentially curative treatment that can achieve a sustained biochemical correction of the metabolic imbalances.

We report a 23-year-old male homozygous for the c.866A > C, p.Glu289Ala mutation of the TYMP gene, who presented with fatty liver and cachexia. Laboratory examinations were unremarkable except for increased transaminase activities. Grade II fibrosis and steatosis was found in an initial and a follow-up liver biopsy 4 years later. Myeloablative conditioning and BMT was performed 10 years after initial presentation due to the progressive weight loss and polyneuropathy. Pre-transplant liver staging was normal except for an elevated transient elastography of 31.6 kPa. Severe ascites developed after transplantation and liver function deteriorated progressively to liver failure. Despite engraftment on day +15, the patient died on day +18 from liver failure. Autopsy revealed micronodular liver cirrhosis, and postmortem diagnosis of acute-on-chronic liver failure was done.

This case illustrates the difficulties and importance of diagnosing liver cirrhosis in MNGIE. Before BMT, patients must be carefully evaluated by transient elastography, liver biopsy, or assessment of hepatic venous pressure gradient. In patients with liver cirrhosis, further studies should evaluate if liver transplantation may be an alternative to BMT. Considerable amounts of thymidine phosphorylase are expressed in liver tissue which may prevent accumulation of toxic metabolites.

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A. Finkenstedt · M. Schranz · W. Vogel · H. Zoller (✉)
Department of Medicine II Gastroenterology and Hepatology, Medical University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria
e-mail: heinz.zoller@i-med.ac.at

S. Bösch
Department of Neurology, Medical University of Innsbruck, Innsbruck, Austria

D. Karall · S.S. Bürgi
Department of Pediatrics I, Medical University of Innsbruck, Innsbruck, Austria

C. Ensinger · M. Drach
Department of Pathology, Medical University of Innsbruck, Innsbruck, Austria

J.A. Mayr
Department of Pediatrics, Paracelsus Medical University, Salzburg, Austria

A.R. Janecke
Division of Human Genetics, Molecular and Clinical Pharmacology, Medical University of Innsbruck, Innsbruck, Austria

D. Nachbaur
University Hospital for Internal Medicine V, Hematology and Oncology, Medical University, Innsbruck, Austria

Introduction

Mitochondrial neurogastrointestinal encephalopathy (MNGIE) is an autosomal recessive mitochondriopathy caused by loss-of-function mutations in the thymidine

phosphorylase gene TYMP. Imbalances in the mitochondrial deoxynucleoside 5'-triphosphate pools result in increased uracil incorporation into the mitochondrial DNA, producing mitochondrial DNA instability and accumulation of mutations. Clinically, the disease is characterized by gastrointestinal dysmotility and cachexia, ptosis, external ophthalmoplegia, and a sensorimotor neuropathy. A leukoencephalopathy is observed in brain magnetic resonance imaging (MRI) (Hirano et al. 2004). Symptoms usually begin between the first and fifth decade of life with a progressive course leading to premature death (Nishino et al. 2000). Bone marrow transplantation (BMT) or allogeneic hematopoietic stem-cell transplantation (HSCT) is the only potentially curative treatment that can achieve genetic correction of the defect in hematopoietic cells and a sustained biochemical correction of the metabolic imbalances (Hirano et al. 2006). To date, HSCT for MNGIE has been reported in 11 patients with varying outcome (Halter et al. 2011; Filosto et al. 2012). A standardized approach to HSCT has been proposed recently to optimize the therapy for patients with MNGIE (Halter et al. 2011). We report a lethal outcome due to acute-on-chronic liver failure from myeloablative conditioning in a patient with MNGIE complicated by liver cirrhosis undiagnosed prior to BMT.

The patient was a 23-year-old male, who presented with fatty liver and cachexia (body mass index 19 kg/m^2). In his family history, two sisters had died at the age of 28 and 30 years, respectively, with a diagnosis of "anorexia nervosa with secondary leukodystrophia." Laboratory examinations were unremarkable except for increased transaminase activities. The liver biopsy showed grade II fibrosis (Metavir) and 70 % steatosis (Fig. 1a). Leukoencephalopathy was found on MRI of the brain. During the next 4 years, the patient developed diarrhea and lost further 10 % of his body weight. A follow-up liver biopsy showed stable steatosis and fibrosis (grade I-II). The patient then presented with ptosis, external ophthalmoplegia, and sensorimotor neuropathy. Based on the family history and clinical presentation, MNGIE syndrome was considered and the diagnosis confirmed by an increased urinary 2-deoxyuridine concentration and homozygosity for the pathogenic c.866A > C, p.Glu289Ala mutation of the thymidine phosphorylase gene in the index patient and an older symptomatic sister. This mutation is known to be restricted to European patients where it is associated with a clinical presentation similar to that of our patient. The prevalence of liver disease has been reported to be < 10 % in MNGIE patients, where exact data in patients homozygous for p.Glu289Ala are lacking. (Garone et al. 2011).

In the light of the progressive course of the disease and the family history, BMT was discussed and offered to the patient as a treatment option. Pre-transplant liver staging included a complete clinical-biochemical and serological evaluation of common metabolic, viral, and immune liver diseases; computed tomography; and ultrasound. Except for transaminase activities lower than twice the upper limit of normal and an elevated transient elastography of 31.6 kPa, all other liver investigations were normal. Ten years after initial presentation, a 28-year-old female 1/12 HLA-antigen-mismatched donor (HLA-C-allele) had been identified and myeloablative conditioning was performed with busulphan 3.2 mg/kg bodyweight in divided doses (day −7 to day −4; total dose 12.8 mg/kg), cyclophosphamide 60 mg/kg bodyweight once daily (day −3 and −2; total dose 120 mg/kg), and anti-thymocyte globulin 2.5 mg/kg bodyweight (day −3 to day −1; total dose 7.5 mg/kg). Standard cyclosporine and methotrexate were given for prophylaxis

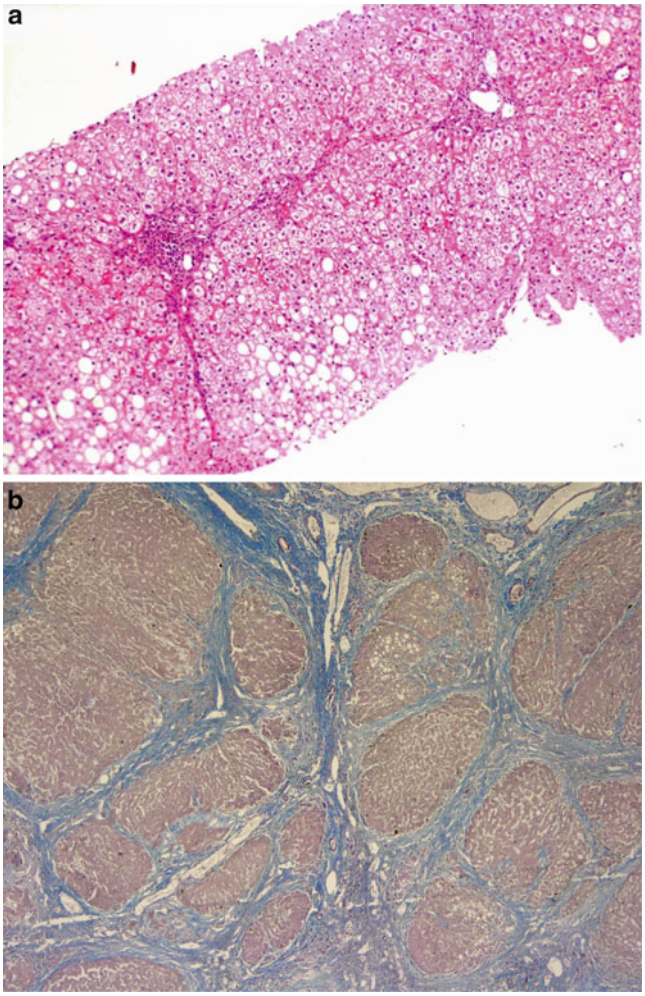

Fig. 1 **Panel a**: Liver biopsy at the time of first presentation 10 years prior to BMT shows a normal liver architecture with fibrosis grade II and 70 % steatosis (hematoxylin and eosin stain). **Panel b**: Postmortem liver histology shows bridging fibrosis and micronodular cirrhosis (chromotrop-anilinblue staining). Pictures were captured using an Olympus BH2 microscope (Olympus, Vienna, Austria) with a Jenoptik Progress C12 digital camera and Progress Capture Pro 2.5 software (Jenoptik, Jena, Germany). Panel a 100x, panel b 40x

against graft-versus-host disease according to the Seattle Protocol (Storb et al. 1989). On day 0, 2.69 x 10^8 nucleated bone marrow cells/kg bodyweight were transplanted. During the next days, the patient developed a progressive paralytic ileus and severe ascites. This was complicated by pneumonia. Despite treatment with antibiotics and steroids, the patient developed an acute respiratory distress syndrome and was mechanically ventilated from day +10 onward. Hemofiltration was started on day +11 due to renal failure. Liver function deteriorated progressively and the patient developed liver failure with intractable lactic acidosis. Despite engraftment on day +15, the patient died on day +18 from multi-organ failure secondary to acute-on-chronic liver failure. Autopsy revealed advanced micronodular liver cirrhosis macroscopically, which was confirmed by post-mortem histology (Fig. 1b). Acute deterioration of liver function after BMT could have been due to vascular liver diseases such as sinusoidal obstruction, or Budd Chiari Syndrome. Alternative causes include acute infection with hepatitis viruses and non-hepatotrophic viruses such as EBV or CMV in the immunocompromised host, all of which were excluded after liver failure was recognized. Hepatotoxic myeloablative conditioning may have been the precipitating event of acute-on-chronic liver failure, although a relation with the infectious complication cannot be excluded.

The present case illustrates the difficulties in detecting advanced-stage liver disease in MNGIE. Liver cirrhosis, which is a rare complication of MNGIE and probably caused by accumulation of toxic intermediates (Shoffner 2011), was excluded by liver biopsy 7 years prior to BMT and remained undetectable upon imaging studies immediately prior to the intervention. The only pre-transplant test suggestive of cirrhosis was an increased transient elastography, but lacking other findings compatible with cirrhosis, a liver stiffness of >30 kPa was not attributed to advanced fibrosis. The presence of cirrhosis was only identified during autopsy. This is in accord with the emerging concept that liver disease staging requires multidimensional assessment by noninvasive and invasive tests (Auberger et al. 2012). The progressive natural course advocates careful evaluation including transient elastography, liver biopsy, or assessment of hepatic venous pressure gradient.

Diagnosis of advanced-stage liver disease will determine the management of patients with MNGIE, and in particular affects the decision of whether BMT/HSCT should be carried out. Considering the hepatotoxicity of drugs required for myeloablative conditioning, the risk for severe complications or liver-related death is significantly increased in patients with liver cirrhosis. Furthermore, clinically relevant mitochondrial toxicity of myeloablative conditioning has been described. Despite the limited experience with stem cell transplantation for MNGIE, conditioning with busulphan and fludarabine seems favorable (Halter et al. 2011). In liver disease patients, even the risk of reduced intensity conditioning might be unacceptably high. Based on the experience from patients with hepatic amyloidosis where liver transplantation has been performed prior to BMT, such an approach could be adopted for MNGIE patients (Kumar et al. 2002). If such a sequential approach is considered, it should be carefully evaluated if liver transplantation alone would also prevent further accumulation of toxic metabolites as considerable amounts of thymidine phosphorylase are expressed in liver tissue (Zimmerman and Seidenberg 1964). If this were the case, subsequent BMT after liver transplantation might no longer be necessary.

In conclusion, despite significant progress in diagnosis and treatment of MNGIE syndrome, the disease remains a challenge. The presence of advanced-stage liver disease should be excluded prior to BMT/HSCT using noninvasive and invasive tests and liver transplantation should be evaluated as an alternative treatment option in selected patients.

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RESEARCH REPORT

Differential Phonological Awareness Skills in Children with Classic Galactosemia: A Descriptive Study of Four Cases

Fiona M. Lewis · David J. Coman · Maryann Syrmis · Sarah Kilcoyne · Bruce E. Murdoch

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Abstract Educational achievement, which for individuals with the metabolic disorder classic galactosemia (GAL) is significantly lower than in the wider population, correlates with self-reported quality of life. Phonological awareness skills underpin the development of literacy, and although literacy is a key contributor to successful academic outcomes, no study to date has investigated phonological awareness skills in children with GAL. This study investigated phonological awareness (PA) in four school-aged children with the disorder, two of whom were siblings. Age range for the children was 7 years 7 months to 9 years 2 months. Each child was assessed with the Phonological Awareness criterion-referenced subtest from the Clinical Evaluation of Language Fundamentals-Fourth Edition. Included in the data for analysis was each child's performance measures obtained from their most recent assessment of cognitive and lexical development. A number of descriptive analyses were undertaken on the data. One child, who met her age criterion for PA, had cognitive and lexical development skills in the average range. The remaining three children failed to meet their age criteria. Although these three children presented with clinically similar cognitive and lexical development skills, disparate PA skills were identified. The PA skills of one of the sibling pair were notably more advanced than his older sibling. The limitations of relying on behavioural test results in children with GAL to predict those most at risk of reduced skill development are discussed in terms future research directions.

Abbreviations

CELF-4	Clinical Evaluation of Language Fundamentals-Fourth Edition
EVT-2	Expressive Vocabulary Test-Second Edition
GAL	Galactosemia
PA	Phonological awareness
PPVT-4	Peabody Picture Vocabulary Test-Fourth Edition
SES	Socio-economic status
STM	Short-term memory

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F.M. Lewis (✉) · B.E. Murdoch
Centre for Neurogenic Communication Disorders Research, School of Health and Rehabilitation Sciences, The University of Queensland, 4072, Brisbane, Australia
e-mail: f.lewis@uq.edu.au

D.J. Coman
Department of Metabolic Medicine, The Royal Children's Hospital, Brisbane, Australia

D.J. Coman
School of Medicine, The University of Queensland, Brisbane, Australia

M. Syrmis · S. Kilcoyne
Department of Speech Pathology, The Royal Children's Hospital, Brisbane, Australia

Introduction

One of the most important skills that a child learns when they start school is how to read (McGeown et al. 2012). Literacy skills are a key contributor to successful academic outcomes (Snow and Beals 2006) and are important functional skills in today's technologically advanced society (Organization for Economic Cooperation and Development 2000). One skill critical to the development of literacy is phonological awareness (Gillon 2004; Ziegler and Goswami 2005; Neuman and Dickinson 2010; Apel et al. 2012), or

the awareness of the sound structure of a spoken word (Gillon 2004).

Phonological awareness (PA) is knowledge that a spoken word is composed of syllables and phonemes (Kovelman et al. 2012) and involves the explicit and deliberate manipulation of components of speech (Castles and Coltheart 2004). PA skills encompass auditory discrimination of familiar sounds, similar sounds, rhyming words, and sounds of letters (Morrow 2007), segmentation of words into individual phonemes, the blending of phonemes into words, and manipulation of the sound structure of words, such as deleting sounds from words and stating what remains (Apel et al. 2012). Growth in PA can be attributed to a number of factors, including age, lexical development, cognitive abilities, and socio-economic status (Lonigan et al. 1998; Nation and Snowling 2004; Lemons and Fuchs 2010).

Based on the recognition that PA precedes and predicts successful transition from language to literacy (Kovelman et al. 2012), investigations of PA skill development have been undertaken in a range of potentially-at-risk paediatric populations including cerebral palsy (Vandervelden and Siegel, 1999), vision impairment (Dodd and Conn 2000), Down syndrome (Hulme et al. 2012), hearing impairment (Briscoe et al. 2001), and following treatment for brain tumours (Docking et al. 2003). Further, proactive intervention programmes aimed at nurturing the maturation of PA (Morrow 2007) have been implemented with successful outcomes for at-risk populations such as children with Down syndrome (Lemons and Fuchs 2010), speech impairment (Al Otaiba et al. 2009), specific language impairment (Collet et al. 2012), minority language speakers (Vadasy and Sanders 2010), foetal alcohol spectrum disorders (Adnams et al. 2007), and emotionally disturbed children (Nelson et al. 2005).

Despite the interest across a broad range of paediatric presentations, no research to date has investigated PA skills in children with metabolic disorders. Classic galactosemia [GAL] is one of a number of metabolic disorders tested through newborn screening, a mass screening programme widely undertaken throughout the world. GAL is the result of an inborn deficiency of the enzyme *galactose-1-phosphate uridyltransferase* [*GALT*] (Bosch 2006). Approximately one child in 53,000 in the United States is born with the disorder (Ridel et al. 2005). Intervention for galactose toxicity is the implementation of a galactose-restricted diet (Hughes et al. 2009). Despite dietary restrictions, long-term complications, including developmental delays (Fridovich and Walter 2008), speech and language deficits (Waisbren et al. 1983; Waggoner et al. 1990; Nelson et al. 1991), and academic difficulties (Bosch et al. 2004) have been reported in children with GAL.

Heterogeneity of developmental abilities and attainment is evident across individuals with GAL (Doyle et al. 2010), but neither genetic nor biochemical measures predict or explain the heterogeneous outcomes for children (Ridel et al. 2005). Given the currently inexplicable outcomes for children with GAL, Bosch et al. (2004) have suggested that a focus of ongoing care should be aimed at supporting children to attain the best achievable quality of life (Bosch et al. 2004). Educational achievement, which for individuals with GAL is significantly lower than in the wider population, correlates with self-reported quality of life (Bosch et al. 2004). There are, thus, psychosocial benefits associated with facilitating academic achievement through the development of literacy skills to approximate as near to normal attainment as possible in children with GAL.

The purpose of this study was largely descriptive in order to provide preliminary data on PA skills in a small group of school-aged children with a diagnosis of GAL. Based on the heterogeneity of developmental achievement observed in children with GAL (Doyle et al. 2010), it was hypothesized that variable PA skill would be noted in the group of children.

Method

Participants

Recruitment of the participants was undertaken through a mass recruitment mail-out to all families with children monitored through the Metabolic Clinic at the Royal Children's Hospital, Brisbane, Australia, who met the study's inclusion criteria of school-aged children with a neonatal diagnosis of classic galactosemia as defined by *GALT* activity < 1.3 units/ml blood, the presence of two known pathogenic mutations in the GALT gene (Duarte variants of GAL were excluded from the study), and English as the language spoken in the family home. The parents of five children responded. One child withdrew from the research prior to assessment. Thus, four children with classic GAL participated in the research.

Child 1

WG, a female, was aged 7 years 7 months at the time of the assessment of PA skills. WG was born at term and breast feeding was initiated. WG presented during the neonatal period with decreasing levels of alertness, hepatic dysfunction, and coagulopathy. She was subsequently identified through newborn screening as having GAL. Dietary restrictions of galactose were initiated through the implementation

Table 1 Neonate's presentation and laboratory findings

Child ID	Gender	*GALT* genotype	Gal-1-P uridyltransferase activity reference range (0.26–0.52)	Age at diagnosis and clinical features at time of diagnosis	Initial treatment
WG	Fe	Q188R/Q188R	<0.01 U/g Hb	Day 5 from newborn screening Poor feeding, jaundice, coagulopathy	Intravenous fluids Fresh frozen plasma to correct coagulopathy Phototherapy Breast feeding ceases, soy formula introduced
BS	Fe	Q188R/Q188R	<0.01 U/g Hb	Day 5 from newborn screening Poor feeding, jaundice, coagulopathy	Intravenous fluids Fresh frozen plasma to correct coagulopathy Phototherapy Breast feeding ceases, soy formula introduced
FP	Fe	Q188R/Q188R	<0.01 U/g Hb	Day 5 from newborn screening Poor feeding, jaundice, coagulopathy	Intravenous fluids Fresh frozen plasma to correct coagulopathy Phototherapy Breast feeding ceases, soy formula introduced
MP	M	Q188R/Q188R	<0.01 U/g Hb	Day 5 from newborn screening	Soy formula introduced from birth as older sibling presented with GAL

GALT galactose-1-phosphate uridyltransferase, *GAL* galactosemia

Table 2 Biographical details of the children with GAL

Child ID	Occ Grp[a]	Age at assessment of phonological awareness	Most recent results from an assessment of cognitive development[b]			Most recent results from an assessment of lexical development[b]	
			VIQ (WPPSI-III)	PIQ	FSIQ	Rec Vocab (PPVT-4)	Exp Vocab (EVT-2)
WG	1	7; 7 years	119	112	118	111	107
MP	1	7; 8 years	77	59	67	75	80
FP	1	8; 5 years	61	61	60	67	71
BS	3	9; 2 years	72	78	73	70	70

GAL classic galactosemia; *Occ Grp* Jones' (2003) parental occupational grouping; *Fe* female; *M* male; age shown in years, months; *VIQ* verbal intelligence quotient; *PIQ* performance intelligence quotient; *FSIQ* full scale intelligence quotient; *WPPSI-III* Wechsler Preschool and Primary Scale of Intelligence-Third Edition; *Rec Vocab* receptive vocabulary; *PPVT-4* Peabody Picture Vocabulary Test-Fourth Edition; *Exp Vocab* expressive vocabulary; *EVT-2* Expressive Vocabulary Test-Second Edition

[a] Jones' Occupational Group 1 = senior management in large business organization, government administration and defence, and qualified professionals; Jones' Occupational Group 3 = tradesperson, clerks, and skilled office, sales and service staff

[b] Test mean = 100; test standard deviation = 15 for all test results provided

of a soy-based diet and neonatal symptoms subsided. Table 1 displays WG's neonatal experience and laboratory data. She remains on a galactose-restricted diet. Results from her most recent assessment of cognitive development [Wechsler Preschool and Primary Scale of Intelligence-Third Edition-Australian Standardised Edition] (Wechsler 2004)] and lexical development [Peabody Picture Vocabulary Test-Fourth Edition [PPVT-4] (Dunn and Dunn 2007); Expressive Vocabulary Test-Second Edition [EVT-2] (Williams 2007)] are shown in Table 2. Her performance scores indicate her cognitive and lexical development was in the high average to above average range for her chronological age.

Child 2

BS, a female, was 9 years 2 months at the time of her involvement in the research. BS was born at term and breast feeding was commenced. Once home, she experienced weight loss and unexplained jaundice. BS was readmitted to hospital with liver disease and failure to thrive. She was

Table 3 Performance by the children with GAL on the CELF-4 Phonological Awareness subtest

Phonological skill assessed	Child ID			
	WG	MP	FP	BS
Syllable blending	5	4	4	5
Rhyme detection	5	2	0	4
Initial phoneme identification	5	5	4	5
Two syllable deletion	5	3	2	4
Rhyme production	5	1	0	1
Syllable segmentation	5	1	0	5
Final phoneme identification	5	5	0	4
Sentence segmentation	4	2	#	3
Three syllable deletion	3	3		2
Phoneme blending	5	4		4
Final syllable deletion	2	2		2
Medial phoneme identification	5	2		2
Initial phoneme substitution	5	1		3
Phoneme segmentation	2	3		1
Initial phoneme deletion	4	2		3
Medial phoneme substitution	4	2		0
Final phoneme substitution	4	0		0
TOTAL SCORE	**73**	**42**	**10**	**48**
Age-criterion score	≥ 46	≥ 46	≥ 58	≥ 61

GAL classic galactosemia; *CELF-4* Clinical Evaluation of Language Fundamentals-Fourth Edition. Maximum score for each subtest = 5; maximum total score = 85

Test discontinuation rule = four incorrect responses on three consecutive item sets

in hospital when the results of newborn screening identified her as having GAL. Dietary restrictions were implemented, and she responded well both clinically and biochemically to the change in diet. BS's neonatal presentation and laboratory data are shown in Table 1. She continues on diet-implemented intervention. As shown in Table 2, BS's most recent assessment of cognitive (Wechsler 2005) and lexical development (Dunn and Dunn 2007; Williams 2007) indicated she had significant cognitive and lexical skill deficits.

Child 3

FP, a female, was 8 years 5 months at the time of her involvement in the study. She was born at term with a birth weight of 5470 grams. She was extremely sick in the neonatal period. Once identified as having GAL through newborn screening, dietary restrictions were implemented. Table 1 presents FP's neonatal experience and laboratory data. FP's diet continues to be galactose restricted. Recent assessment of FP's cognitive (Wechsler 2004) and lexical (Dunn and Dunn 2007; Williams 2007) development, as displayed in Table 2, indicated she had significant deficits in cognitive and lexical development.

Child 4

MP, a male, is a younger sibling of FP. He was aged 7 years 8 months at the time of his involvement in the study. He and his dizygotic twin brother were born at 33 weeks. MP's birth weight was 2300 grams. Due to the family history of GAL, intake was galactose restricted from birth for both infants. MP, but not his twin, was identified with GAL through newborn screening. Restrictions in MP's diet continued upon confirmation and he remains on diet-implemented treatment for GAL. MP's neonatal presentation and subsequent laboratory data is shown in Table 1. His most recent assessment of cognitive (Wechsler 2004) and lexical development (Dunn and Dunn 2007; Williams 2007) indicated he was performing in the low range cognitively and experiencing delays in receptive and expressive vocabulary skill development (refer to Table 2).

Measures

The Phonological Awareness criterion-referenced subtest from the Clinical Evaluation of Language Fundamentals-Fourth Edition [CELF-4] (Semel et al. 2003) was administered to the children. Criterion-referenced scores allow comparison of the child's performance to an objective and absolute standard (criterion) of performance. Criterion-referenced scores indicate the cut-off point considered typical performance for the child's chronological age. Raw scores obtained from measurement of skills not dependent upon learning but rather upon developmental maturity, such as PA, tend to deviate greatly from the normal distribution. Criterion-referenced scores, however, are not dependent upon an approximately normal distribution of raw scores (Semel et al. 2003). The child either meets the age-based criterion score or does not meet the criterion-based score.

Procedure

The research was given institutional and ethical approval from the Behavioural and Social Sciences Ethical Review Committee at the University of Queensland and the Royal Children's Hospital and Health Services District Ethics Committee. Each child provided a signed informed assent and the child's parent provided a signed informed consent prior to acceptance into this study. Each child was individually assessed at the University of Queensland or in their home.

Descriptive Analyses

A number of descriptive approaches were used to examine the performance of the four children. Firstly, to investigate PA skill development in the children with GAL, the age-criterion score as provided by the CELF-4 test manual (Semel et al. 2003) was used to determine which of the four children met their age criterion. For any child failing the age criterion for PA skill development, a percentage correct of the total required to meet the criterion was calculated. For example, if a child's Total Score on the CELF-4 Phonological Awareness subtest (Semel et al. 2003) was 26 and the age criterion for the child was 42, the child's percentage would be 62 %. A subjective figure of $\geq$ 90 % of the score required to meet an age criterion was chosen as indicative of a notable performance despite not meeting the age criterion. Secondly, to investigate the performance by the siblings MC and FC, a subjective figure of $\geq$25 % was chosen as indicative of a notable distinction between the siblings' Total Scores from the CELF-4 Phonological Awareness subtest. Thirdly, to investigate factors that may have influenced the children's performance on the CELF-4 Phonological Awareness subtest, the children's age-corrected standard scores on the assessments of cognitive (Wechsler 2004, 2005) and lexical development (Dunn and Dunn 2007; Williams 2007), as displayed in Table 1, were examined. A score difference $>$ 1.5 *SD* of the test mean was chosen to indicate a clinically significant difference between the individual children on each performance score provided by the tests.

Results

As shown in Table 3, one of the four children with GAL (Child WG) met the age criterion for PA skill. Of the three children failing their age criterion on PA skill development, MC's total score was 91 % of the score required to meet his age criterion, indicating a notable performance despite not meeting his age criterion ($\geq$ 90 % of the score required to meet an age criterion). FC's total score of 17 % of the score required to meet her age criterion and BS's total score of 79 % of the score required for her to meet her age criterion were not identified as notable performances ($<$90 % of the score required to meet an age criterion). In regard to the performance by the siblings, there was a notable distinction between the two siblings' PA skills, with MC outperforming his older sister FC ($\geq$25 % difference in total scores).

Based on a score difference of $>$ 1.5 *SD* of the test mean, there was a clinically significant difference between Child WG's performance scores reflecting cognitive and lexical development and the three other children with GAL, with Child WG outperforming the remaining three children on each test outcome measure. There were no clinically significant differences between the performance scores reflecting the cognitive and lexical development of MP, FP, or BS (all scores between the three children within 1.5 *SD* of each other).

Discussion

This study investigated PA skills in four children with the metabolic disorder GAL. Three of the four children failed to meet the age criterion for PA skill development. As predicted, there was marked heterogeneity noted between the individual children, a finding which offers further support for the variations in individual outcomes previously reported across a range of neurocognitive and academic measures in children with GAL (Doyle et al. 2010). Further, and in support of previous findings (Hughes et al. 2009), despite a similar profile on a number of pertinent indices, considerable variation in PA skill between the sibling pair was noted.

Child WG, with cognitive and lexical development skills in the average range, presented with age-appropriate PA skills. The three remaining children (Child MP, Child FP, and Child BS) all presented with cognitive and lexical development skills in the low range and all failed to meet the criterion expected for their age. Nonetheless, the skill of these three children differed considerably. Child MP's phonological awareness skills approximated his age criterion (91 % of score required), Child BS's performance placed her within 79 % of the score required for age appropriateness, while Child FP's performance indicated severely restricted PA skill.

Although growth in PA is influenced by age, lexical development, and cognitive abilities (Lonigan et al. 1998; Nation and Snowling 2004; Lemons and Fuchs 2010), no clear profile of PA skill associated with GAL was evident in the data from the three children failing to meet their respective age criteria. MP, who notably outperformed FP and BS, was the youngest of the three children. Lexical development of the three children was clinically similar as were their measures of cognitive skill. Socio-economic status (SES) likewise influences the growth of PA (Lonigan et al. 1998), and Jones' (2003) parental occupational codings were used in this study as an indicator of SES, and again there was no clear profile to predict performance. Child BS's parental occupational coding was lower than Child FP, yet BS was notably more skilled than FP on the PA tasks.

The inclusion in the study of the sibling pair with similar IQs and performance scores on measures of lexical development effectively controlled a number of the variables discussed above that are known to influence the

development of PA. Although FP's status as the older sibling would suggest her PA skills would be more developed than her sibling MP, MP's skills were notably more advanced than his older sister. The finding of vastly contrasting PA skills between siblings with GAL is consistent with previous research findings where intra-family variability has been reported (Lambert and Boneh 2004; Hughes et al. 2009; Coman et al. 2010, Doyle et al. 2010; Bosch 2011).

Siblings FP and MP experienced contrasting neonatal histories. Galactose was introduced to FP in the neonatal period. She subsequently presented with features of galactose intoxication including poor feeding lethargy and hepatic dysfunction and was eventually identified with GAL through newborn screening. In contrast, galactose restriction was implemented at birth for MP, thus avoiding the diverse system failure associated with the accumulation of galactose and its by-products in the blood and tissues (Fridovich and Walter 2008). Research to date suggests that differential developmental outcomes observed in the disorder are not related to conditions during the neonatal (e.g., age at diagnosis and restriction of galactose intake or severity of neonatal crisis) or postnatal period [e.g., adherence to diet] (Cleary et al. 1995; Bosch 2011). Rather, developmental outcome may be influenced by prenatal conditions (Fridovich and Walter 2008, Potter et al. 2008; Berry and Elsas 2011) such as harmful intrauterine levels of galactitol causing early but static neural injury (Ridel et al. 2005). Exposure to galactose in the neonatal period may magnify the galactose toxicity in individual children (Berry and Elsas 2011).

Harmful intrauterine conditions for both siblings, compounded by FP's galactose neurotoxicity in the neonatal period may account for the differential outcome for the siblings, but not the intact developmental outcomes for Child WG, who also experienced serious galactose toxicity in the neonatal period. Another theory of poor ongoing development in children with GAL, despite neonatal changes to diet, is a complication of dietary compliance itself. An over-restriction of galactose can potentially interfere with myelin development by secondary impairments in N-glycosylation (Hughes et al. 2009; Coman et al. 2010; Coss et al. 2010, 2012). Epigenetic effects may subsequently determine the variable developmental outcomes (Coman et al. 2010), such as those observed in this study.

Proactive interventions designed to facilitate the maturation of PA in at-risk children have shown to be efficacious with at-risk groups of children (Nelson et al. 2005; Adnams et al. 2007; Vadasy and Sanders 2010; Collet et al. 2012). With 75 % of the children with GAL investigated in this study presenting with diminished PA skills, a means of identifying which children with GAL require proactive support would be beneficial. The preliminary findings of this study failed to identify behavioural factors supporting the differential performance scores of the children with GAL. PA, however, can be viewed as a subset of implicit phonological processing skills such as verbal short-term memory (STM) and rapid automatized naming (Alloway et al. 2004; Melby-Lervåg et al. 2012). STM deficits have been described in individuals with GAL (Kaufman et al. 1995; Manis et al. 1997; Antshel et al. 2004) and individual STM skill may account for the performance outcomes observed in the three children with similar cognitive measures but with discrepant PA skills.

A theoretical framework of PA skill assessment has been proposed, whereby assessment should include not only detection, isolation, and manipulation of sub-word phonological segments at the syllabic, onset-rime, and/or phonemic level, as undertaken with the Phonological Awareness criterion-referenced subtest from the CELF-4 (Semel et al. 2003), but also conversion or the ability to use PA skills, such as, for instance, grapheme-phoneme conversion rules required for processing non-words (Dodd et al. 1996). The use of a more comprehensive PA assessment tool, such as the Queensland University Inventory of Literacy (Dodd et al. 1996), which provides a broad base of quantitative and qualitative data, may clarify the differential outcomes observed in this study.

The pathophysiology of neurodevelopmental impairments in GAL, including the PA deficits identified in the children in this study, remains unknown, but abnormalities associated with the disorder detected through brain imaging include cerebral atrophy, abnormal white matter signal, diffuse white matter abnormalities, and delayed myelination (Nelson et al. 1992; Kaufman et al. 1995; Wang et al. 2001). Kovelman et al. (2012), through functional magnetic resonance imaging (fMRI) during an auditory word rhyming task, identified the left dorsolateral prefrontal cortex as the neural correlate of PA and suggested that the site may play a critical role in the development of PA for spoken language. Advanced neurophysiological (e.g., electroencephalography) and imaging (fMRI, diffusion tensor imaging) studies that investigate neural activity related to cognitive function (see Timmers et al. 2011) are required to further elucidate the current unpredictable long-term complications of the disorder, for, as shown in this study, behavioural testing alone offers limited predictive value for identifying which children with GAL need proactive support.

Clinical Implications and Conclusion

This descriptive study of the PA skills of four children with GAL revealed widely disparate performance scores. Three of the four children presented with clinically similar cognitive and lexical development skills, but displayed

dissimilar PA skill. In the current absence of therapeutic strategies to prevent *GALT* deficiency (Bosch 2006) and the current restricted understanding of the cognitive pathophysiological processes associated with GAL (Timmers et al. 2011), the focus of ongoing care should be aimed at supporting children with GAL to attain the best achievable quality of life (Bosch et al. 2004). Due to marked variability noted in PA skills in the four children investigated in this study, specific individualized testing and support for skill development should be implemented for all children with the disorder (Kaufman et al. 1995) in order to optimize their literacy development.

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CASE REPORT

Dihydropteridine Reductase Deficiency and Treatment with Tetrahydrobiopterin: A Case Report

Curtis R. Coughlin II · Keith Hyland · Rebecca Randall · Can Ficicioglu

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Abstract Dihydropteridine reductase (DHPR) deficiency is a genetic disorder of tetrahydrobiopterin (BH4) regeneration and may present with hyperphenylalaninemia, microcephaly, hypotonia, mental retardation, and convulsions. BH4 is an essential cofactor for the hydroxylation of aromatic amino acids and a deficiency of BH4 results in decreased synthesis of dopamine and serotonin. We present a 27-month-old female patient with DHPR deficiency who was treated with L-dopa/carbidopa (2 mg/kg, four times per day), 5-hydroxytryptophan (2 mg/kg, four times per day), folinic acid (10 mg/day), and BH4 supplementation (20 mg/kg, twice a day). Although remarkable clinical improvement with normal plasma phenylalanine (Phe) levels and increased phenylalanine tolerance was noted 1 month after the treatment, CSF neurotransmitter metabolites did not improve. BH4 supplementation was increased to 40 mg/kg/day and the CSF study was repeated 1 month later. There was no significant change of CSF neurotransmitters, BH4 or BH2 levels but plasma Phe level was within normal range. Surprisingly, she had developmental improvement noted at 1-month and 3-month visits following an augmented neurotransmitter and BH4 treatment. She was able to pull herself to the standing position and sit down on her own. She was also noted to be more alert and responsive following treatment. Her expressive language did not improve, although her receptive language was markedly improved. The above treatment improved patient's clinical findings, normalized blood Phe levels, and increased Phe tolerance in the diet, but neither 20 nor 40 mg/kg/day BH4 supplementation corrected neurotransmitter or BH4 levels or increased BH2 level in CSF. Further studies are needed to find the optimal management plan for patients with DHPR deficiency.

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C.R. Coughlin II · C. Ficicioglu
Section of Biochemical Genetics, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

C.R. Coughlin II
Section of Clinical Genetics and Metabolism, University of Colorado, Aurora, CO, USA

K. Hyland
Medical Neurogenetics, Atlanta, GA, USA

R. Randall · C. Ficicioglu
University of Pennsylvania School of Medicine, Philadelphia, PA, USA

C. Ficicioglu (✉)
The Children's Hospital of Philadelphia, Section of Biochemical Genetics, 34th& Civic blvd. 9S23, 19104, Philadelphia, PA, USA
e-mail: Ficicioglu@email.chop.edu

Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
5-MTHF	5-Methyltetrahydrofolate
BH4	Tetrahydrobiopterin
CSF	Cerebral spinal fluid
DHPR	Dihydropteridine reductase
HVA	Homovanillic acid
NO	Nitric oxide

Introduction

Dihydropteridine reductase (DHPR) deficiency (OMIM: 261630) is an autosomal recessive disorder in the regeneration pathway of tetrahydrobiopterin (BH4). Individuals with DHPR deficiency may present with hyperphenylalaninemia, microcephaly, hypotonia, mental retardation, and convulsions (Blau et al. 1996; Ponzone et al. 2004). DHPR

deficiency is encoded by the gene *QPDR* and missense mutations, insertions/deletions, and splice site mutations in the *QPDR* gene have been associated with DHPR deficiency (Dahl et al. 1987; Dianzani et al. 1998; Howells et al. 1990).

Although there is no curative treatment for DHPR deficiency, a low phenylalanine diet, L-dopa, and 5-hydroxytryptophan supplementation are the mainstay of treatment to keep phenylalanine levels within normal range and to increase the level of neurotransmitters. Folinic acid supplementation, which is also utilized as DHPR, is associated with the maintenance of appropriate folate levels, and patients with DHPR deficiency may have low folate (5-Methyltetrahydrofolate) levels in CSF (Irons et al. 1987; Smith et al. 1985). BH4 is not generally used to treat DHPR deficiency, as large quantities are required to normalize peripheral phenylalanine levels in the absence of the recycling enzyme (Kaufman et al. 1982). The effect of large dose BH4 on central amine metabolism and on CSF BH4 levels in DHPR deficiency has not been reported. In this report, we present a patient with DHPR deficiency who had significant clinical improvement after BH4 supplementation.

Case Report

The patient had elevated phenylalanine detected on newborn screening and was subsequently diagnosed with DHPR deficiency based on decreased activity of DHPR measured on a second newborn screening filter card. Molecular genetic analysis of the *QPDR* gene was performed in peripheral blood and revealed a novel homozygous splice site mutation (c.199-1g>t) consistent with the biochemical diagnosis of DHPR deficiency.

The patient presented to our institution at 27 months of age for further evaluation and treatment. Since initial diagnosis, she had been on a phenylalanine-restricted diet, folinic acid (3 mg/day), and L-dopa (1.4 mg/kg/day). She was reported to have global developmental delay, hypotonia, and convulsions for which she received levetiracetam in the past.

At her initial evaluation in our clinic, she had a mildly elevated plasma phenylalanine level of 170.4 (reference range 23–95 nmol/ml). Her protein intake was 2.5 g/kg and 44% (1.1 g/kg) of the protein was from an incomplete protein in the form of a metabolic formula (free of phenylalanine). Her total phenylalanine intake was 300 mg/day (approximately 25–30 mg/kg phenylalanine). The patient was unable to sit on her own or pull herself to a sitting position. She had minimal expressive or receptive language skills, although she was reported to react with familiarity toward her family members. She had a normal electroencephalogram.

We switched her from L-dopa (1.4 mg/kg/day) and folinic acid (3mg/day) to carbidopa/levodopa (25/100) 2 mg/kg/ day four times daily and folinic acid 10 mg/day. We also added 5-hydroxytryptophan (10 mg/mL) 2 mg/kg/day four times daily. After 2 weeks of this augmented neurotransmitter therapy, we also added sapropterin dihydrochloride (KUVAN), a derivative of BH4, at a dose of 20 mg/kg/day for 2 months. This dosage was then increased to 40 mg/kg/day. The patient had noted developmental improvement 1 month after initiation of BH4 supplementation. Although she remained hypotonic, she was able to pull herself to the standing position and sit down on her own. Her expressive language did not improve, although her receptive language was markedly improved. She was noted to be more alert and responsive after 1 month of treatment with high doses of BH4 supplementation (40 mg/kg/day). Throughout the 3 months of treatment, she was slowly weaned from incomplete protein source while simultaneously increasing complete protein sources. The patient was discharged with similar protein intake at 2.5 g/kg and no longer required an incomplete protein source (medical formula) with all of the protein coming from complete protein. The patient continued to increase her complete protein sources, including animal protein, and her phenylalanine levels remained below 120 nmol/ml.

Biochemical Results

CSF neurotransmitter metabolites and CSF amino acid analysis were performed three times: (1) 2 weeks after the augmented neurotransmitter therapy but prior to BH4 supplementation; (2) after 65 days on BH4 at 20 mg/kg/day; and (3) after 30 days on BH4 at 40 mg/kg/day. Despite BH4 supplementation and neurotransmitter therapy, tetrahydrobiopterin, 5-hydroxyindoleacetic acid, and homovanillic acid remained below reference ranges (Table 1). She had elevated dihydrobiopterin (BH2) before BH4 therapy. There was no significant increase in BH2 levels on BH4 supplementation; plasma phenylalanine level normalized and dietary phenylalanine tolerance increased on 20 mg/kg/day of BH4 therapy.

Discussion

Although BH4 supplementation may increase phenylalanine tolerance and lower plasma phenylalanine levels in patients with DHPR deficiency, the effectiveness of BH4 therapy in the correction of CSF neurotransmitter metabolites is not apparent. Nor is it clear if BH4 supplementation would increase levels of 7, 8-dihydrobiopterin (BH2), and increase further nitric oxide (NO) uncoupling and oxidative stress.

Table 1 Metabolites measured in plasma and cerebral spinal fluid (CSF) following treatment with sapropterin dihydrochloride (BH4)

		65 days BH4 (20 mg/kg)	30 days BH4 (40 mg/kg)	
Metabolite	Neurotransmitter therapy[a] Prior to BH4 supplementation	Neurotransmitter[b]+ BH4 (20 mg/kg/day) therapy	Neurotransmitter[b]+ BH4 (40 mg/kg/day) therapy	Reference ranges
5-MTHF, CSF	100	100	95	40–150 nmol/L
5-HIAA, CSF	23	8	18	74–345 nmol/L
HVA, CSF	108	100	137	233–928 nmol/L
3-O-methyldopa, CSF	201	616	292	< 150 nmol/L
Neopterin, CSF	15	10	9	7–65 nmol/L
Tetrahydrobiopterin, CSF	12	12	16	18–50 nmol/L
Dihydrobiopterin, CSF	28	31	27	2.2–13 nmol/L
Phenylalanine, CSF	34.8	133.9*	11	5–25 nmol/mL
Phenylalanine, plasma	170.5	75.5*	60.0	23–95 nmol/mL
Tyrosine, CSF	9.6	12.4	9.1	4–26 nmol/ml
Tyrosine, plasma	82.8	49.2	36.4	22–102 nmol/ml

5-MTHF 5-Methyltetrahydrofolate, *5-HIAA* 5-hydroxyindoleacetic acid, *HVA* homovanillic acid

*Plasma phenylalanine level was measured 1 week before CSF phenylalanine

[a] The patient was on folinic acid (10 mg), carbidopa/levodopa (2 mg/kg/day), and 5-hydroxytryptophan (2 mg/kg/day)

[b] The patient remained on folinic acid, carbidopa/levodopa, and 5-hydroxytryptophan

Isolated case reports have suggested that BH4 may cross the blood–brain barrier and have therapeutic potential for individuals with primary BH4 deficiency. Initial therapy with BH4 supplementation in two DHPR-deficient patients resulted in increased pterins (BH4 and 6-MPH4) in CSF suggesting that BH4 may cross the blood–brain barrier (Kaufman et al. 1982). Treatment with BH4 was reported in another individual with DHPR deficiency for 12 months without progression of neurologic symptoms suggesting that monotherapy with BH4 may be a sufficient treatment, although homovanillic acid and 5-hydroxyindoleacetic acid levels were decreased after discontinuation of neurotransmitter therapy (Ponzone et al. 1993). Animal studies have provided evidence that larger doses of BH4 supplementation result in elevated pterin concentrations in the brain and suggested that BH4 may enter the brain when appropriate dosage is utilized (Brand et al. 1996). A minimal dose of 20 mg/kg/day of BH4 may be required before changes in neurotransmitter metabolites are noted (al Aqeel et al. 1992; Kapatos and Kaufman 1981).

We present a patient with DHPR deficiency that was noted to have neurologic improvement after the initiation of BH4 supplementation, although neurotransmitter therapy was augmented with the addition of both carbidopa (the patient remained on L-dopa) and 5-hydroxytryptophan. The patient was trialed on 20 mg/kg/day and 40 mg/kg/day of BH4 supplementation. Plasma phenylalanine normalized from 170.5 to 75.5 nmol/mL (reference range 23–95 nmol/mL) with 20 mg/kg/day of BH4 supplementation and remained within the normal range when the dose of BH4 supplementation was increased (Table 1). She had elevated BH2 level in CSF and it did not increase on BH4 treatment. Despite clinical improvement after BH4 therapy and neurotransmitter supplementation, there were no significant changes in CSF neurotransmitter metabolites.

It is unclear whether the patient's clinical improvement was a result of BH4 supplementation or the initiation of L-dopa/carbidopa and 5-hydroxytryptophan. BH4 supplementation did improve the patient's phenylalanine tolerance and the patient was able to eat a regular diet. High doses of BH4 supplementation may be necessary to cross the blood–brain barrier and provide a therapeutic effect for individuals with an error in BH4 metabolism.

BH2 production is higher than normal in individuals with DHPR deficiency because of the enzyme defect. Some of the symptoms might be caused by increased BH2 levels and decreased BH4/BH2 ratio, which will result in NO uncoupling and increased production of superoxides. There is a concern that BH4 supplementation can further increase BH2 production, which will cause decreased BH4/BH2 ratio in patients with DHPR deficiency. On the other hand, one can speculate that BH4 supplementation may actually increase BH4 availability, which can function as a cofactor for hydroxylases, an electron donor to nitric oxide synthase, and improve NO coupling. In this case, we showed that BH4 treatment did not increase BH2 in CSF. Further

studies are needed to prove that BH4 supplementation does not decrease BH4/BH2 ratio further and find the optimal management plan for patients with DHPR deficiency.

Synopsis

A patient with DHPR deficiency was treated with high doses of BH4 supplementation resulting in clinical improvement despite limited improvement in CSF metabolites.

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Hyperphenylalaninemia, BH4-deficient, C; HPABH4C; 261630

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CASE REPORT

Stem Cell Transplantation for Adult-Onset Krabbe Disease: Report of a Case

Madeleine E. Sharp · Cornelia Laule · Stephen Nantel · Burkhard Mädler · Ritu B. Aul · Samuel Yip · Sandra Sirrs

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Abstract Krabbe disease is an autosomal recessive demyelinating lysosomal storage disorder caused by a deficiency of galactocerebrosidase. The adult-onset variant is very rare. Hematopoietic stem cell transplantation (HSCT) is reported to be successful in treating infants with Krabbe disease prior to the onset of symptoms, but there are no reported cases of its use for adult-onset disease. We report the first follow-up data for a patient with adult-onset Krabbe disease who underwent HSCT at age 41, 16 years after the onset of symptoms. HSCT resulted in a sustained normalization of peripheral GALC enzyme activity, halted the progression of symptoms at 24 months post-allograft, and led to improvements in gait and balance. Serial imaging also confirmed that no significant progression of demyelination has occurred. Although long-term follow-up is needed to confirm the effects of HSCT, our 24-month results suggest that HSCT is a viable therapeutic option for symptomatic patients with adult-onset Krabbe disease.

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M.E. Sharp · S. Yip
Department of Medicine, Division of Neurology, University of British Columbia, Vancouver, BC, Canada

C. Laule
Department of Pathology & Laboratory Medicine, Department of Radiology, University of British Columbia, Vancouver, BC, Canada

S. Nantel
Department of Medicine, Division of Hematology, University of British Columbia, Vancouver, BC, Canada

B. Mädler
Department of Neurosurgery, University of Bonn Medical Centre, Bonn, Germany

R.B. Aul
Department Medical Genetics, University of British Columbia, Vancouver, BC, Canada

S. Sirrs
Department of Medicine, Division of Endocrinology and Metabolic Diseases, University of British Columbia, Vancouver, BC, Canada

M.E. Sharp (✉)
Gordon & Leslie Diamond Health Care Centre, Room 8219, 8th Floor, 2775 Laurel Street, Vancouver V5Z 1M9 BC, Canada
e-mail: madeleinesharp@gmail.com

M.E. Sharp
Neurological Institute, Columbia University Medical Centre, 3rd floor, 710 West 168th Street, New York, NY 10032, USA

Introduction

Krabbe disease, or globoid cell leukodystrophy, is an autosomal recessive demyelinating lysosomal storage disorder caused by a deficiency of galactocerebrosidase (GALC). The accumulation of psychosine results in death of oligodendrocytes and Schwann cells, both essential to myelin formation. Infantile- and juvenile-onset disease result in rapid neurological decline and death in most in the first few years of life. The adult-onset variant, which is the rarest, has a much milder and more protracted course typically presenting with slowly progressive spastic quadriplegia, bulbar signs, and demyelinating peripheral neuropathy (Kolodny et al. 1991; Suzuki 2003). Though the diagnosis is based on reduced GALC activity, MRI serves to support the diagnosis in late-onset cases by demonstrating regions of increased T_2 signal in the pyramidal tracts, the posterior corpus callosum, and the parietooccipital white matter (Loes et al. 1999).

Therapeutic developments have focused on hematopoietic stem cell transplant (HSCT) strategies whereby the newly derived white blood cells (WBC) restore GALC

levels thus halting accumulation of toxic metabolites (Sakai 2009). The evidence in support of HSCT comes from treatment of the infantile- and juvenile-onset disease. Outcomes have been most encouraging in patients treated prior to the development of neurological symptoms (Escolar et al. 2005; Krivit et al. 1998; Sakai 2009); however, there is some concern that most of these children do eventually develop symptoms (Duffner 2009). The largest study of symptomatic later-onset Krabbe disease describes only four patients. Following HCST, all patients had either improved or at least stabilized on neurological and MRI assessments (Krivit et al. 1998).

Even fewer data are available on the role of HSCT in the management of adult patients. Only one other adult patient has been reported to undergo HSCT for the management of Krabbe disease. Following HSCT at 24 years of age, she showed marked clinical improvement and had no progression of white matter abnormalities on MRI over a 7-year follow-up period (Lim et al. 2008). This patient had onset of symptoms at 3 years of age, while our patient developed symptoms in her 20s. Thus we present the first reported case of successful HSCT in a patient with adult-onset disease and who, to our knowledge, is also the oldest patient with Krabbe disease to be transplanted.

Report of a Case

The patient presented with spasticity, appendicular ataxia, dysarthria, and emotional lability that started in her 20s and progressed slowly over a 15-year period. White matter changes on MRI led to a possible diagnosis of primary progressive multiple sclerosis. She was seen at our center at 41 years of age at which point she had marked spasticity, dysmetria, bilateral foot drops, and ambulated with a walker. Krabbe disease was confirmed with a markedly reduced GALC activity of 0.2 nmol/h/mg protein in WBC (normal 2.1–10.44 nmol/h/mg protein using the substrate HMU-beta-Gal). Our patient was found to be a compound heterozygote for two mutations in trans: c.857G>A/p.G286D (previously described as p.G270D), a known pathogenic mutation (Furuya et al. 1997), and c.349A>G/p.M117V (previously p.M101V), a novel mutation found in several other patients with late-onset disease (De Gasperi et al. 1999). At the time of diagnosis, she had normal cerebrospinal fluid protein (261 mg/L, normal<450 mg/L), normal bilateral visual evoked potentials, and a normal EEG. Nerve conduction studies revealed a length-dependent axonal neuropathy that remained stable over the course of follow-up and was thought to be related to her diabetes. There was no demyelinating neuropathy (normal motor conduction velocities in the arm, 51–56 m/s, and in the legs, 41–45 m/s), which is the typical pattern of peripheral nerve involvement in Krabbe disease (Siddiqi et al. 2006). Lower extremity somatosensory evoked potentials revealed bilateral symmetrically delayed cortical responses (P40 latency 46.8–47.1 ms, normal<43.5 ms) with normal peripheral responses. Though the upper extremity responses were normal, this result was reproducible with repeated testing over 10 years and, though not definitive, suggests a delay in the dorsal columns of the spinal cord. Brain MRI revealed minimal progression over a 7-year period of bilateral symmetric T_2 hyperintensities within the corticospinal tracts, cerebellar peduncles, and periventricular white matter (Fig. 1a). Findings on diffusion tensor imaging (DTI) and myelin water imaging were in support of a demyelinating process.

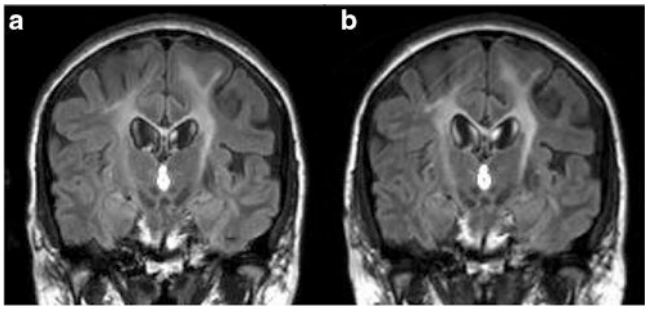

Fig. 1 Serial MRI follow-up. Coronal FLAIR images obtained (**a**) prior to HSCT and (**b**) 24-month post-HSCT showing symmetrical confluent high-signal intensity changes along the corticospinal tracts with no progression of WM abnormality

The patient underwent 10/10 HLA-matched sibling allogenic blood stem cell transplant following a reduced-intensity conditioning regimen (fludarabine/melphalan/alemtuzumab), which did not cause neurological deterioration. The donor sibling was negative for both mutations. She received prophylaxis for graft versus host disease (GVHD) with cyclosporine delivered at 3 mg/kg/day and methotrexate 5 mg/m^2 on days +1, +3, and +6. Her post-transplant course was relatively uncomplicated, and she was discharged on day +16 from hospital. She developed no significant GVHD. GALC activity was normalized at 1-year post-allograft (4.7 nmol/h/mg protein) and, though still within normal limits, has trended down to 2.7 nmol/h/mg protein at 24 months post-allograft. Though too early to determine if this trend is significant in our patient, it has been documented by others that the GALC enzyme activity peaked within 2 years of the transplantation and then decreased but remained within normal limits over the course of a 3- to 8-year follow-up period (Krivit et al. 1998). Full donor chimerism was confirmed on day +61 (myeloid 98% donor/lymphoid 95% donor) and maintained at 24 months (myeloid 97% donor/lymphoid 77% donor).

At 24 months post-allograft, on our assessments, her dysarthria is improved, her gait is less wide based, and she

can tandem walk without support. Spasticity and hyperreflexia are unchanged. She can now walk unaided for 750 m and, with the help of a cane, she can even hike, an activity she could not enjoy pre-transplant. Her mood lability is also improved. Electrophysiological testing, including nerve conduction studies and somatosensory evoked potentials, is unchanged. Follow-up conventional and advanced MRI metrics at 6, 12, and 24 months post-allograft indicate the demyelinating process has not progressed (Fig. 1b), but given the slow rate of change on imaging pre-transplant, only ongoing follow-up will determine whether this trend is a significant change from her baseline.

In this case, HSCT resulted in improvements in gait and balance. Demyelination on serial imaging has not progressed. Although long-term follow-up is needed to confirm the effects of HSCT, our 24-month results suggest that HSCT is a viable therapeutic option for symptomatic patients with adult-onset Krabbe disease.

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RESEARCH REPORT

Detection by Urinary GAG Testing of Mucopolysaccharidosis Type II in an At-Risk Spanish Population

Laura López-Marín · Luis G. Gutiérrez-Solana · Luis Aldamiz-Echevarria Azuara · Rogelio Simón de las Heras · Anna Duat Rodríguez · Verónica Cantarín Extremera

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Abstract Hunter syndrome (Mucopolysaccharidosis type II) is an inherited lysosomal storage disorder with potentially severe degenerative consequences. Clinical diagnosis is not easy, although biochemical confirmation is straightforward, and sometimes patients are diagnosed at a late age. It is widely believed, for inborn errors of metabolism in general, that early diagnosis and management is of paramount importance for improving the prognosis of the disease. The objective of this study was to identify specific populations at risk of suffering from Hunter syndrome. Urine samples were obtained from children between the ages of 0 to 18, belonging to known risk groups of mucopolysaccharidosis (MPS) type II, for the semi-quantitative (GAG test) and quantitative determination of glycosaminoglycans (GAG). One case of Hunter syndrome was found among the 130 samples that were collected and analysed. This study supports the feasibility of early diagnosis and the usefulness of screening tests for MPS II in specific paediatric populations.

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L. López-Marín (✉) · L.G. Gutiérrez-Solana · A.D. Rodríguez · V.C. Extremera
Neuropaediatric Unit, Hospital Infantil Universitario Niño Jesús, Menéndez Pelayo 65, 28009, Madrid, Spain
e-mail: llopez.hnjs@salud.madrid.org

L.A.-E. Azuara
Metabolism Laboratory, Hospital de Cruces, Plaza de Cruces s/n, 48903, Barakaldo, Spain

R.S. de las Heras
Neuropaediatric Unit, Hospital 12 de Octubre, Avda. de Córdoba s/n, 28041, Madrid, Spain

Introduction

Hunter syndrome (Mucopolysaccharidosis type II – MPS II; OMIM 309900) is a genetic storage disease in which the deficiency of the iduronate-2-sulphatase lysosomal enzyme produces a progressive deposition of glycosaminoglycans (mucopolysaccharides) in various organs, leading to degenerative symptoms. It follows an X-linked inheritance (Lonergan et al. 2004; Lyon et al. 2006; Martin et al. 2008; Wraith et al. 2008a).

It is a rare disease, with significant geographical and ethnical variations in the incidence. In studies carried out in different populations, its estimated incidence ranges from one case per 49,000 to one case per 526,000 male live births, and in Europe from one case per 72,000 to one case per 77,000 male live births (Lin et al. 2009; Baehner et al. 2005; Nelson et al. 2003).

Although traditionally patients with Hunter syndrome have been classified into "attenuated" or "severe" subtypes, on the basis of life expectancy and presence or absence of central nervous system (CNS) involvement, the disease should be considered as a continuous spectrum of phenotypes between the two extremes (severe form and attenuated form), being more severe with an early onset of presentation of clinical manifestations (Guelbert et al. 2011; Wraith et al. 2008a). Patients usually appear normal at birth. In patients with the severe form, the age at diagnosis is usually between 18 months and 4 years of age, and CNS involvement causes cognitive impairment and profound mental retardation (Holt et al. 2011a).

The phenotype is characterised by coarse facies, infection and obstruction of the upper airways, hypoacusis, inguinal and umbilical hernia, joint contractures, skeletal

abnormalities (dysostosis multiplex: thoracolumbar kyphosis, bell-shaped thoracic deformity, subluxated hips, valgus knees), hepatosplenomegaly, cardiac valve disease (Kampmann et al. 2011) and slowly progressive mental deterioration with behavioural alterations between the ages of 2 and 6 (del Toro-Riera 2007; Holt et al. 2011b). Diarrhoea, possibly due to infiltration of the autonomic nervous system, can be a chronic management problem. Neuroimaging may reveal increased lateral ventricles, increased perivascular spaces, leukodystrophy and hydrocephalus. Some patients may present with spinal cord compression with quadriplegia (Gutierrez-Solana 2008; Finn et al. 2008; Holt et al. 2011a, b; Manara et al. 2011). The natural course of the disease leads to death by the age of 10–15.

Most of the patients with the attenuated form are diagnosed between the ages of 4 and 8 years. Early facial appearance is normal and facial dysmorphism is usually mild and more slowly progressive. The most apparent symptoms are limited joint mobility, contractures, carpal tunnel syndrome (Kwon et al. 2011), hernias and hepatosplenomegaly. It differs from the severe forms in the absence of neurodegeneration. However, it should be taken into account that some children may present a slight developmental delay due, at least in part, to external factors such as hypoacusia, sleep apnoeas or visual alterations (Gutierrez-Solana 2008; del Toro-Riera 2007; Burton and Giugliani 2012). Typical bone abnormalities are present but are less conspicuous than in the severe forms. In addition to cardiac valve and respiratory disease, the attenuated form may include hypoacusis, retinopathy and, occasionally, spinal cord compression due to cervical spinal stenosis (Matheus et al. 2004). Some patients may survive for more than 60 years, but others die early, even in the second decade of life, due to heart disease, lung infections or obstruction of the airways (Wraith et al. 2008a; Jones et al. 2009).

The majority of children with Hunter syndrome appear healthy at birth, but typical signs and symptoms appear in the early stages of life that could allow for the identification of patient groups at risk of developing the disease. These include patients suffering from thoracolumbar kyphosis (del Toro-Riera 2007; Wraith et al. 2008b), recurrent inguinal and umbilical hernias (Kliegman et al. 1996; Mendelson et al. 2010) and nodular skin lesions (Demitsu et al. 1999; Schwartz et al. 2007).

Objective

The objective of this study was to identify MPS II patients from population groups considered to have a possible higher incidence of children suffering from Hunter syndrome:

- Patient group with thoracolumbar kyphosis of unknown aetiology (in the first 3 years of life)
- Groups of children with bilateral inguinal hernias, recurrent hernias, umbilical hernia that require surgery or those hernias requiring surgery where the surgeon believes there are signs of connective tissue involvement
- Group of patients with typical nodular skin lesions, for example, cobblestone, hypopigmented, ivory or skin-coloured lesions around the shoulder area, upper limbs, and the lateral portion of the thighs, neck and chest

Any of these groups, either alone, in combination with each other or with other MPS signs and symptoms (recurrent respiratory symptoms, facial dysmorphia, hepatosplenomegaly, cardiac valve disorders, etc.) would increase the index of suspicion for the diagnosis of MPS II.

Patients and Methods

Study Population

The study population included children from the ages of 0 to 18 belonging to the above-mentioned “MPS II” risk groups. Patients were selected retrospectively (where data were available) and prospectively, at the Paediatric Trauma, Paediatric Surgery and Paediatric Dermatology Departments of four Spanish reference centres (Hospital de Cruces in Bilbao, Hospital Infantil Universitario Niño Jesús and Hospital Universitario 12 de Octubre in Madrid and Hospital Virgen del Rocío in Seville).

The inclusion criteria were children under the age of 3 suffering from thoracolumbar kyphosis of unknown cause; children with recurrent or bilateral inguinal hernias or with umbilical hernia requiring surgery; children undergoing hernia surgery where the surgeon detected possible signs of connective tissue involvement; and male patients with typical nodular lesions (hypopigmented or skin coloured, cobblestone appearance in the shoulder area, upper limbs, outer part of buttocks, neck or chest). The medical history and examination of children with kyphosis should not be consistent with other known paediatric causes of kyphosis, such as congenital malformation, infection, malignancy, osteogenesis imperfecta, rickets or neurofibromatosis type 1.

This study was conducted in compliance with the recommendations of the Declaration of Helsinki and the Good Clinical Practice (GCP) Guidelines. The study was submitted to the appropriate Independent Ethics Committee for approval, and complied with Spanish Act No. 15/1999 on Personal Data Protection in relation to the confidentiality of patient data. In all cases, signed informed consent was obtained from patients and/or their parents or guardians.

Table 1 Distribution of subjects by hospital department

Urinary quantitative GAG	Negative ($n = 103$)	Inconclusive ($n = 15$)	Positive ($n = 12$)	Total ($n = 130$)
Trauma	18 (17.48 %)	2 (18.75 %)	1 (8.33 %)	21 (16.15 %)
Surgery	85 (82.52 %)	13 (81.25 %)	11 (91.66 %)	109 (83.85 %)
Dermatology	0	0	0	0

Methods

All subjects identified in the "MPS II" risk groups underwent a screening test using a first morning urine sample, which were sent to Hospital de Cruces (Barakaldo), for the determination of glycosaminoglycans (GAG) (semi-quantitative using the colorimetric method and quantitative using ultraviolet-visible spectrophotometry). In the case of detection of increased excretion of urinary GAG, an enzymatic assay of a blood sample on absorbent paper (dried blood spot) was performed to determine the activity of iduronate-2-sulphatase. In all patients with increased levels of GAG and normal activity of iduronate-2-sulphatase, tests were carried out, looking for another MPS.

The GAG test was performed using a colorimetric method based on the change in colour produced by the complexes formed between methylene blue (DMB) and GAG (Lage et al. 2011). A solution of DMB, ethanol and sodium formate was prepared, whose concentrations were previously optimised and adjusted to an acidic pH that also required optimising. This solution was encapsulated in transparent vials, to which first morning urine was then added using a syringe. If the concentration of GAG in the urine is high, as occurs in patients with MPS II, the blue colour of the DMB turns to purple when mixed with urine. By contrast, when adding urine from a healthy individual to DMB, the colour does not change and remains blue.

The quantitative determination of urinary GAG was performed using ultraviolet-visible spectrophotometry. As such, a dilution curve of chondroitin sulphate 100 mg/ml (by diluting 5, 10, 25, 50 and 100 µl in 500 µl of water), a blank (500 µl of water) and the patient samples for analysis (100 µl of urine diluted in 500 µl of water) were used. Afterwards, 2.5 ml of DMB is added and readings are taken on the differences in absorbance between 520 and 600 nm.

The semi-quantitative and quantitative techniques do not always give matching results. In this case, the determinant is the result of the ultraviolet-visible spectrophotometry. If a positive result in this determination was obtained, the analysis was repeated on a new urine sample to confirm the positive result, prior to continuing on to the enzymatic assay.

For the enzymatic assay, we determined the enzymatic activity of iduronate-2-sulphatase in blood on filter paper (Voznyi et al. 2001). This determination is based on the reaction of a substrate containing 4-methylumbelliferone-iduronate-2-sulphatase to the test sample in a first incubation and then adding the enzyme α-iduronidase in a second incubation, releasing fluorescent 4-methylumbelliferone, thus measuring the fluorescence emitted with the fluorometer (360–450 nm).

Statistical Analysis

This study was designed to provide descriptive information and as such the statistical analysis was descriptive in nature. Categorical variables were described according to the number and percentage of subjects within each category. The mean, standard deviation, median and minimum and maximum values were used for the description of continuous variables.

Results

From May 2007 to May 2011, a total of 130 samples were collected from four reference centres. Most of these patients (112 subjects) were recruited at the surgery departments: 45.7 % were included for umbilical hernia, 51.4 % for bilateral inguinal hernia and 2.8 % for recurrent inguinal hernia.

After analysis of the 130 urine samples, there were 12 positive GAG tests and 15 inconclusive, while the remaining 103 were negative. Table 1 shows the distribution of subjects by department, mainly surgical (109 subjects, 85 %). Table 2 shows the age and gender of the subjects participating in the study based on whether the result was negative, inconclusive or positive. It should be noted that the mean age of positive subjects was significantly lower than the age of the remaining subjects. Similarly, in subjects with positive results, the percentage of male children was higher than that of female children (67 % vs. 33 %, respectively). Moreover, there are high mean concentrations of GAG in positive subjects.

All patients with a negative GAG test showed quantitative GAG values within the normal range. Of those with

Table 2 Demographics of patients

Urinary quantitative GAG	Negative ($n = 103$)	Inconclusive ($n = 15$)	Positive ($n = 12$)	Totals ($n = 130$)
Mean age in years (range)	4.41 (0.17–16.51)	4.19 (0.33–12)	3.43 (0.03–14)	4.22 (0.03–16.51)
Gender				
Male, *n* (%)	63 (61.16 %)	9 (60.00 %)	8 (66.67 %)	73 (55.15 %)
Female, *n* (%)	40 (38.83 %)	6 (40.00 %)	4 (33.33 %)	50 (38.46 %)

inconclusive GAG tests, 14 out of 15 showed quantitative GAG values within the normal range, whereas of those with positive GAG tests, 3 out of 12 had quantitative values within normal limits. Enzymatic determination was carried out on seven out of the ten patients with increased GAG levels. No enzymatic assay was carried out on three patients because they were lost to follow-up. There were three cases with high quantitation (two with positive GAG tests and one with inconclusive GAG test) where values were within the normal range upon repeating the test, after which no further tests were carried out. In all patients with increased levels of GAG and normal activity of iduronate-2-sulphatase, tests were carried out, looking for another MPS, with normal results in all cases. All results of the enzymatic assay were normal except one case that was eventually diagnosed with Hunter syndrome. In this case, the enzymatic deficiency was confirmed in fibroblasts.

Table 3 includes quantitative and enzymatic determination data for subjects with positive or inconclusive results.

Discussion

This is one of the first MPS II screening studies to take place in Spain aimed at the early detection of MPS II. The objective of any screening programme is to reduce morbidity and mortality caused by the disease in the tested population, by providing the opportunity for early treatment of detected cases. Early diagnosis is thought to be of great importance in diseases such as Hunter syndrome, the outcome of which is usually fatal at an early age for the most severe forms (Mendelson et al. 2010). Although Hunter syndrome is a rare condition, its severity makes it very important as a health problem. This is because it could cause premature death, severe neurological disorders, mental retardation and poor overall quality of life, dependency on others, institutionalisation, higher healthcare costs and, therefore, notably higher family, social and economic burdens (Imarzumi et al. 1994).

Inguinal hernia is very common in childhood, occurring in 10–20 of every 1,000 people. It is predominant in males by a 4:1 ratio. Half of these cases manifest in the first year of life, usually within the first 6 months. Some 60 % occur on the right side, 30 % on the left and 10 % are bilateral. In some cases, inguinal hernia may be familial but not associated with familial MPS II. Premature infants have a nearly 30 % incidence of suffering from inguinal hernia, while the incidence is higher in infants with connective tissue disorder such as Ehlers-Danlos syndrome and MPS (Hunter-Hurler) (Mendelson et al. 2010).

We have found 1 enzymatically confirmed case of Hunter syndrome among the 109 subjects examined with hernias, which is significantly higher than that reported among the general population, which is approximately 1 case per 72,000–77,000 male live births in Europe (Baehner et al. 2005). This case was a 3-year-old boy (at the time of analysis) with umbilical hernia who was operated on at the age of 2 months for bilateral inguinal hernia. At the time of diagnosis, he had already suffered moderate bilateral hypoacusis of conduction due to bilateral seromucous otitis, mild bilateral carpal tunnel syndrome, mild hepatomegaly and emerging joint contractures. His psychomotor development was normal, except for a slight language delay. He is currently being treated with enzyme replacement therapy (ERT) with idursulfase (Elaprase®, Shire Human Genetic Therapies, Inc., Lexington, MA) at the standard dose of 0.5 mg/kg, for the past 14 months. Urine GAG values are almost normalised (17.3 mg GAG/mmol Cr for normal levels between 7.9 and 16.2) and hepatomegaly has been significantly reduced (initial liver volume of 652cc, compared with 600cc after 6 months of treatment). He underwent surgery being submitted to adenoamigdalectomy and placement of transtympanic drainage, resulting in improved hearing and normalisation of language. He is now 5 years old and is scholarised in the year of studies in accordance with his age. No behavioural problems have been developed. The results of the neuropsychological assessments carried out were within normal limits (total intellectual coefficient of 111). He seems to have an attenuated form of the disease. The genetic study is currently still pending.

Within the kyphosis group, we found one subject with very high GAG values (364.09 mg GAG/mmol Cr for normal values between 9.5 and 25.7), which may indicate that the patient suffers from MPS. This case was a 4-year-

Table 3 Analysis of subjects with positive or inconclusive results

	GAG test	Quantitative determination (mg GAG/mmol Cr)*	Enzymatic determination (iduronate-2-sulphatase)
Male, 8 months Thoracolumbar kyphosis	Inconclusive	14.18 (normal)	Not carried out
Male, 6 years 6 months Bilateral inguinal hernia	Inconclusive	10.76 (normal)	Not carried out
Male, 3 years Bilateral inguinal hernia	Inconclusive	16.97 (normal)	Not carried out
Male, 2 years Umbilical hernia	Inconclusive	13.27 (normal)	Not carried out
Male, 1 year Thoracolumbar kyphosis	Inconclusive	13.76 (normal)	Not carried out
Male, 3 years Umbilical hernia	Inconclusive	10.84 (normal)	Not carried out
Male, 6 years Umbilical hernia	Inconclusive	12.46 (normal)	Not carried out
Male, 5 years Umbilical hernia	Inconclusive	14.87 (normal)	Not carried out
Male, 2 years Bilateral inguinal hernia	Inconclusive	16.66 (normal)	Not carried out
Female, 12 years Umbilical hernia	Inconclusive	42.93 (increased)	Not carried out
Female, 4 months Bilateral inguinal hernia	Inconclusive	24.09 (normal)	Not carried out
Female, 4 years Umbilical hernia	Inconclusive	14.20 (normal)	Not carried out
Female, 5 years 6 months Umbilical hernia	Inconclusive	13.08 (normal)	Not carried out
Female, 3 years Bilateral inguinal hernia	Inconclusive	9.68 (normal)	Not carried out
Female, 8 years Umbilical hernia	Inconclusive	7.59 (normal)	Not carried out
Male, 3 years Bilateral inguinal hernia	Positive	58.79 (increased)	1.9 μmol/l h (NR: 9–29) Hunter syndrome
Male, 2 years 5 months Bilateral inguinal hernia	Positive	34.27 (increased)	Normal
Male, 1 year 6 months Umbilical hernia	Positive	89.64 (increased)	Normal
Male, 2 years Umbilical hernia	Positive	126.98 (increased)	Normal
Male, 3 months Bilateral inguinal hernia	Positive	25.40 (normal)	Not carried out
Male, 4 years Umbilical hernia	Positive	11.62 (normal)	Not carried out
Male, 10 days Bilateral inguinal hernia	Positive	70.85 (increased)	Normal
Male, 4 years Umbilical hernia	Positive	19.80 (normal)	Not carried out
Female, 3 years Umbilical hernia	Positive	42.94 (increased)	Not carried out
Female, 3 years Umbilical hernia	Positive	58.29 (increased)	Normal
Female, 4 years Thoracolumbar kyphosis	Positive	364.09 (increased)	Not carried out
Female, 14 years Umbilical hernia	Positive	18.82 (increased)	Normal

*Age (normal range): <1 year (13.3–36.3); 1–2 years (8.1–35.3); 3–4 years (9.5–25.7); 5–6 years (7.9–16.2); 7–10 years (6.7–15.5); 11–16 years (3.3–13.7); 17–18 years (2.4–9.0)

old girl, living in Angola, who was admitted in our hospital due to respiratory decompensation. The physical examination showed macroglossia, severe kyphoscoliosis and serious psychomotor delay. A urine sample was collected for GAG determination. Unfortunately, when the result was obtained, several days later, the family had returned to Angola, where it has not been possible to continue the study.

In this study, we were unable to include any children from dermatology visits with typical nodular lesions, probably because the target population is very limited and very specific to Hunter syndrome. However, this is a group requiring a GAG analysis, as the literature already reports.

Recent publications indicate that GAG test allows for rapid detection, confirms or refutes a suspicion and reduces the number of individuals who require further analysis (Lage et al. 2011). It is, therefore, a method that could be used as initial screening, but as it can give rise to false positive or false negative, it is also recommended to carry out the quantitative urine GAG test (Martin et al. 2008; Baldellou VÄzquez and García Jiménez 2006; Wraith et al. 2008a).

For the definitive diagnosis of MPS II, it is necessary to prove deficient activity of the enzyme iduronate-2-sulphatase in leucocytes, fibroblasts or plasma, in the presence of normal activity of at least one other sulphatase. Genetic analysis to identify the IDS gene mutation associated with MPS II is now also commonly performed, but the actual diagnosis remains an enzymatic one (Scarpa et al. 2011; Martin et al. 2008; Guelbert et al. 2011).

Nowadays, ERT is available for the treatment of patients with MPS II. Idursulfase is a glycoprotein similar to the human iduronate-2-sulphatase produced by genetic engineering in a human cell line. The treatment reduces the size of the spleen and liver and normalises GAG excretion in urine, it improves obstructive sleep apnoea and lung function, it decreases the number of ENT (ear, nose, and throat) infections and it improves quality of life (Muenzer et al. 2006, 2007, 2011a, b). It also has a positive effect on growth, especially when treatment begins before the age of 10 (Schulze-Frenking et al. 2011; Alcalde-Martín et al. 2010). Another recent report suggests that it may be possible for early treatment to slow or prevent the development of irreversible manifestations and therefore modify the natural history of MPS II (Tylki-Szymanska et al. 2012). Another recent study of enzyme replacement therapy with idursulfase showed beneficial effects after only 8 months of treatment in patients under the age of 5 showing neurological abnormalities and other signs and symptoms of being severely affected by the disease (Alcalde-Martín et al. 2010). Thus, enzyme replacement therapy has the potential to benefit many patients with MPS II, especially if started early in the course of the disease (Wraith et al. 2008a).

The eventual goal for the early treatment of MPS II is newborn screening (Marsden and Levy 2010). Assays for lysosomal enzyme activity, including I2S activity, in dried blood spots that may be suitable for newborn screening have been developed (Gelb et al. 2006; Wolfe et al. 2011; Sista et al. 2011). A pilot population-based newborn screening using any of these methods has not yet been reported. In addition, controversy exists on the appropriateness to perform these programmes in MPS as there is not a good genotype-phenotype correlation, with the exception of certain mutations-deletions or rearrangements of the IDS gene that completely abolish I2S transcript production will result in the severe phenotype (Burton and Giugliani 2012). It is currently being discussed if it is ethical and if is justifiable submitting a child, who may have an attenuated form of the disease, to weekly infusions from the first few weeks of life, with the risk for subsequent complications. For the time being, and while addressing all these issues, it remains in the hands of primary care paediatricians and paediatric specialists to recognise and refer patients with suspected MPS II as early as possible.

The patient found in this study, at the time of diagnosis, in addition to hernias presented early stage contractures in hands and elbows, and discrete hepatomegaly which was unnoticed on medical examinations carried out so far. His facial appearance was not striking but characteristic. These data could possibly have been enough to suspect MPS if these have been evaluated by a neuropaediatrician or a paediatrician expert in metabolic diseases, but neither his primary care paediatrician nor the surgeons being visited had referred him to a specialist until the time of the screening. It is common for children with MPS to have been examined in several hospital departments before reaching the neurologist (surgery, rheumatology, trauma, ear, nose, and throat) (Muenzer et al. 2006). There is often a delay of several years between the onset of signs and symptoms and diagnosis. This is particularly true for patients with the attenuated phenotype, as the disease onset can be insidious. Such delays increase the risk of irreversible organ damage and may decrease the benefit of ERT (Muenzer et al. 2009; Vieira et al. 2008; Wraith et al. 2008a). It is thus necessary that physicians who usually visit these patients know about these diseases and the signs and symptoms for suspecting the existence of MPS. In this sense, the diffusion of groups at risk can be a great help.

The determination of GAG in a single urine sample is a painless, low-cost method that is suitable as a screening test during routine hospital visits. We therefore believe it is worth requesting the test at the slightest suspicion. We recommend the development of a simple referral protocol for suspected MPS II, based on European Guidelines (Working Group for Rare Diseases 2011) and local protocols (GonzÄlez-Meneses et al. 2010), at each centre (especially in surgery departments) in order to aid in the diagnosis, treatment and appropriate follow-up of this patient group. Nevertheless, we believe that further studies, more prolonged and with a higher number of patients, need to be conducted to be able to establish the cost-effectiveness of those programmes.

In summary, our study has demonstrated that MPS screening in these patient groups, with such an easy technique as the determination of GAG in urine, may be a simple but useful approach that allows for the early detection of patients suffering from MPS II and offers an individualised and appropriate follow-up and treatment of these patients.

Synopsis

One case of Hunter syndrome was found among 109 patients with hernias in the first screening study in Europe aimed at the early detection of MPS II.

All authors have made a substantial contribution to the study and all have approved the final draft.

The author who serves as guarantor for the article is Laura López-Marín.

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

This study was conducted in compliance with the recommendations of the Declaration of Helsinki and the Good Clinical Practice (GCP) Guidelines. It was submitted to the appropriate Independent Ethics Committee for approval and complied with Spanish Act No. 15/1999 on Personal Data Protection in relation to the confidentiality of patient data. In all cases, signed informed consent was obtained from patients and/or their parents or guardians.

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RESEARCH REPORT

Simple, Fast, and Simultaneous Detection of Plasma Total Homocysteine, Methylmalonic Acid, Methionine, and 2-Methylcitric Acid Using Liquid Chromatography and Mass Spectrometry (LC/MS/MS)

Xiaowei Fu · Yan-Kang Xu · Penny Chan · Paul K. Pattengale

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Abstract Cobalamin (Vitamin B12) plays an essential role both in the conversion of methylmalonyl-CoA to succinyl-CoA and in the synthesis of methionine (Met) from homocysteine (Hcy). Elevations of total homocysteine (tHcy), Met, methylmalonic acid (MMA), and 2-methylcitric acid (2MCA) are indicative of disorders in these related pathways, and can clinically present as methylmalonic acidemia, cobalamin defects or deficiency, propionic acidemia, homocystinuria, and hypermethioninemia. We have developed a fast, sensitive, and simple method for the simultaneous detection of plasma tHcy, MMA, Met, and 2MCA using liquid chromatography mass spectrometry (LC/MS/MS). All analytes were directly determined without the need of derivatization. Both positive and negative modes were used to achieve the best sensitivity and specificity. The two stereo isomers of 2MCA (2S, 3S) and (2R, 3S) were successfully separated and were designated as 2MCA1 and 2MCA2. The assays were linear up to a concentration of 800 μMol/l for tHcy, 2,000 μMol/l for Met, 80 μMol/l for MMA, 40 μMol/l for 2MCA1, and 40 μMol/l for 2MCA2 (80 μMol/l for total 2MCA), respectively. The recovery was between 84.42 % and 120.05 %. The intra-assay coefficient of variations (CVs) ranged from 2.1 % to 6.9 % (n = 20), and the inter-assay CVs ranged from 2.7 % to 11.6 % (n = 20). Reference intervals were established and verified (n = 125). A total of 15 patients with variable disorders in related pathway were successfully confirmed. The assay can be performed either in diagnostic laboratories or as second-tier, follow-up test in newborn screening laboratories.

A fast, sensitive, and simple LC/MS/MS method was developed successfully for the simultaneous detection of plasma total homocysteine, methylmalonic acid, methionine, and 2-methylcitric acid for diagnosis of disorders in related pathways.

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X. Fu (✉) · P.K. Pattengale
Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, United States
e-mail: xfu@chla.usc.edu

X. Fu · Y.-K. Xu · P. Chan · P.K. Pattengale
Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, 4650 Sunset Boulevard MS#32, Los Angeles, CA 90027, United States

Abbreviations

2MCA	2-methylcitric acid
Cbl	Cobalamin
GC/MS	Gas chromatography–mass spectrometry
Hcy	Homocysteine
HPLC	High performance liquid chromatography
LC/MS/MS	Liquid chromatography-mass spectrometry
Met	Methionine
MMA	Methylmalonic acid
MTHFR	Methylenetetrahydrofolate reductase
SA	Succinic acid
TCEP-HCL	Tris 2-carboxyethyl phosphine hydrochloride
tHcy	Total homocysteine

Introduction

Cobalamin (Vitamin B12) plays an essential role both in the conversion of methylmalonyl-CoA to succinyl-CoA, and in the synthesis of methionine (Met) from homocysteine (Hcy) (Fig. 1). Two enzymatic reactions in mammalian

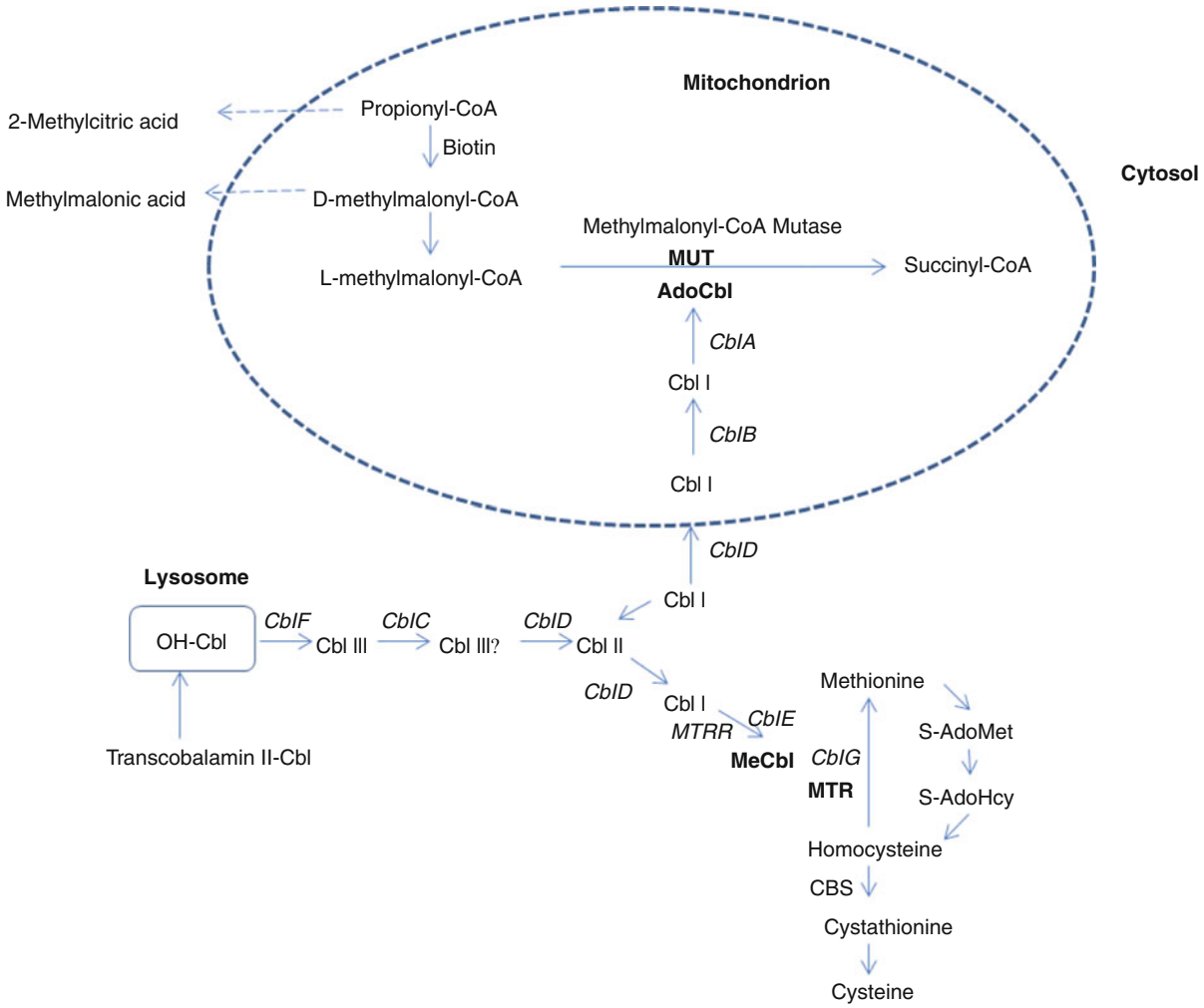

Fig. 1 Metabolic pathways involving Cobalamin (Cbl) for MMA, tHcy, and Met. The enzyme propionyl-CoA carboxylase converts propionyl-CoA into D-methylmalonyl-CoA, which is biotin dependent, and then is racemized into L-methylmalonyl-CoA and isomerized into succinyl-CoA, a Krebs cycle intermediate. Adenosylcobalamin (AdoCbl) is the cofactor of the methylmalonyl-CoA mutase (MUT) reaction; methylcobalamin (MeCbl) is the cofactor of the methionine synthase (MTR) reaction. Abbreviations used: *Cbl* cobalamin, *OH-Cbl* hydroxycobalamin, *AdoCbl* adenosylcobalamin, *MeCbl* methylcobalamin, *MTR* methionine synthase, *MTRR* methionine synthase reductase, *CBS* cystathionine β synthase, *S-AdoMet* S-adenosylmethionine, *S-AdoHcy* S-adenosylhomocysteine

cells require cobalamin as a cofactor. Adenosylcobalamin is the cofactor for methylmalonyl-CoA-mutase (MUT, EC 5.4.99.2), and methylcobalamin is a cofactor for methionine synthase (MTR, EC 1.16.1.18). Disorders of intracellular cobalamin metabolism may impair the function of either or both enzymes.

Acquired cobalamin deficiencies are very common worldwide (Obeid 2012), especially in elderly subjects (Andrès et al. 2004). Vegetarians are prone to develop cobalamin deficiencies, which often go unrecognized because of subtle clinical manifestations. Asymptomatic newborns with nutritional cobalamin deficiencies secondary to maternal cobalamin deficiencies have also been reported (Campbell 2005; Lemieux et al. 1992). Cobalamin levels have also been assessed in infants, children, and adolescents (Monsen and Ueland 2003). Cobalamin deficiency in infancy and childhood may cause irreversible neurologic damage (Requejo et al. 1997, Rasmussen et al. 2001, Garewal et al.1988). The concentration of tHcy in serum and plasma is increased in both folate and cobalamin deficiencies. MMA is another sensitive and specific marker of cobalamin function. These functional measurements are therefore useful in the identification of subclinical cobalamin states and reflect early changes in cobalamin status. tHcy is

also elevated in pathologic states such as renal failure, thyroid dysfunction, and cardiovascular diseases (Monsen and Ueland 2003). Elevation of MMA and 2MCA can also be detected in end-stage renal failure patients (Henning et al. 1999).

There are a number of inherited defects that cause cobalamin deficiency or defects in the pathways of cobalamin absorption, transport, metabolism, or utilization (Fig. 1), such as congenital pernicious anemia (intrinsic factor deficiency, OMIM 261000) and Imerslund-Gräsbeck syndrome (OMIM 261100); transcobalamin II deficiency (OMIM 275350) and methylmalonic acidemia (OMIM 251000) due to methylmalonyl-CoA mutase (EC 5.4.99.2) deficiency (mut0, mut-); and intracellular cobalamin metabolism, identified by complementation class as Cbl C (OMIM 277400), Cbl D (OMIM 277410), including Cbl D variant 1 and variant 2, Cbl F (OMIM 277380), Cbl E (OMIM 236270), and Cbl G (OMIM 250940) (Obeid 2012).

Disorders that can cause elevated MMA alone are methylmalonyl-CoA mutase deficiencies (mut0, mut-), and adenosyl-Cbl-specific deficiencies, such as Cbl A, Cbl B, and Cbl D-variant 2 deficiencies. Combined methyl-Cbl and adenosyl-Cbl deficiencies, such as Cbl C, Cbl D, and Cbl F can cause elevations of both MMA and tHcy. Imerslund-Gräsbeck syndrome and transcobalamin II deficiency can also cause elevations of both MMA and tHcy. Disorders causing primarily hyperhomocysteinemia are classic homocystinuria (OMIM 606664) (cystathionine beta-synthase deficiency, CBS, EC. 4.2.1.22), and methyl-Cbl-specific deficiencies, such as Cbl D-variant 1, Cbl E, and Cbl G, as well as methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) deficiency (OMIM 236250).

Hyperhomocysteinemia can also result from folate deficiency, Vitamin B12 deficiency, or mutations in enzymes in remethylation enzymes. Elevation of tHcy is also a known risk factor for premature cardiovascular disease.

At present, several genetic conditions leading to abnormal methionine elevations are known. They are methionine adenosyltransferase (MAT, EC 2.5.1.6) I/III deficiency (OMIM 250850), homocystinuria due to cystathionine beta-synthase deficiency, deficiencies of glycine *N*-methyltransferase (GNMT, EC 2.1.1.20), (OMIM #614300), deficiencies of *S*-adenosylhomocysteine hydrolase (AHCY, EC 3.3.1.1) (OMIM 613752), and deficiencies of citrin (OMIM 605814, OMIM 603471) (Mudd 2011). Hypermethioninemia is also suggestive of mitochondrial dysfunction and liver diseases (Mudd 2011).

Several inborn errors of methylmalonic acid, methionine, and cobalamin metabolism are included in the supplemental newborn screening program recommended by ACMG (American College of Medical Genetics). These conditions rely on abnormal levels of propionylcarnitine (C3-acylcarnitine) and/or methionine. Unfortunately, these markers lack both disease specificity and poor diagnostic sensitivity (Turgeon et al. 2010; Ueland et al. 1993), and can sometimes lead to false-positive results. Therefore, measurement of specific markers such as tHcy, MMA, and 2MCA are more desirable. Marca G et al. (2007) reported a rapid second-tier test for measurement of 3-OH-propionic and MMA in dried blood spots to reduce the false-positive rate for propionylcarnitine. Turgeon et al. (2010) also reported a second-tier assay to determine tHcy, MMA, and 2MCA in dried blood spots by LC/MS/MS.

Many methods have been described for measurement of plasma MMA and/or tHcy by LC/MS/MS and GC/MS. Blom et al. (2007) reported an assay for MMA without derivatization by LC/MS/MS. Tomaiuolo et al. (2006) reported a method for determination of plasma tHcy by isotope dilution and electrospray-tandem mass spectrometry. Windelberg et al. (2005) reported an automated assay for the determination of MMA, tHcy, and related amino acids by GC/MS. Kushnir et al. (2001) reported an assay of dicarboxylic acid including MMA by tandem mass spectrometry. A simultaneous determination of total homocysteine, MMA, and possible Met using LC/MS/MS without derivatization was described by Hempen et al. (2008).

We therefore have developed a simple, fast LC/MS/MS assay for the simultaneous determination of tHcy, Met, MMA, and 2MCA in plasma or serum. Furthermore, all analytes are directly measurable without the need for an additional derivatization step, and both positive and negative ion modes were used to achieve the best sensitivity and specificity. This method consolidates all four analytes thus allowing for the diagnosis of disorders in related pathways. In addition, this test can also be used as a second-tier or confirmatory test for positive newborn screening.

Materials and Methods

Chemicals such as DL-D_8-homocystine and D_3-MMA were obtained from Cambridge Isotope Laboratories. TCEP-HCL was purchased from Thermo Scientific. DL-homocysteine, L-Met, and MMA were purchased from Sigma-Aldrich. 2MCA was purchased from CDN Isotope Laboratories, Canada. Other reagents, such as HPLC grade water, formic acid-optima LC/MS, and methanol-optima LC/MS were purchased from Fisher Scientific.

Preparation of Calibrators and Controls

Stock solutions of Hcy, Met, MMA, and 2MCA were prepared in water at concentrations of 1.0, 0.2, 1.22 and 1.0 mg/ml, respectively. The combo internal standard solution including DL-D_8-homocystine and D_3-MMA was prepared at a concentration of 50 ng/100 µl. PSHM (plasma/serum/H_2O mixture solution) was prepared in V:V:V=25:25:150. A standard stock solution was prepared by adding 1.351 ml of 1.0 mg/ml Hcy, 29.844 ml of 0.2 mg/ml Met, 0.097 ml of 1.22 mg/ml MMA, and 0.410 ml of 1.0 mg/ml 2MCA, and filled up to a volume of 50 ml with H_2O. Working standard solutions were prepared by diluting 3:4, 1:2, 1:4, 1:20, and 1:40 from stock solutions with PSHM as calibrators. Three levels of controls were prepared by 1:4, 1:20, and 1:40 dilutions from the standard stock solution with PSHM. The three levels of controls were also used for the imprecision study.

Samples

Diagnosed patients' plasma samples sent to the lab for follow up were used to evaluate this method.

Methods

100 µl aliquots of EDTA or heparinized plasma or serum samples were placed into 12 x 75 mm glass test tubes and 250 µl of HPLC grade water was added to each. 100 µl of combo IS was then added to each tube and mixed gently for 3 s. Then 50 µl of TCEP-HCL (1g/34.8 ml in water) was added and mixed gently for 3 s. The mixtures were vortexed twice for 15 s at a medium speed and incubated for 15 min each at room temperature. The solutions were transferred with plastic transfer pipette into Amicon Ultra 0.5 ml 10K Da and then centrifuged at 13,500 rpm for 10 min. The filtrates were transferred into HPLC vials, and 10 µl was injected into the instrument.

MS/MS Procedures

A triple-quadrupole MS/MS system (Applied Biosystem/MDS SCIEX API 4000 Qtrap) was coupled with a Shimadzu HPLC system and a Leap technologies auto sampler. All MS investigations were carried out with a turbo ion spray source operated in both positive mode and negative mode under multiple reaction monitoring (MRM) conditions. MRM transition of m/z 136.1/90.1 was monitored for tHcy, m/z 140.1/94.1 was monitored for D_4-homocysteine (reduced form of D_8-homocystine), and m/z 150.1/104.1 was monitored for Met, respectively. tHcy and Met were measured in positive ESI mode (run time 0–2.04 min) and quantified using D_4-homocysteine. MRM transition of m/z 117.1/73.0 was monitored for MMA, m/z 204.9/125.0 for 2MCA1 and 2MCA2. MRM transition of m/z 120.1/76.0 was monitored for D_3-MMA. MMA and 2MCA were measured in negative ESI mode (run time 2.05–6.0 min) and quantified using D_3-MMA. 10 µl was injected into the LC column with the mobile phase flow of 0.7 ml/min. The LC separation was carried out on a reversed-phase C18 column (Phenomenex, Kinetex 2.6u C18 100A; 100 × 4.6 mm). Gradient elution of the analytes was achieved using a program with mobile phase A (aqueous 0.2% formic acid) and mobile phase B (0.2% formic acid in methanol) as follows: 3% B from 0 min to 4.4 min; 90% B from 4.5 min to 5 min; then back to 3% B from 5 min to 6 min.

Results

Linearity and Imprecision

Plasma standards of MMA, tHcy, Met, and 2MCA at five different concentrations of spiked analytes were used. They were as follows: tHCy: 150, 100, 50, 10, and 5 µMol/l; Met: 600, 400, 200, 40, and 20 µMol/l; MMA: 15, 10, 5, 1, and 0.5 µMol/l; total 2MCA: 30, 20, 10, 2, and 1 µMol/l. The results showed reproducible signals with a linear response (tHcy $R^2 = 0.9978$; Met $R^2 = 0.9962$; MMA $R^2 = 0.9992$; 2MCA1$R^2 = 0.9973$; 2MCA1$R^2 = 0.9976$. $n = 3$ for each analyte). The determination is linear up to a concentration of 800 µMol/l for tHcy, 2,000 µMol/l for Met, 80 µMol/l for MMA, 40 µMol/l for 2MCA1, and 40 µMol/l for 2MCA2 (80 µMol/l for total 2MCA), respectively. If the amount is greater than the levels mentioned, the specimen is diluted accordingly and reanalyzed for an accurate calculation in the linear range. Intra-assay imprecision for tHcy, Met, MMA, and 2MCA1 and 2MCA2 was determined by assaying the same 20 replicates of 3 concentrations in 1 day (Table 1). Inter-assay imprecision was determined by assaying the same samples on 20 different days at 3 concentrations by 2 different technologists over a period of 2 months (Table 1).

Method Comparison

A correlation study was performed by determining tHcy (0.06–179 µMol/l) and Met (2.07–229 µMol/l) in 40 plasma samples by means of the new LC/MS/MS method in comparison with the HPLC assays routinely used in the lab. An X-Y plot showed excellent correlation between the two methods (Fig. 2).

Table 1 Imprecision for tHcy, Met, MMA, and 2MCA (CV%)

	Concentration (μMol/l)	Intra-assay precision ($n = 20$)	Inter-assay precision ($n = 20$)
tHcy	50	4.9 %	7.1 %
	10	4.4 %	6.8 %
	5	4.1 %	6.5 %
Met	200	6.9 %	11.6 %
	40	4.6 %	10.9 %
	20	3.5 %	8.4 %
MMA	5	2.1 %	2.7 %
	1	3.9 %	4.4 %
	0.5	4.9 %	5.9 %
2MCA1	5	2.4 %	4.1 %
	1	2.7 %	6.0 %
	0.5	7.5 %	9.9 %
2MCA2	5	2.2 %	9.5 %
	1	2.7 %	10.3 %
	0.5	4.5 %	8.1 %

Recovery

Recovery was evaluated by the analysis of three different levels of tHcy, Met, MMA, and 2MCA diluted with PSHM (plasma/serum/H_2O mixture solution) (Table 2). Recovery was defined as follows: final concentration – endogenous concentration/added concentration. The recoveries are shown in Table 2. Recovery study for MMA was also carried out by comparing the reference material (UTAK lab, Valencia, CA) at two levels, 0.2 and 1 μMol/l. The recoveries were 112 % and 105 %, respectively ($n = 5$).

Limit of Detection

The detection of limit was carried out by preparing and measuring the lowest standard solution that had been diluted with water by factors of 1 (undiluted), 2, 4, 8, and 14. The limit of detection for tHcy, Met, MMA, 2MCA1, and 2MCA2 was determined to be 0.3, 1.25, 0.03, 0.03, and 0.03 μMol/l, respectively.

Reference Intervals

Reference intervals were established and verified by analyzing 125 anonymous individual male and female plasma samples (ages 7 months to 20 years) with normal chemistry results, and which would otherwise be discarded. The concentration distribution in these specimens were calculated and evaluated with EP evaluator software. The histogram was obtained to ensure normal distribution.

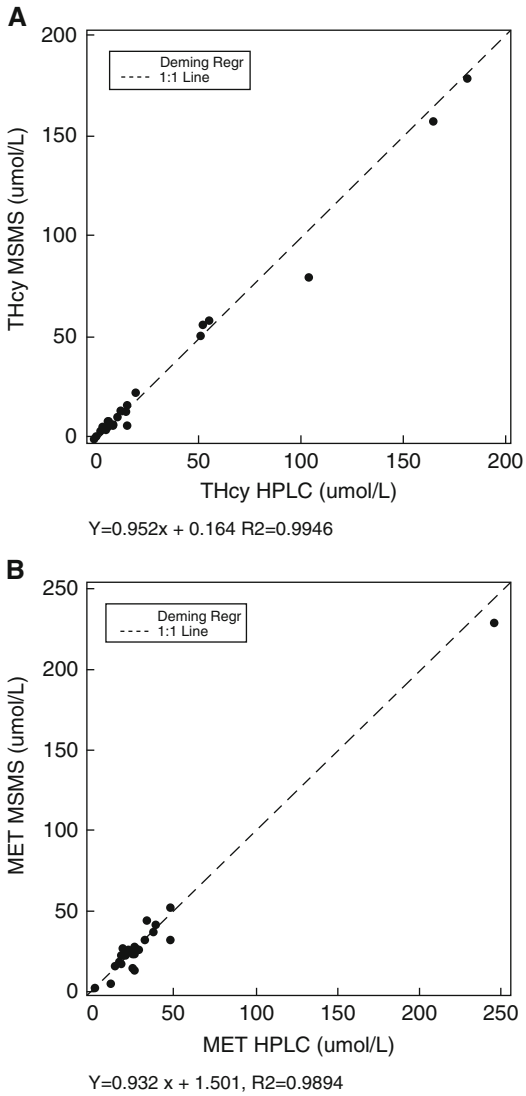

Fig. 2 Passing and Bablok regression lines for tHcy (A) and Met (B) in the correlation study of the LC/MS/MS method with the HPLC method ($n = 40$)

Outliers were excluded. A central 95 % interval was used as reference interval. The reference intervals are 2.47–12.51 μMol/l for tHcy, 6–53 μMol/l for Met, 0.05–0.29 μMol/l for MMA, and 0.02–0.26 μMol/l for 2MCA (total), respectively. The reference intervals were similar to those reported by others (Obeid 2012; Rasmussen et al. 2001; Allen et al. 1993).

Patients' Results

Fifteen diagnosed patient specimens that were sent to the lab for follow up were analyzed to evaluate this method (Table 3). Two diagnosed classical homocystinuria patients

Table 2 Analytical recovery of the assay

Concentration, μMol/l				
	Expected concentration	Endogenous mean (n=3)	Detected mean (n=5)	Recovery* (%)
tHcy	200	1.17	170.0	84.42
	40	1.17	38.78	94.03
	4	1.17	4.88	92.75
Met	400	4.50	478.0	118.38
	80	4.50	96.60	115.13
	8	4.50	13.42	111.55
MMA	20	0.04	23.76	118.61
	4	0.04	4.84	120.05
	0.4	0.04	0.51	118.00
2MCA1	20	0.10	18.00	89.49
	4	0.10	3.78	91.95
	0.4	0.10	0.49	96.00
2MCA2	20	0	18.90	94.50
	4	0	3.53	88.25
	0.4	0	0.35	87.75

*Recovery: Detected concentration - endogenous concentration/ expected concentration%

Table 3 Diagnosed patients and elevations of abnormal compounds (μMol/l)

Normal level	Hcy 2.5–12.5	Met 6–53	MMA 0.05–0.29	2MCA 0.02–0.26
Homocystinuria ($n = 2$)	92.4–103	419–759	NL*	NL
MMA ($n = 3$)	NL	NL	439–944	8.8–32.9
Cobalamin C defect ($n = 5$)	17.4–114	NL	0.58–46.3	0.15–1.12
PPA ($n = 3$)	NL	NL	NL	9.21–34.7
Cobalamin deficiency ($n = 1$)	NL	NL	0.88	NL
MTHFR ($n = 1$)	117	29.3	NL	NL

*NL: Normal values

showed elevations of tHcy and Met. Three methylmalonic acidemia patients showed significant elevations of MMA and 2MCA. Five diagnosed cobalamin C defect patients showed elevations of MMA, tHcy, and slight elevation of 2MCA. Three diagnosed propionic acidemia patients showed elevation of 2MCA with a normal MMA level. One patient with MTHFR showed elevation of tHcy with normal Met. This patient was receiving Met supplementation. One patient with Vitamin B12 deficiency showed elevation of MMA, but normal tHcy. Figure 3 depicts chromatograms from a patient with propionic acidemia (B), a normal individual (C), a patient with a Cbl C defect (D), and a standard solution (A) for comparison.

Discussion

The primary objective of this study was to develop a simple, fast LC/MS/MS method for the simultaneous and quantitative determination of tHcy, Met, MMA, and 2MCA. The method was developed, validated, and successfully applied to the analysis of clinical samples. All four analytes are directly measurable without the need for an additional derivatization step. The simultaneous measurement is a significant improvement in the diagnosis of disorders in related pathways.

Analytes were measured in 6-min intervals between injections. tHcy (m/z 136/90.1) and Met (m/z 150.0/104.1) were quantified using D_4-homocysteine (m/z 140.1/94.1) in positive mode (0–2.04 min). MMA (m/z 117.1/73.0) and two isomers of 2MCA (m/z 204.9/125.0) were measured in negative mode (2.05–6.0 min) and quantified using D_3-MMA (120.1/76). Both negative and positive modes were used to achieve the best sensitivity and specificity. The chromatography is carried on by gradient program through Kinetex-C18 column. All five peaks were successfully separated. Succinic acid has the same transition with MMA (m/z 117.1/73.0), and it appeared in negative mode (2.57 min). Because there are two isomers of 2MCA, the sum of the two was reported.

The ACMG recommends newborn screening for propionic acid, Met, and Cbl metabolism in their panel of 29 conditions. The elevation of propionylcarnitine (C3-acylcarnitine) can indicate propionic acidemia (OMIM #606054), methylmalonic acidemia, or congenital cobalamin defect or deficiency. Increased C3-acylcarnitine and abnormally low concentrations of Met are also observed in newborns with Vitamin B12 deficiency (Turgeon et al. 2010). If C3-acylcarnitine is elevated in newborn screening, then multiple tests, such as urinary organic acids analysis, plasma tHcy, Met, and/or plasma MMA are needed for the differential diagnosis between methylmalonic acidemia, propionic acidemia and cobalamin defects and deficiencies. Our assay is able to differentiate between methylmalonic acidemia and propionic acidemia by simultaneous measurement of MMA and 2MCA. This assay can also detect tHcy, MMA, and Met simultaneously, which enables identification of patients with remethylation defects, such as Cbl D-Varl, Cbl E, Cbl G, and MTHFR deficiency, all of which

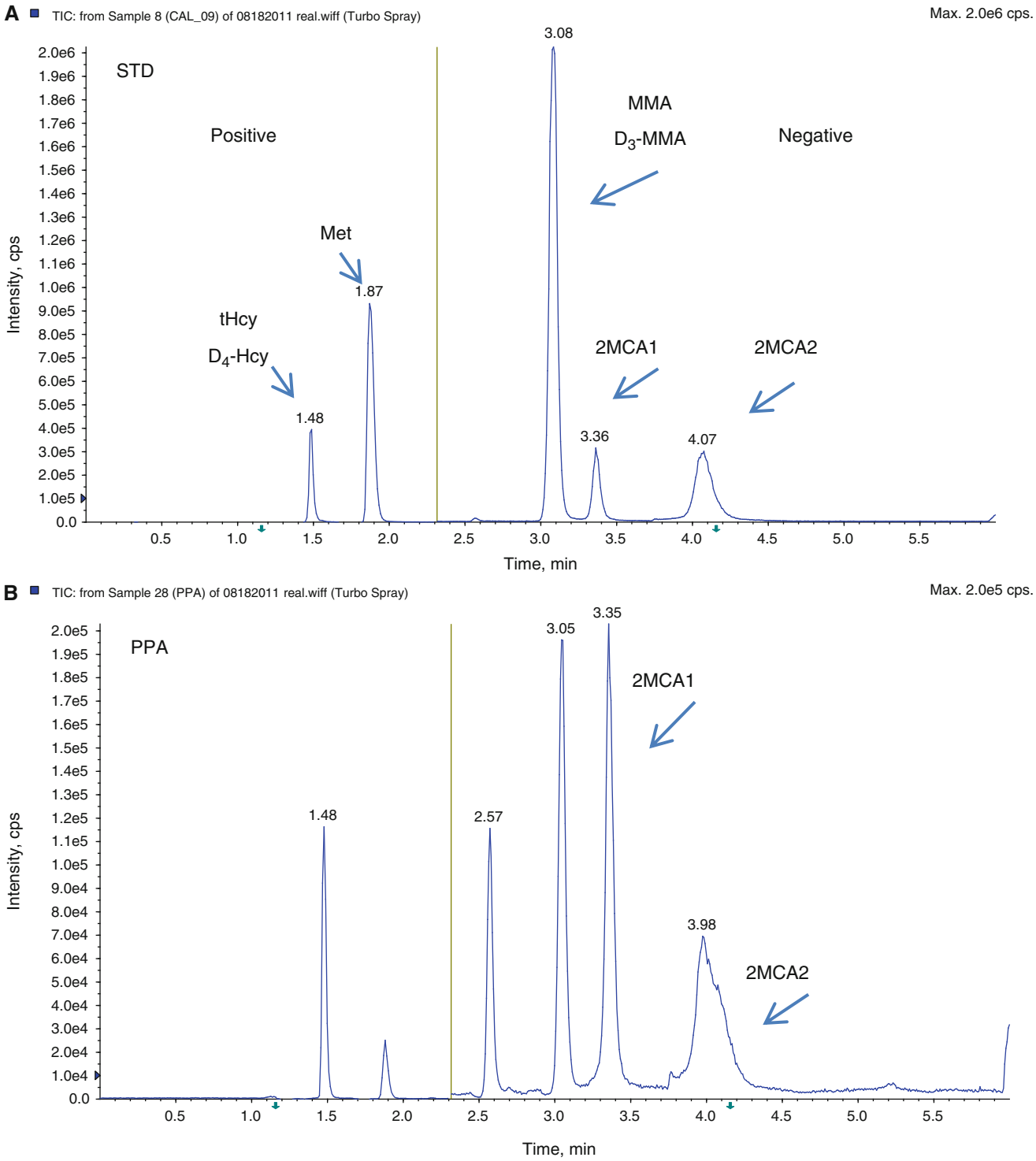

Fig. 3 (continued)

are characterized by low Met and increased tHcy (Turgeon et al. 2010).

The reference ranges were verified using 125 plasma specimens from Children's Hospital of Los Angeles patients with normal chemistry values. Additionally, the reference interval for 2MCA was established. To our knowledge, this is the first 2MCA reference interval using LC/MS/MS in plasma or serum specimens, which is very similar with that analyzed by GC/MS (Allen et al. 1993). Turgeon et al. (2010) reported a method to measure 2MCA in dried blood spots. We developed a LC/MS/MS method to detect 2MCA, along with MMA, which can provide additional

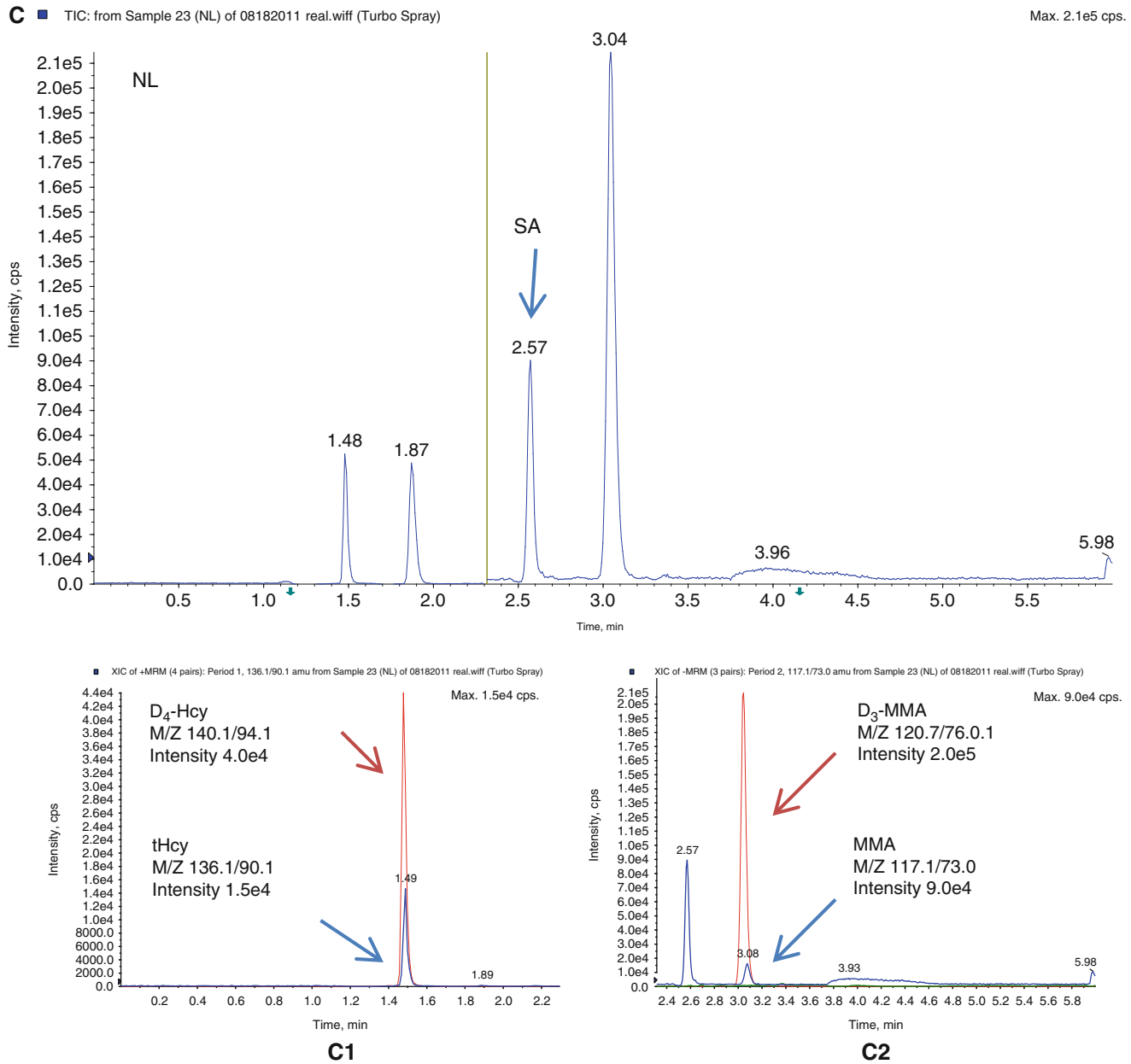

Fig. 3 (continued)

diagnostic information for propionic acidemia, methylmalonic acidemia, and also can be used as a confirmatory test for elevated C3 as observed in newborn screening.

A total of 15 patients diagnosed with disorders in these pathways were reanalyzed when follow-up specimens were received, and all were successfully reconfirmed. One MTHFR patient showed elevated tHcy with a normal Met level. This patient had been receiving Met supplementation.

The assay described can be performed either in diagnostic laboratories or as second-tier, follow-up test in newborn screening laboratories. Our assay is characterized by simple sample preparation (without derivatization), rapid chromatography, high specificity, and small sample size. Simultaneous determination of MMA and tHcy in the same serum or plasma specimen is useful in the diagnosis of both cobalamin and folate deficiencies. The inclusion of methionine may give additional information and can also be diagnostic for methionine disorders. Simultaneous determination of 2MCA provides a differential diagnosis of methylmalonic acidemia versus propionic acidemia and is especially useful for follow-up of newborns screened with elevations of C3-acylcarnitine. A dried blood spot method is currently being evaluated in our laboratory.

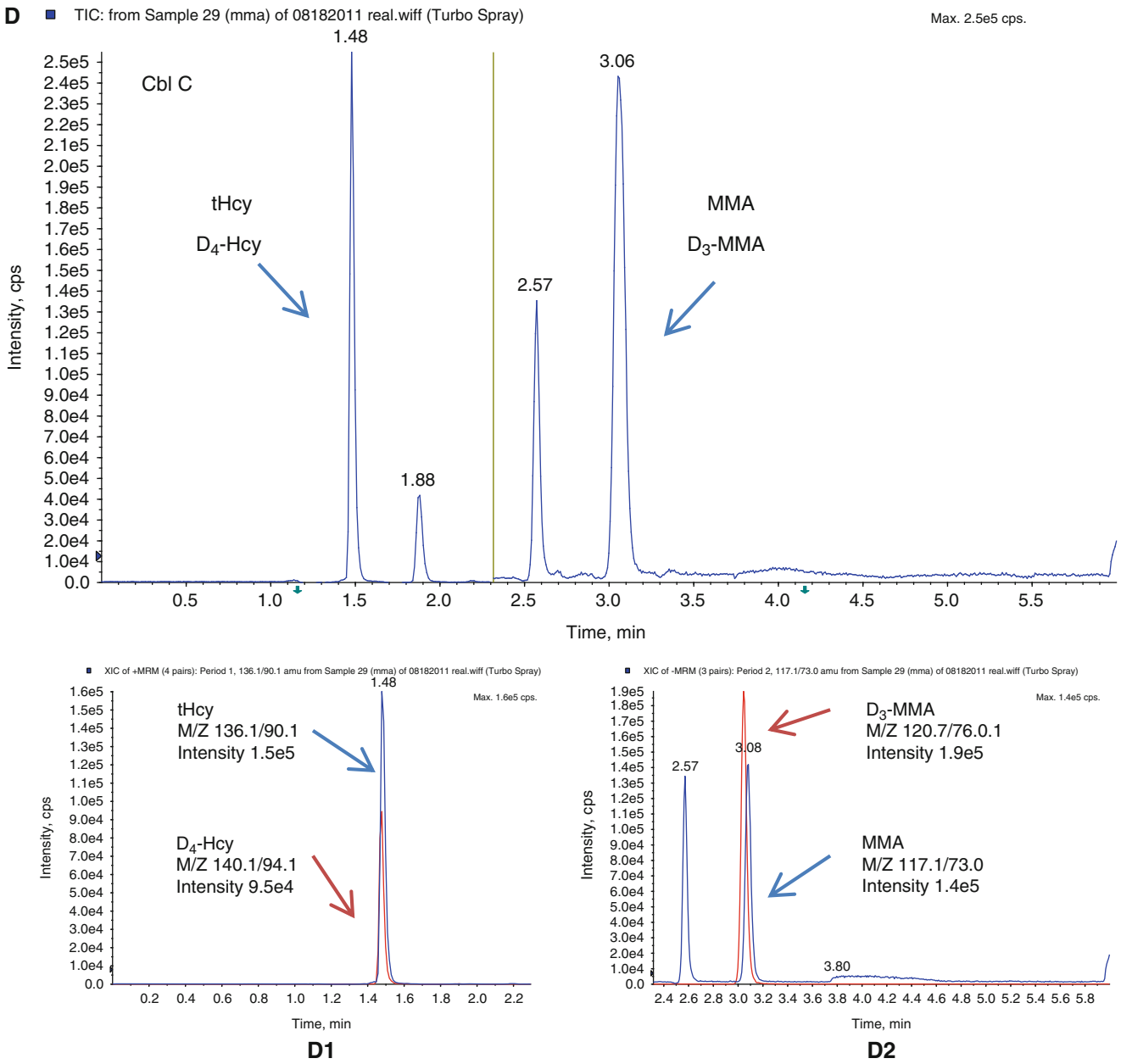

Fig. 3 Multiple reaction monitoring chromatograms of (**A**) standard solution (STD); (**B**) a patient with propionic acidemia (PPA); (**C**) a normal individual (NL), and (**C1**) scan intensity of tHcy (*blue*) and internal standard D4-Hcy (*red*) in this normal individual, and (C2) scan intensity of MMA (*blue*) and internal standard, D3-MMA (*red*) in this normal individual; (**D**) a patient with cobalamin C defect (Cbl C), and (**D1**) scan intensity of tHcy (*blue*) and internal standard D4-Hcy (*red*) in this Cbl C defect patient, and (**D2**) scan intensity of MMA (*blue*) and internal standard D3-MMA (*red*) in this Cbl C defect patient. *tHcy* total homocysteine, *D4-Hcy* D4-homocysteine, *MMA* methylmalonic acid, *D3-MMA* D3-methylmalonic acid, *2MCA1* 2-methylcitric acid 1, *2MCA2* 2-methylcitric acid 2, *SA* succinic acid. The *yellow line* in the middle represents the switch from positive ESI mode to negative ESI mode. The run time is 6 min

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CASE REPORT

An Exceptional Family with Three Consecutive Generations Affected by Wilson Disease

James T. Bennett · Kathleen B. Schwarz · Phillip D. Swanson · Si Houn Hahn

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Abstract Wilson disease (WD) is a disorder of copper transport that can cause hepatic and neuropsychiatric symptoms. Because of its broad spectrum of clinical manifestations that can present in almost any decade of life, a high degree of clinical suspicion is needed for diagnosis. We present an exceptional family with three consecutive generations affected by WD. Autosomal recessive disorders are not typically present in consecutive generations, but this can occur, particularly when carrier frequencies are as high as in WD. This point is of critical importance in counseling families affected by WD. This case also highlights the importance of genetic testing in confirming the diagnosis of WD, particularly when there is a positive family history. To our knowledge, this is the first report of WD in three consecutive generations.

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J.T. Bennett · S.H. Hahn
Department of Pediatrics, University of Washington School of Medicine, Seattle Children's Hospital, Seattle, USA

P.D. Swanson
Department of Neurology, University of Washington School of Medicine, Seattle, USA

K.B. Schwarz
Division of Pediatric Gastroenterology and Nutrition, Department of Pediatrics, Johns Hopkins University Medical Institutions, Seattle, USA

S.H. Hahn (✉)
B6594 Biochemical Genetics, 4800 Sand Point Way NE, Seattle, WA 98105, USA
e-mail: sihoun.hahn@seattlechildrens.org

Abbreviations
WD Wilson disease

Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper transport. The causative gene, *ATP7B*, was identified in 1993 and remains the only gene associated with this disorder (Bull et al. 1993; Petrukhin et al. 1993; Tanzi et al. 1993; Yamaguchi et al. 1993). *ATP7B* is required for efficient transport of copper from hepatocytes into the biliary system (Sato and Gitlin 1991). In the absence of ATP7B function, there is toxic accumulation of copper in various body tissues, resulting in a wide variety of symptoms, including acute and chronic hepatitis, liver failure, and neurologic dysfunction. The wide spectrum of clinical manifestations, which can present in almost any decade of life, can make diagnosis challenging. However, early and accurate diagnosis is critical as oral chelating agents such as penicillamine and trientine, or agents that reduce intestinal copper absorption such as zinc, are simple, effective, and can prevent the development of neurologic and hepatic symptoms (Roberts and Schilsky 2008). The diagnosis of WD has traditionally been made by identifying low serum ceruloplasmin levels and elevated urine and hepatic copper levels, but because the biochemical values can overlap with those of healthy controls, the sensitivity and specificity of these tests can be problematic. For this reason, direct sequencing of the ATP7B gene has been suggested to be a critical component of the diagnosis of WD (Bennett and Hahn 2011). Indeed the sensitivity of ATP7B sequencing is estimated to be greater than 95% (http://www.ncbi.nlm.nih.gov/books/NBK1512/). The utility of direct gene sequencing is even greater in cases

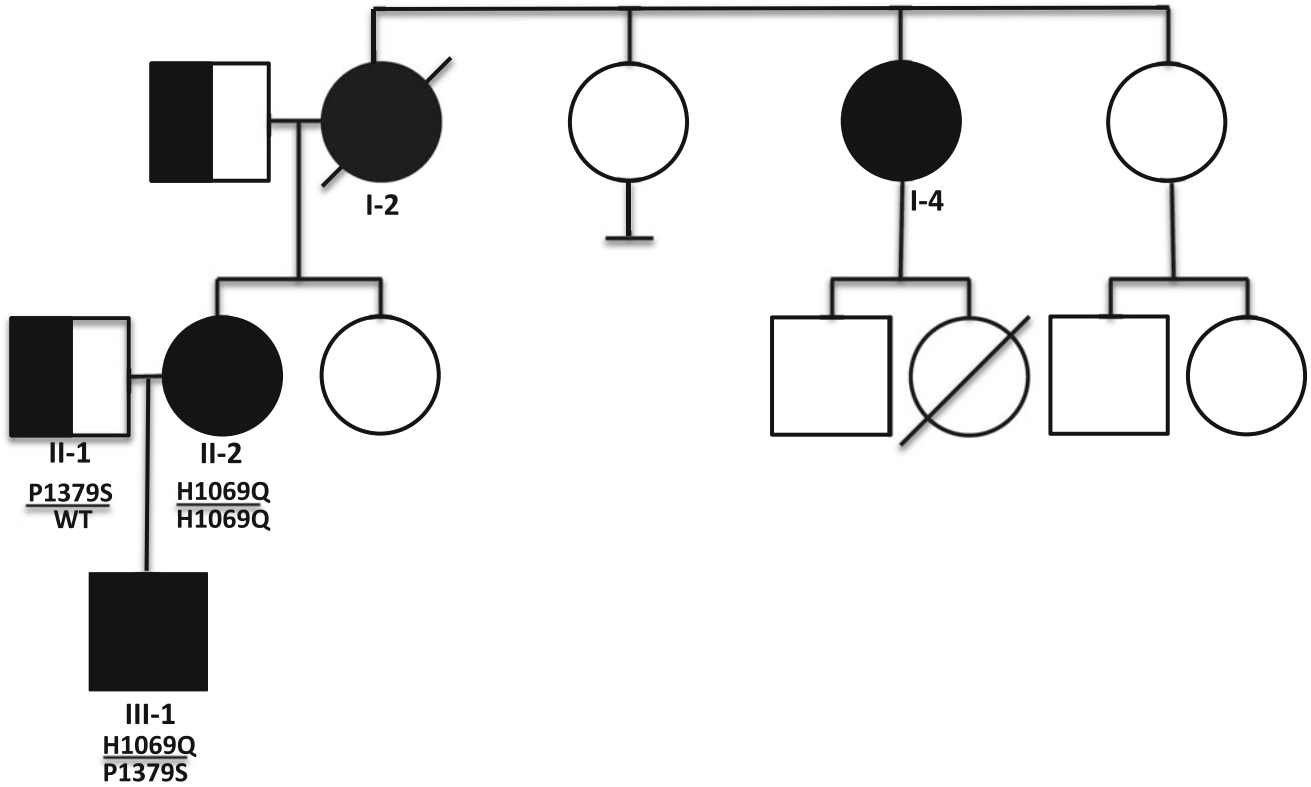

Fig. 1 Individuals affected with Wilson disease are fully filled in with *black*; obligate carriers are indicated by *half-filled symbols*. ATP7B genotypes are also indicated below each individual, when known

with a positive family history, as presymptomatic patients can be easily identified without ambiguity or invasive liver biopsy, allowing therapy to begin and prevent symptoms from ever developing. The ease and effectiveness of treating presymptomatic patients has prompted proposals to include WD in newborn screening (Kroll et al. 2006; deWilde et al. 2008).

In this report, we describe an exceptional family with three consecutive generations affected by WD. Although this is not a typical pattern for autosomal recessive disorders, it can occur, particularly when the carrier frequency is as high as is seen in WD. In addition, this family highlights the importance of genetic testing in diagnosing Wilson disease, as gene testing was used to confirm the diagnosis in two asymptomatic individuals who will, because of that test, be maintained on effective, life-saving therapies.

Case Report

The proband (II-2, see Fig. 1) presented for management of WD after moving cross-country at 28 years of age. She was diagnosed at 12 years of age after undergoing a diagnostic workup because her mother (I-2) had died of WD. At that time, the proband had mild elevations in her liver function tests (AST/ALT = 57/110 IU/L) and an elevated urine copper (220 ug/24 h, normal range 15–60). Her ceruloplasmin was normal (26 mg/dL, normal range 21–53), and liver biopsy showed mild focal portal inflammation and occasional hepatocytes positive for copper staining. She was started on penicillamine and has remained asymptomatic, without hepatic or neurologic symptoms.

Her mother (I-2) developed symptoms of liver failure, including ascites and esophageal varices, at age 39 years. At that time, she had an elevated urine copper (202 ug/24 h) and normal ceruloplasmin (24 mg/dL). Liver biopsy showed macronodular cirrhosis with marked hepatocellular copper accumulation. A slit lamp exam revealed Kayser-Fleischer rings. She was treated with penicillamine but died from complications of liver failure within 6 months of her presentation. Her three sisters and two daughters were screened for WD at that time. One of her sisters (I-4) had an elevated urine copper (464 ug/24 h) as well as hepatocellular copper accumulation on liver biopsy. She was 45 years old at that time. She developed signs of liver failure but responded to penicillamine. She is currently 63 years old and asymptomatic. The remaining family members' screens were normal. Genetic testing was not performed as the gene for WD had not yet been identified.

Table 1 Laboratory values for individuals in this family affected by Wilson disease

	AST (U/L)	ALT (U/L)	Urine copper (ug/24 h)	Ceruloplasmin (mg/dL)
Normal range	5–41	6–40	15–60	21–53
I-2	Unknown	Unknown	202	24
I-4	Unknown	Unknown	464	Unknown
II-2	57	110	220	26
III-1	45	22	Not done	30

To confirm II-2's diagnosis, we analyzed the sequence of ATP7B. She was homozygous for p.H1069Q, the most common WD associated allele (Thomas et al. 1995). Her treatment was changed from penicillamine to zinc acetate because she was trying to get pregnant.

A year later, II-2 became pregnant. Her husband's carrier testing showed that he is a heterozygote for a known pathogenic mutation (p.P1379S) in ATP7B (Cox et al. 2005). The family was appropriately counseled that there was a 50 % chance of their child being affected with WD. Prenatal testing was not pursued, but postnatal genetic testing showed that their newborn son (III-1) is a compound heterozygote for p.H1069Q/p.P1379S. He is currently 21 months of age, healthy, and developing normally. He has not yet been started on any dietary restriction and was started on low-dose zinc treatment at 16 months of age. At 1 year of age, prior to starting zinc treatment, he had a very mildly elevated AST with a normal ALT and ceruloplasmin (see Table 1).

In this exceptional family, there are four individuals in three consecutive generations affected with WD. To our knowledge, this has not been previously reported. There is no known consanguinity in this family.

Discussion

The availability of safe and effective treatment for WD highlights the importance of early and accurate diagnosis. However, there are reports of patients being misdiagnosed with WD and inappropriately treated, which has resulted in symptoms of copper deficiency (Kumar et al. 2003). When our proband (II-2) initially presented to us, she had never had any signs or symptoms of hepatic or neurologic dysfunction, and we did not initially have records of her childhood testing. Since WD does not usually present in consecutive generations, we wondered if her diagnosis was correct. Confirmatory genetic testing allowed unambiguous confirmation of her diagnosis and provided clear guidance that continued therapy for WD was indicated.

The presence of multiple affected generations is atypical for autosomal recessive disorders. In the absence of consanguinity, the chances of it occurring are related to the population carrier frequency. The prevalence of WD has been estimated to be 1/30,000, though it can be significantly higher in some populations (Dedoussis et al. 2005). This family highlights the fact that, with an estimated carrier frequency of 1/90 (Olivarez et al. 2001), WD is not rare. Given this carrier frequency, the chance of an individual with WD having an affected child is one half of one percent (½ × 1/90), and the chance of having an affected child and grandchild is ~0.003 % (½ × 1/90 × ½ × 1/90). There is one previous report of WD occurring in two generations, but consanguinity was present (Firneisz et al. 2001). Our case is, to our knowledge, the only report of WD occurring in three consecutive generations. It also underscores the utility of ATP7B sequence analysis in confirming a diagnosis of WD, particularly when there is a positive family history. As Table 1 indicates, all the individuals in this family with WD had normal ceruloplasmin levels. Although variants of uncertain significance remain a complication of ATP7B sequencing, the biochemical tests of WD can also be misleading (Bennett and Hahn 2011).

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Author Contributions

JB, KS, PS, and SH saw patients and contributed clinical information. JB and SH wrote the manuscript.

Disclosures

There are no conflicts of interest.

Take-Home Message

Providers must counsel their patients with Wilson disease carefully about recurrence risks since there can be vertical transmission of WD due to the fact that the carrier frequency is fairly high.

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CASE REPORT

Sight-Threatening Phenylketonuric Encephalopathy in a Young Adult, Reversed by Diet

S. Rubin · A.L. Le Piffer · M.B. Rougier · M.N. Delyfer · J.F. Korobelnik · I. Redonnet-Vernhet · C. Marchal · C. Goizet · S. Mesli · C. Gonzalez · H. Gin · V. Rigalleau

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Abstract Phenylketonuria (PKU) leads to severe neurological disorders in childhood, shunned by the diet. The long-term prognosis after diet diversification at adolescence is uncertain. We report a case of cortical blindness in a young patient regressive 1 month after the diet was resumed.

Mr M., 25 years old, had PKU detected at birth. He maintained good serum levels of Phenylalanine (Phe) (120–300 μmol/L) during childhood and got a normal intellectual development. During adolescence he diversified his diet but maintained low meat and fish intake; Phe was ~1,200 μmol/L with no symptoms. In 2009, the patient stopped the low-Phe amino acid substitutes due to weariness. On June 27, 2011, he consulted for a decrease of visual acuity progressing for 6 months. Ophthalmologic examination found that visual acuity was 2/10 in two eyes associated to a central visual field defect. The visual evoked potentials were altered. MRI showed bilateral and symmetric occipital FLAIR hyperintensities. On admission in the Nutrional Unit on June 29, 2011, blood pressure was 120/70 mmHg, there was no other neurological abnormality. Phe was at 1,512 μmol/L, and not responsive to BH4. He was then treated with a very low-Phe diet with an amino acid substitute, and he obtained Phe between 120 and 300 μmol/L. Visual acuity was suddenly restored on August 1, 2011, with a dramatic attenuation of the MRI hyperintensities.

Our observation shows that the withdrawal of the diet and substitutes exposes to serious neurological complications in adults that may reverse with a fast nutritional support.

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S. Rubin (✉) · C. Gonzalez · H. Gin · V. Rigalleau
Service de Nutrition, CHU de Bordeaux, Avenue de Magellan, 33604, Pessac, France
e-mail: Sebastien.rubin@chu-bordeaux.fr

A.L. Le Piffer · M.B. Rougier · M.N. Delyfer · J.F. Korobelnik
Ophthalmology, CHU de Bordeaux, Bordeaux, France

I. Redonnet-Vernhet · S. Mesli
Biochemistry, CHU de Bordeaux, Bordeaux, France

S. Rubin · A.L. Le Piffer · J.F. Korobelnik · C. Goizet · C. Gonzalez · H. Gin · V. Rigalleau
Université Bordeaux Segalen, Bordeaux, France

M.N. Delyfer · J.F. Korobelnik · I. Redonnet-Vernhet · V. Rigalleau
Centre INSERM U897-Epidémiologie-Biostatistique, INSERM, ISPED, Bordeaux, France

C. Marchal
Neurology, CHU de Bordeaux, Bordeaux, France

C. Goizet
Genetics, CHU de Bordeaux, Bordeaux, France

A 25-year-old man was referred to the ophthalmic department in June 2011 for a progressive painless bilateral visual loss of few months' duration. After correction, visual acuity was 20/200 in both eyes. Slit lamp and fundus examinations were normal, no optic nerve atrophy was observed. The visual field examination showed a large bilateral central scotoma and pattern reversal visual evoked potentials were flat in the different spatial frequencies. The diagnosis of cortical blindness was evoked, confirmed by Cerebral Magnetic Resonance Imaging which disclosed symmetric high signal intensity on T2-weighted images in the periventricular white matter and cortico-subcortical occipital lobes (Fig. 1a).

As his only past medical history was phenylketonuria (PKU), the patient was quickly transferred to the nutrition department on June 29, 2011. PKU had been diagnosed at birth, immediately treated by diet, and phenylalanine levels

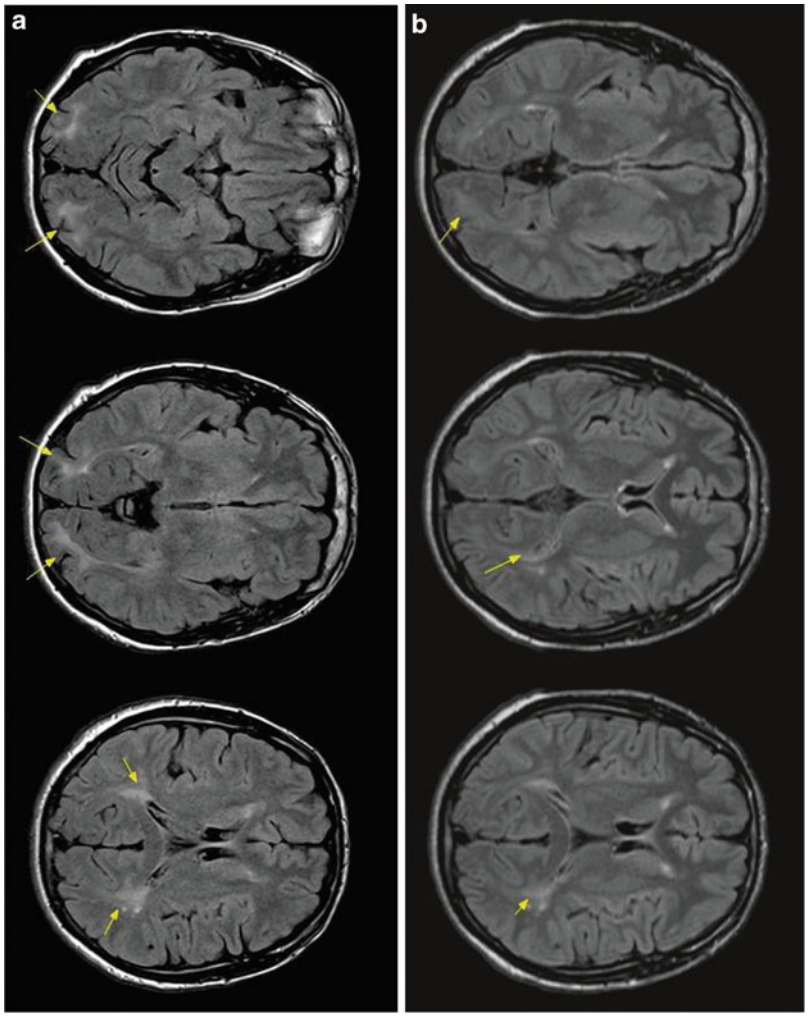

Fig. 1 MRI T2 weighted images (**a**) MRI on admission. High signal intensity of white matter in occipital lobes and periventricular areas. (**b**) MRI after 5 weeks of optimal diet. Signals were decreased

(Phe) were within target levels (120–300 μmol/L) during childhood. During adolescence dietary compliance was less good, although he still consumed Phe-free substitutes. Phe levels increased to ~1,200 μmol/L with no appearance of symptoms. He stopped the Phe-free substitutes during the summer of 2009.

On admission, Phe was 1,512 μmol/L. He was then treated with a low-Phe diet (300 mg/day) and amino acid substitutes; Phe were monitored daily by Guthrie tests. After 1 week, Phe reached a plateau at 400 μmol/L and the patient reported a slight visual improvement. The diet was strengthened (Phe 240 mg/day), the target Phe level (<300 μmol/L) was obtained on July 25, 2011, and the patient was discharged. A few days later, on waking he realized his sight had returned.

On August 01, 2011, the visual acuity was 20/20 in both eyes. Visual field examination showed normalization in his right eye, with a persistent central scotoma in his left eye. Pattern reversal visual evoked potentials improved as P-100 reappeared in large size patterns. On MRI examination, the white matter abnormalities in the occipital lobes were drastically reduced (Fig. 1b).

We report the first case of a young patient who lost vision after withdrawing from the low-Phe diet, and regained it by change in diet. Screening by Guthrie test and a prompt phenylalanine-free diet can prevent the dramatic neurological consequences of PKU in childhood (Blau et al. 2010 Oct 23). In many cases, the patients stop adherence to a strict diet during adolescence, and do well. However, in adults, poorly controlled PKU can be complicated by seizures or cognitive defects and white matter injury (Thompson et al. 1990 Sep 8). Two cases of cortical blindness have been reported. The first patient also had spastic tetraplegia and seizures; he died during follow-up (Kornguth et al. 1992 Nov 1). The second also had impaired gait and spasticity (Ishimaru et al. 1993). None of

these symptoms reversed as in our patient. The main mechanism of the neurological deterioration in untreated PKU in childhood is the reduction in cerebral protein synthesis, particularly myelin protein, due to low transport of Large Neutral Amino Acids (LNAA), as Phe has the most affinity for LNAA type 1 Transporter (LAT-1). High Phe level is also responsible of an impaired cholesterol synthesis (one of the primary constituents of myelin lipid) by activity reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme of cholesterol synthesis. The disease causes a neurotransmitter reduction (dopamine and catecholamines by competition between brain Phe and Tyrosine for hydroxylation by tyrosine hydroxylase and serotonin by unclear mechanisms). In adults treated during childhood like our patient, mechanism of white matter pathology is principally a cytotoxic edema partially due to a worse activity of Na-K ATPase in PKU. (Anderson and Leuzzi 2010) Consequence of this is the swelling of myelin sheaths. This would affect relaxation times and restrict diffusivity on MRI (Vermathen et al. 2007).

These mechanisms are potentially reversible with Phe restriction (de Groot et al. 2010) which we instigated promptly in our case. Our patient's history argues for maintenance of a strict diet to control PKU in adults.

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RESEARCH REPORT

Cardiac Ultrasound Findings in Infants with Severe (Hurler Phenotype) Untreated Mucopolysaccharidosis (MPS) Type I

L. Schroeder · P. Orchard · C.B. Whitley · J.M. Berry · J. Tolar · W. Miller · E.A. Braunlin

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Abstract *Background:* Serious cardiac valve disease and left ventricular hypertrophy occur in most untreated older children with severe mucopolysaccharidosis type I. Although it is assumed that early intervention prevents these processes, evaluation of cardiac findings in these infants has not yet been reported.

Methods: We reviewed echocardiograms of 13 untreated infants < 1 year of age with severe mucopolysaccharidosis type I who had undergone evaluation for hematopoietic cell transplantation. We recorded left ventricular chamber dimensions, septal and posterior wall thicknesses, ventricular function, and aortic sinus diameters. We evaluated mitral and aortic valves for increased thickness, regurgitation, and stenosis.

Results: Average age (7M, 6F) was 221 (range 25–347) days. Left ventricular chamber dimension was ≥2 SD of normal in 3/13; wall thicknesses were ≥2 SD of normal in 2/13 infants. Systolic function was normal. Mitral valves were thickened in all infants; mitral regurgitation was present in 9/13, but significant in only three infants. Aortic valves were thickened in 10/13, but no infant had significant aortic regurgitation. Neither mitral nor aortic stenosis occurred. Aortic roots were dilated to ≥2 SD of normal in 5/13.

Conclusions: Characteristic cardiac features of severe mucopolysaccharidosis type I can be seen in infancy. Mitral and aortic valve thickening are nearly universally present, even in the youngest infants. In 20–30 % of infants, other abnormalities such as left ventricular dilation, increased wall thickness, and mild mitral/aortic regurgitation may occur. Aortic root dilation is a frequent finding. Early intervention with enzyme replacement therapy may minimize the incidence and severity of cardiac findings in these infants.

Summary: Serious cardiac valve disease and left ventricular hypertrophy occur in most untreated older children with severe mucopolysaccharidosis type I. Although it is assumed that early intervention prevents these processes, evaluation of cardiac findings in these infants has not yet been reported. In our study of 13 infants with severe untreated MPS I < 1 year of age, mitral and aortic valve thickening was nearly universally present and aortic root dilation was frequent. Despite this, we found a lower incidence of left ventricular hypertrophy and both a lower incidence and milder expression of mitral and aortic valve dysfunction than previously reported in older children. These findings suggest that earlier intervention, including neonatal screening, may be of benefit to children with severe MPS I.

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L. Schroeder · P. Orchard · C.B. Whitley · J.M. Berry · J. Tolar · W. Miller · E.A. Braunlin
Department of Pediatrics, University of Minnesota, Minneapolis, MN, USA

E.A. Braunlin (✉)
Department of Pediatrics, Division of Pediatric Cardiology, University of Minnesota, 2450 Riverside Ave., Minneapolis, MN 55454, USA
e-mail: braun002@umn.edu

Abbreviations

ASE	American Society of Echocardiography
BSA	Body surface area
ERT	Enzyme replacement therapy
GAG	Glycosaminoglycan
HCT	Hematopoietic cell transplantation
IVSd	Interventricular septal thickness in diastole
LVID	Left ventricular internal dimension in diastole
LVIS	Left ventricular internal dimension in systole
IVSd	Intraventricular septal wall thickness in diastole

LVPWd	Left ventricular posterior wall thickness in diastole
MPS I	Mucopolysaccharidosis Type I
SD	Standard deviation
SF	Shortening fraction

Introduction

Severe mucopolysaccharidosis type I (MPS I, OMIM # 607014) is a lethal autosomal recessive disorder that is caused by mutation of the genes encoding α-L-iduronidase (IDUA), an enzyme responsible for the degradation of glycosaminoglycan (GAG). GAGs are ubiquitous and essential components of virtually all mammalian tissue (Neufeld and Muenzer 2001). GAG deposition in severe MPS I may occur in the central nervous system leading to blindness, deafness, increased intracranial pressure, and mental retardation; in the joints and bones leading to short stature, dysostosis multiplex, and stiff and poorly mobile joints; and in the airways and soft tissues leading to sleep apnea and other respiratory difficulties. GAG deposition in the heart is associated with myocardial hypertrophy, narrowing of the coronary arteries, aortic root dilation, dilated cardiomyopathy, and thickened aortic and mitral valves resulting in stenosis and/or regurgitation (Braunlin 2012).

The progressive nature of severe MPS I is well established (Neufeld and Muenzer 2001), and it is becoming evident that neither hematopoietic cell transplant (HCT) nor enzyme replacement therapy (ERT) can reverse some pathological processes such as dysostosis multiplex (Weisstein et al. 2004; Sifuentes et al. 2007), corneal clouding (Gillingsrud et al. 1998), and cardiac valve abnormalities (Braunlin et al. 2003, 2006) in older children. The initiative for earlier diagnosis (e.g., newborn screening) and treatment of severe MPS I has been motivated by recent case reports which have shown that ERT, when given to infants less than 1 year of age, may prevent, delay, or attenuate the course of the disease (Gabrielli et al. 2010; McGill et al. 2010; Tylki-Szymanska et al. 2012). The success of this initiative will depend upon the incidence and severity of cardiac pathology found in untreated infants < 1 year of age with severe MPS I; evaluating these issues forms the basis of this study.

Methods

Patients. This study was reviewed by the Institutional Review Board of the University of Minnesota and deemed an exempted study. The files of the University of Minnesota Bone Marrow Transplant Database were reviewed for patients with severe MPS I who were (1) treated with HCT; (2) had undergone mutation analysis; (3) had an initial echocardiogram obtained at < 1 year of age prior to any therapy (e.g., ERT); and, (4) had an echo available for review. Additional parameters assessed included urinary GAG analysis and leukocyte enzyme analysis. Since 2005, all patients referred for HCT have undergone mutation analysis and cardiac ultrasounds have been digitally recorded and stored. Height and weight were recorded within 24 h of performance of ultrasound and body surface area (BSA) was calculated according to the method of Haycock (Lopez et al. 2010). Cardiac ultrasounds were obtained in un-sedated infants using either Sonos 5500 or iE33 cardiac ultrasound equipment (Phillips Corporation, Bothell, Washington, USA) with 8 and 12 MHz transducers. All echocardiograms were reviewed by two of the authors (EB, JMB) and compared to the reports generated for the medical record. Interpretation differences between the original report and the reviews for this study were minimal and resolved by discussion between the two authors.

Chamber dimensions and function. All studies included two-dimensional imaging and M-mode measurement of left ventricular chamber dimensions in diastole and systole (LVID and LVIS, respectively) and left ventricular septal and posterior wall thicknesses in diastole (IVSd and LVPWd, respectively). Shortening fraction (SF) was calculated following standard guidelines (Lopez et al. 2010). BSA-based Z-scores were determined for each infant's cardiac dimensions (LVID, LVPWd, and IVSd) using standard methods (http://parameterz.blogspot.com), where, for a given BSA, a Z-score of 0 indicates the mean value, and a Z-score of +2 indicates a value 2 standard deviations greater than expected.

Cardiac valves. Detailed two-dimensional and M-mode imaging of mitral and aortic valves was performed to evaluate for increased thickness, which was graded as absent or present. Spectral and color-flow Doppler interrogation of aortic and mitral valves was performed in standard views (Lai et al. 2006). Mitral and aortic valve regurgitation was qualitatively graded from color-flow images as absent, trace (central color jet <5 % diameter and length of the left atrium or ventricle, respectively), mild (central color jet between 5 % and 20 % diameter and length of left atrium or ventricle, respectively), or moderate (central color jet >20 % diameter and length of left atrium or ventricle, respectively). The presence of mitral or aortic stenosis was determined by comparison of pulsed-Doppler measurement of peak E-wave flow signal across the mitral valve and peak systolic flow signal across the aortic valve to established normal pediatric values (Hatle and Angelsen 1985).

Aortic root. Measurement of the aortic root at the aortic sinuses was performed during peak systole (inner wall to inner wall) by two-dimensional imaging according to ASE

Table 1 Demographics, urinary GAGs, leukocyte α-L-iduronidase levels, and IDUA mutations of infants <1 year of age with severe untreated MPS I

Patient	Gender	Age at echo (Days)	u-GAG	IDUA activity (nmol 4MU/h/mg protein)	Mutation 1	Mutation 2
1	M	134	1645.44*	0	Q70X	35del12
2	M	262	114.7**	0	W402X	W402X
3	F	212	135.4**	0	W402X	W402X
4	M	319	172.4**	0	Y167X	W402X
5	M	323	186**	2	W402X	W402X
6	F	347	1300**	0.7	W402X	W402X
7	M	320	N/A	1.1	Q70X	Q70X
8	F	178	116**	0.5	Q70X	W402X
9	F	57	N/A	0.6	Q70X	Q70X
10	M	256	311**	0	Q70X	c386-2A>G
11	F	180	80.9**	0.5	Q70X	W402X
12	M	256	366-488*	0	Q70X	C386-2A>G
13	F	25	N/A	0.5	W402X	W402X

*mg GAG/g creatinine

**mg GAG/mmol creatinine

guidelines (Lopez et al. 2010). BSA-based Z-scores were calculated from each of these measurements, as previously described.

Statistics. Analysis of differences between groups were made using either Fisher's exact test for analysis of 2 x 2 contingency tables and unpaired Student *t*-test for continuous variables with significance found when $p < 0.05$ (http://graphpad.com).

Results

Between 2005 and 2012, there were 13 infants (6F, 7M) ranging from 25 to 347 (mean 221) days of age who presented for evaluation prior to HCT and all fulfilled the criteria for this study (Table 1). Males were older, but not significantly so, than females (267 ± 67 vs 165 ± 116 days, $p = 0.0757$). The mean heights of both male and female infants (72.1 ± 2.2 and 65.4 ± 9.0 cm, respectively) were at the 75th percentile for age. The mean weights for males and females (8.8 ± 1.4 kg and 7.3 ± 2.4 kg, respectively) corresponded to the 50th and 75th percentiles for age. Urinary GAG excretion ($N = 10$) was elevated and leukocyte IDUA levels ($N = 13$, range 0–2 nmol 4MU/h/mg protein) were markedly low (Table 1) consistent with the diagnosis of severe MPS I. IDUA analysis demonstrated mutations known to be associated with severe MPS I: 7 infants were homozygous for either W402X or Q70X; 2 were compound heterozygous for W402X/Q70X; 2 were compound heterozygous for Q70X/c386-2A>G; and one each with Q70X/35del12 and W402X/Y167X.

Table 2 Mean and range for Z-scores of left ventricular dimensions and shortening fraction (SF) from cardiac ultrasounds in 13 infants <1 year of age with severe untreated MPS I

Parameter (number measured)	Mean value	Range of individual values
Mean Z-score LVID (13)	1.26 ± 0.86	0.45–3.37
Mean Z-score LVPWd (13)	0.97 ± 1.2	−1.11–2.61
Mean Z-score IVSd (13)	0.38 ± 1.05	−1.47–2.25
Mean Z-score Aortic Sinus (13)	1.55 ± 0.92	0.1–2.48
Mean SF (13)	35.1 ± 4.8	29–45

Mean Z-scores for left ventricular chamber dimensions (LVID), left ventricular wall thicknesses (LVPWd, IVSd), and aortic sinus internal dimensions were all within 2 standard deviations of normal (Table 2); mean shortening fraction was within normal limits. Seven infants had values more than 2 standard deviations greater than normal for one or more of the dimensions (LVID, LVPWd, IVSd, or Aortic Sinus diameter), but none were significantly related to gender, age (≤180 D vs >180 D), or specific mutation (data not shown). The child with the greatest single Z-score (Case 7, LVID-Z-score of 3.37) had a SF at the lower limits of normal, but left ventricular performance appeared depressed and this was confirmed by a depressed measured ejection fraction of 36 %.

Table 3 Summary of mitral/aortic valve findings seen on cardiac ultrasounds in infants <1 year of age with severe untreated MPS I

	Number affected	Doppler velocity mean (range)
Mitral valve		
Incidence thickened mitral valve	13/13 (100 %)	-
Incidence of mitral regurgitation		-
None	4/13 (31 %)	
Trace	6/13 (46 %)	
Mild or moderate	3/13 (23 %)	
Mitral valve stenosis	0/13 (0 %)	
E-wave flow signal (m/s)	13	0.85 ± 0.16 (0.6–1.1)
Aortic valve		
Incidence thickened aortic valve	10/13 (77 %)	-
Incidence aortic regurgitation		-
None	8/13 (62 %)	
Trace	5/13 (38 %)	
Aortic valve stenosis	0/11 (0 %)	
Peak systolic flow signal (m/s)	11	0.84 ± 0.22 (0.49–1.14)

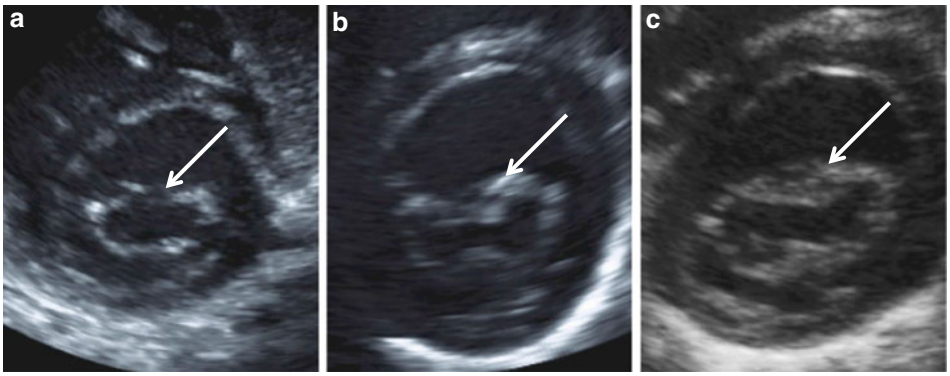

Fig. 1 (**a**–**c**). Two-dimensional cardiac ultrasound images of mitral valve in diastole from (**a**) normal 7 month old infant; (**b**) 25 day old infant with severe untreated MPS I, and (**c**) 256 day old infant with severe untreated MPS I. Note increased thickening of mitral valve, especially in central portion of leaflets (*arrows*) when compared to normal valve

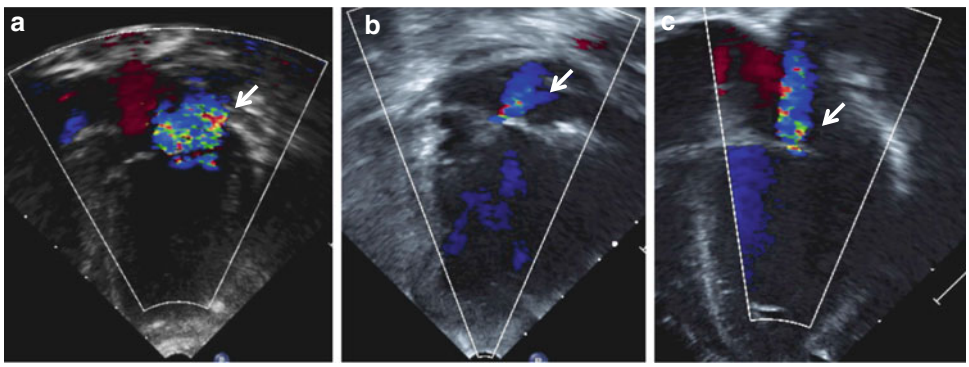

Fig. 2 (**a**–**c**). Color Doppler images from the left ventricle showing moderate mitral regurgitation (*arrows*) in: (**a**) a 212 day infant; and mild mitral valve regurgitation from: (**b**) a 212 day old infant and (**c**) a 25 day old infant, all with untreated severe MPS I

Increased mitral valve thickness was universally present (Table 3, Fig. 1a–c). Despite this finding, mitral regurgitation was either absent or insignificant (trace) in 10/13 (77 %) patients. Significant (mild, or mild to moderate) mitral regurgitation was documented in 3/13 (23 %) infants (Fig. **2**a–c), and was found as early as 25 days of age. Mitral valve stenosis was not identified. Normal mitral inflow patterns (E wave >A wave) were present in all infants (data

not shown); mean E-wave inflow velocity of 0.85 m/s (equivalent to a peak inflow gradient of 2.9 mmHg) was well within the normal limits of 1.3 m/s (6.8 mmHg) recognized in pediatrics (Hatle and Angelsen 1985).

Increased aortic valve thickness occurred in 10/13 infants (77 %) (Table 4, Fig. 3a–c), but aortic regurgitation was either absent (8 infants; 62 %) or insignificant/trace (5 infants; 38 %) (Fig. 4). Aortic valve stenosis was not present. The mean value for peak flow across the aortic valve of 0.84 m/s (equivalent to a gradient of 2.8 mmHg) was well within normal limits of 1.8 m/s (13 mmHg) recognized in pediatrics (Hatle and Angelsen 1985).

There were five infants who were ≤180 days of age at first echo (Table 4). The mean Z-score for IVSd was significantly less for these infants ($p = 0.0215$) when compared to infants >180 days of age, but there was no other significant difference between these two groups for any other parameters (LVID, LVPWd, aortic sinus diameter, or left ventricular function). There was no significant difference in the incidence of Z-scores more than 2 standard deviations greater than normal nor was there a significant difference in the incidence of significant mitral regurgitation between the two groups (data not shown).

Table 4 Comparison of mean Z-scores of cardiac dimensions and cardiac function between infants >180 days and those ≤180 days of age with severe untreated MPS I

Parameter	≤ 180 D ($N = 5$)	> 180 D ($N = 8$)	*p*-value
Age	114.8±70.7	286.9±46.6	**0.0002**
BSA	0.33±0.05	0.44±0.03	**0.0005**
Mean Z-score LVID	0.97±0.66	1.44±0.96	0.3568
SF	33.6±2.7	36±5.7	0.4043
Mean Z-score LVPWd	0.57±1.27	1.22±1.17	0.3727
Mean Z-score IVSd	−0.42±0.93	0.88±0.80	**0.0215**

Discussion

Previously, little information was available regarding the cardiac status of infants with severe untreated MPS I. In this report, we describe a lower incidence of ventricular hypertrophy and both a lower incidence and a milder expression of cardiac valve dysfunction than previously reported in older children with untreated MPS I (Leal et al. 2010; Wippermann et al. 1995). Comparison of the incidence of ventricular hypertrophy between infants and older children is best made with Leal's study, since it also uses Z-scores. Left ventricular hypertrophy occurred in 2/13 (15 %) infants from our study as compared to 5/6 (83 %) in older children (Table 5). Unfortunately, Leal's study does not differentiate between severe and attenuated MPS I, so it may not be representative for either the incidence of hypertrophy or valve findings in patients with a clear severe phenotype. The incidence of cardiac valve findings in Wippermann's study of older children with severe MPS I is a good comparison to our infant study since color Doppler was used in all of his echocardiograms. While increased mitral valve thickness occurred universally in both our infants and Wippermann's older children, only 3/13 (23 %) of our infants had mitral regurgitation compared to 10/12 (82 %) of his older children. Mitral regurgitation was only mild or mild/moderate in our three infants, but was moderate or severe in the majority of his older children. Mitral stenosis did not occur in our infants but was seen in 2/12 (17 %) of this older cohort. While increased aortic valve thickness occurred commonly in both our infants and Wippermann's older children, significant aortic valve regurgitation or stenosis did not occur in our infants but was present in 4/12 (25 %) of the older children.

Although cardiac pathology appeared milder, clinical expression of MPS was already evident in infancy as supported by the presence of increased mitral valve

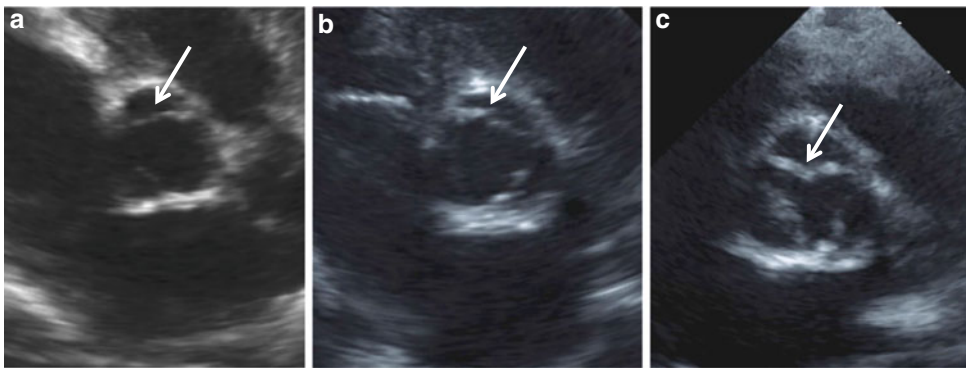

Fig. 3 (**a**–**c**). Two-dimensional short axis images of: (**a**) normal aortic valve in 3 month old infant; and thickened aortic valves in (**b**) 25 day old infant and (**c**) 256 day old infant, both with severe untreated MPS I. Compare the thin, nearly transparent aortic leaflets (*arrow*) in (**a**) with those of (**b**) and (**c**)

thickness in all infants (Braunlin et al. 2011). In addition, significant mitral valve regurgitation was present in 23 % of these infants and was seen as early as 25 days of life. Although our numbers are small, there did not appear to be a relationship between valve regurgitation and early or late infancy, gender, or mutation. It is unknown at present whether early intervention can arrest or reverse the pathological process within these very young cardiac valves.

While the mean left ventricular chamber dimensions and left ventricular wall thicknesses were all within 2 SD of normal, there were outliers (infants with Z-scores >2 SD normal). Two infants had wall thickness Z-scores >2 SD of normal and three others had Z-scores for left ventricular chamber dimension >2 SD of normal. Similarly, mean shortening fraction was normal in all infants although the measured ejection fraction in a single infant (Case #7) was moderately depressed. These parameters are known to respond favorably to either ERT or HCT (Braunlin et al. 2003, 2006; Wiseman et al. 2012) and this was true as well for Case 7 whose ejection fraction normalized after a period of ERT. Surprisingly, dilation of aortic sinus diameter to >2 SD of normal occurred in 5/13 (38 %) infants within the first year of life. The arrest or reversibility of this finding with early treatment is unknown.

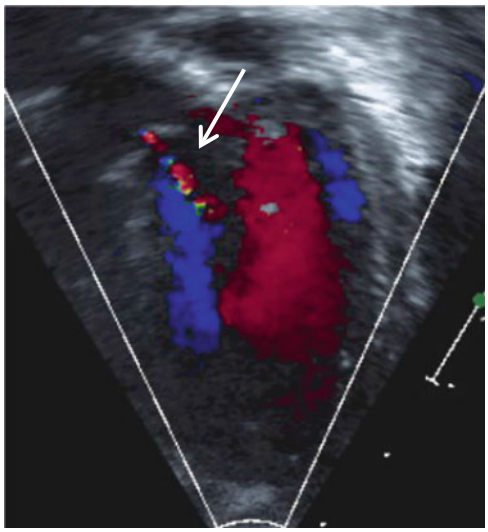

Fig. 4 Color Doppler image from left ventricle showing trace aortic regurgitation in 178 day old infant with severe untreated MPS I. Note narrow central jet (*arrow*) extending into left ventricular outflow tract only

The presence of significant coronary artery involvement is an important feature of severe MPS I even in infancy (Brosius and Roberts 1981; van den Broek et al. 2011) but has not been investigated in this, or other, reported series. It has been shown that critical coronary artery disease can occur very early in life in severe MPS I and may be a limiting factor even in the early treatment of this syndrome (van de Broek et al. 2011).

The incidence and severity of ventricular hypertrophy, depressed function, and valve pathology in MPS has been the subject of many reports over the past 35 years (Dangel 1998; Schieken et al. 1975; Gross et al. 1988; Fesslova et al. 2009; Mohan et al. 2002). It is important to recognize that during this time echo technology has evolved from the initial grainy M-mode images seen in 1975 to the high-resolution two-dimensional images of today. In addition, both spectral and color Doppler were developed, with color Doppler being the more recent technology. The presence of

Table 5 Comparison of infants with severe, untreated MPS I cardiac data with published studies of older cohorts

	Current study	Leal et al. 2010	Wippermann et al. 1995
Mean age (years)	0.6	6.8	3.8
Incidence Z-score > 2 SD wall thickness	2/13 (15 %)	5/6 (83 %)	Not reported
Mitral valve			
Incidence thickened mitral valve	13/13 (100 %)	5/6 (83 %)	12/12 (100 %)
Incidence of mitral regurgitation			
None	4/13 (31 %)	1/6 (17 %)	2/12 (17 %)
Trace	6/13 (46 %)	0/6 (0 %)	0/12 (0 %)
Mild	3/13 (23 %)	5/6 (83 %)	4/12 (33 %)
Moderate	0/13 (0 %)	0/6 (0 %)	4/12 (33 %)
Severe	0/13 (0 %)	0/6 (0 %)	2/12 (17 %)
Aortic valve			
Incidence thickened aortic valve	10/13 (77 %)	5/6 (83 %)	6/12 (50 %)
Incidence aortic regurgitation			
None	8/13 (62 %)	3/6 (50 %)	8/12 (67 %)
Trace	5/13 (38 %)	0/6 (0 %)	0/12 (0 %)
Mild	0/13 (0 %)	3/6 (50 %)	3/12 (25 %)
Moderate	0/13 (0 %)	0/6 (0 %)	1/12 (8 %)
Severe	0/13 (0 %)	0/6 (0 %)	0/12 (0 %)

cardiac valve stenosis or regurgitation can be difficult to assess by spectral Doppler, but both are easily identifiable by color Doppler. Thus, when reporting the incidence of valve dysfunction in MPS, studies such as Wippermann's utilizing color Doppler for all individuals within the study are likely the most accurate. Similarly, the use of Z-scores to assess the difference of a particular value from normal has been a relatively recent addition to the complete pediatric cardiac echo report (Lai et al. 2006) enabling an assessment not only of the presence of an abnormality but also its extent. Studies such as Leal's use these metrics to permit a more accurate comparison of abnormalities of chamber dimensions and wall thicknesses between studies from various institutions.

For the younger patients with severe MPS I included in this study, the incidence of pathology was less than that of older patients and the severity milder, but it was not absent. The outcome of cardiac valve pathology when treatment with ERT and HCT is begun early in life is currently unknown. The absence of cardiac valve disease after ERT was started at 5 months of age and continued for 5 years as described in the infant reported by Gabrielli et al., while encouraging, may be related in part to the fact that attenuated, rather than severe, MPS I was present in this child. In severe MPS I, there may be some infants with early-onset valve regurgitation or rapidly progressing coronary disease, in whom even early treatment may be too late; however, it would seem reasonable to think that intervention within the first year of life, facilitated by newborn screening, will improve long-term outcomes in severe MPS I for most infants.

References to Electronic Database

MPS I, OMIM phenotype 607014, Locus MIM number 252800

Contributions of Individual Authors

Elizabeth Braunlin conceived the project, participated in the analysis of the data, provided the interpretation of the data, and is the guarantor of the study.

Luke Schroeder participated in the data collection and interpretation as well as critical revisions of the draft of the article.

Chester B. Whitley performed the mutation analysis for the infants and participated in the critical review of the manuscript.

James M. Berry performed the infant cardiac ultrasounds, provided interpretation of the data, and critical review of the manuscript.

Paul Orchard, Weston Miller, IV, and Jakub Tolar participated in the analysis and interpretation of data and critical revisions of the manuscript for intellectual content.

Guarantor

Dr. Elizabeth Braunlin will serve as the guarantor for the manuscript.

Competing Interest Statement

Drs. Elizabeth Braunlin, Paul Orchard, Jakub Tolar, and Chester B. Whitley have received funds for speaking, reimbursement for speaking and traveling to symposia from Genzyme (Orchard, Whitley) and Biomarin (all).

Dr. Braunlin is a consultant on a research study of MPS IV for Biomarin. Dr. Orchard receives research funding from Genzyme.

Dr. Whitley receives research funding from Genzyme and Biomarin.

Biomarin and Genzyme produce enzyme replacement therapy for lysosomal storage diseases. The conclusion of this study, earlier treatment is helpful, may increase the amount of enzyme these companies can sell.

Drs. Schroeder and Miller and Mr. James Berry have no financial interests to declare.

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Details of Ethics Approval

The Institutional Review Board at the University of Minnesota granted approval to access the patient information used in this study.

Patient Consent Statement

Patient consent was obtained prior to accessing any confidential patient information.

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RESEARCH REPORT

A Novel Double Mutation in the *ABCD1* Gene in a Patient with X-linked Adrenoleukodystrophy: Analysis of the Stability and Function of the Mutant ABCD1 Protein

Masashi Morita • Junpei Kobayashi • Kozue Yamazaki • Kosuke Kawaguchi • Ayako Honda • Kenji Sugai • Nobuyuki Shimozawa • Reiji Koide • Tsuneo Imanaka

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Abstract We diagnosed an adrenomyeloneuropathy (AMN) patient with a double novel missense mutation, c.284C>A (p.A95D) and c.290A>T (p.H97L) in a single *ABCD1* allele. In skin fibroblasts from the patient, no ABCD1 protein was detected by immunoblot analysis, and the C24:0 β-oxidation activity was decreased to a level at which the ABCD1 protein was absent. To determine the responsible gene mutation in the patient, we constructed three kinds of mutated *ABCD1* gene expression vectors (c.284C>A, c.290A>T or c.284C>A/c.290A>T) and transfected them into CHO cells stably expressing GFP-SKL (CHO/GFP-SKL cells) or CADDS fibroblasts lacking the *ABCD1* gene. ABCD1 (p.H97L) displayed the correct peroxisomal localization in CHO/GFP-SKL cells, but ABCD1 (p.A95D) and ABCD1 (p.A95D/p.H97L) were diffuse in the cytosol. Furthermore, ABCD1 (p.H97L) was detected by immunoblot analysis and restored the C24:0 β-oxidation activity in the CADDS fibroblasts, as the wild type ABCD1 did. On the other hand, ABCD1 (p.A95D) and ABCD1 (p.A95D/p.H97L) were not detected and the C24:0 β-oxidation activity was not restored. These results clearly show that c.284C>A is the responsible gene mutation, whereas c.290A>T is a novel polymorphism.

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M. Morita · K. Yamazaki · K. Kawaguchi · T. Imanaka (✉)
Department of Biological Chemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama, Japan, Japan
e-mail: imanaka@pha.u-toyama.ac.jp

J. Kobayashi · R. Koide
Department of Neurology, Tokyo Metropolitan Neurological Hospital, 2-6-1 Musashidai, Fuchu, Tokyo 183-0042, Japan

A. Honda · N. Shimozawa
Division of Genomics Research, Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

K. Sugai
Department of Child Neurology, National Center of Neurology and Psychiatry, 4-1-1 Kodaira, Tokyo 187-8551, Japan

Introduction

X-linked adrenoleukodystrophy (X-ALD) (MIM 300100) is a neurodegenerative disease with an incidence of approximately 1:15,000–30,000 males (Kemp et al. 2001; Takemoto et al. 2002; Suzuki et al. 2005). The biochemical characterization of the disease is based on the accumulation of pathognomonic amounts of saturated very long chain fatty acids (VLCFA, >C22) in tissues, including the brain white matter and the adrenal glands as well as skin fibroblasts (Dubois-Dalcq et al. 1999; Smith et al. 1999; Bezman et al. 2001; Moser et al. 2004). The accumulation of VLCFA in X-ALD has been linked to mutations in the *ABCD1* gene [MIM 300371] located at Xq28 (Migeon et al. 1981). The gene codes for an mRNA of 4.3 kb, with a protein of 745 amino acids that is referred to as ABCD1/ALDP (Mosser et al. 1993; Kemp et al. 2011). ABCD1 is a 75 kDa peroxisomal membrane protein that belongs to the ATP-binding cassette protein family and is thought to be involved in the transport of saturated very long chain acyl-CoA into peroxisomes (van Roermund et al. 2008; van Roermund et al. 2011; Morita and Imanaka 2012). Dysfunction of the ABCD1 protein results in a reduction of peroxisomal VLCFA β-oxidation, which leads to the

accumulation of VLCFA. Biochemical diagnosis of this disease is based on the measurement of plasma VLCFA. However, it has not yet been made clear how the accumulation of VLCFA in tissues is involved in the pathogenesis and progress of the disease.

X-ALD has a widely varied disease phenotype, including childhood cerebral ALD, adolescent cerebral ALD, adrenomyeloneuropathy (AMN), and Addison's disease (Moser et al. 2007). The only effective therapy for the cerebral form of ALD is hematopoietic stem cell transplantation at the early stages of the manifestation of the cerebral symptoms. Most AMN patients manifest slowly progressive gait disturbances as the initial symptom. The mean age of onset of AMN is reportedly 30.2 (13–51) years in Japan (Takemoto et al. 2002), and about half of these patients exhibit cerebral involvement approximately 10 years after onset. Therefore, it is necessary to determine the ABCD1 mutations in the presymptomatic diagnosis of X-ALD by extended familial screening of the probands.

Thus far, more than 1,200 *ABCD1* gene mutations have been reported by mutation analysis (see the X-ALD mutation database listed at http://www.x-ald.nl). Missense mutations comprise more than 60% of them. Among the missense mutations, about in approximately 50% of them mutant ABCD1 proteins were not detected and in ~15% of them the protein levels were reduced in X-ALD fibroblasts, based on immunofluorescence and/or immunoblot analysis. It is thus likely that most of the mutant ABCD1 are misfolded and do not insert into peroxisomal membrane correctly, which subsequently results in degradation by a protein quality control-related mechanism. We previously showed that certain mutant ABCD1 proteins are degraded by proteasomes or additional protease(s) before or after transport to peroxisomes (Takahashi et al. 2007).

To date, 13 instances of multiple mutations have been reported in the X-ALD database. Among them, only two double mutants (c.38A>C and c.649A>G, p.N13T and p.K217E as well as c. 707G>A and c.1534G>A, p.R236H and p.G512S, respectively) have been partially characterized (Dvorakova et al. 2001; Guimaraes et al. 2002). In this study, we identified a novel double missense mutation, c.284C>A and c.290A>T in the *ABCD1* gene in a patient with clinical symptoms compatible with AMN. This mutation in exon 1 of the *ABCD1* gene leads to an amino acid exchange of alanine to aspartic acid (p.A95D) and histidine to leucine (p.H97L). In this study, we investigated whether these amino acid substitutions affect the function of the ABCD1 protein. The results show that c.284C>A (p.A95D) is a novel disease-causing mutation and c.290A>T (p.H97L) is a novel polymorphism.

Materials and Methods

Materials

[1-^{14}C]lignoceric acid (53 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). ECL Plus, a Western blotting detection system, and Fluorolink Cy3 labeled goat anti-rabbit IgG, were purchased from GE Healthcare (Buckinghamshire, England). The mouse anti-human ALDP/ABCD1 monoclonal antibody (MAB2162) was purchased from Millipore (Billerica, MA). The rabbit anti-PMP70/ABCD3 antibody was raised against the COOH-terminal 15 amino acids of rat PMP70/ABCD3 (Imanaka et al. 1996). The rabbit anti-catalase antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA).

Cell Culture

CHO-K1 cells were cultured in F12 medium with 10% FCS containing streptomycin and penicillin at 37°C and 5% CO_2. CHO/GFP-SKL cells, which were prepared by the transfection of a pEGFP/SKL vector into CHO cells, were cultured in the same medium containing G418. Human skin fibroblasts from a healthy individual, an ALD patient and a contiguous ABCD1 DXS1357E deletion syndrome (CADDS) patient were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. CADDS were characterized by deletions that extended into the promoter region of ABCD1 and the neighboring gene (DXS1357E) (Corzo et al. 2002).

ABCD1 Gene Analysis

PCR products of the *ABCD1* gene were amplified by means of a modification of the method of Boehm et al. (Boehm et al. 1999) using genomic DNA extracted from blood of the patient that covered all of the coding regions and exon-intron junctions of the *ABCD1* gene. DNA analysis was based on informed consent.

VLCFA Analysis

VLCFA in serum from the patient were determined using gas chromatography/mass spectrometry (GC/MS), as described previously (Takemoto et al. 2003).

Plasmid Constructions and Transfection

pcDNA4His/ABCD1 was prepared as described previously (Takahashi et al. 2007). The human *ABCD1* gene was amplified with a PCR protocol using pcDNA3.1+/ABCD1 (kindly provided by Dr. K. Kamijo, Tohoku University) as

the template. The amplification product was cloned into a pcDNA4HisMax vector by TA-cloning, as described in the manufacturer's instructions. pcDNA4/mutant ABCD1 vectors with the target mutations (c.284 C>A and/or c290 A>T) were prepared with a QuickChange Site-Directed mutagenesis kit (Stratagene, La Jolla, CA) using the pcDNA4/ABCD1 plasmid as the template. The mutations in the constructions were confirmed by DNA sequencing on an ABI PRISM 310 DNA sequencer (Perkin Elmer Life Science, Wellesley, MA). The primers used for the generation of the mutant ABCD1 constructs were as follows: the ABCD1-C284A-F primer: gagacggggctgctggacctgcactcgg, the ABCD1-C284A-R primer: ccgagtgcaggtccagcagccccgtctc, the ABCD1-A290T-F primer: gctgctggccctgctctcggccgccttgg, the ABCD1-A290T-R primer: ccaaggcggccgagagcagggccagcag, the ABCD1-C284A, A290T-F primer: gagacggggctgctggacctgctctcggccgccttg, and the ABCD1-C284A, A290T-R primer: caaggcggccgagagcaggtccagcagccccgtctc.

Transfection of the pcDNA4/mutant ABCD1 vectors into CHO/GFP-SKL cells and human skin fibroblasts was performed using Effectene Transfection Reagent (Qiagen, Valencia, CA) and by electroporation with a Gene Pulser (Bio-Rad), respectively (Takahashi et al. 2007). Two or three days after transfection, cells were fixed for immunofluorescence analysis and harvested for immunoblot analysis or fatty acid β-oxidation assay.

Immunofluorescence

Immunofluorescence analysis was performed as described previously (Takahashi et al., 2007). The cells were fixed with 3% formaldehyde and permeabilized with 1% Triton X-100 or 30 μM digitonin. The primary antibodies used were a rabbit anti-human catalase polyclonal antibody or a rabbit anti-rat ABCD3 polyclonal antibody and a mouse anti-human ABCD1 monoclonal antibody.

Fatty Acid β-Oxidation

β-oxidation was measured essentially as described by Watkins et al. (1991). Human skin fibroblasts were suspended in 0.25 M sucrose containing 1 mM EDTA, and 10 mM Tris–HCl, pH 8.0 were incubated with [1-^{14}C] lignoceric acid solubilized in 0.05 ml of α-cyclodextrin (10 mg/ml) in 10 mM Tris–HCl, pH 8.0 for 60 min at 37°C. The reactions were terminated by the addition of 0.05 ml of 1 N KOH and perchloric acid was added to a final concentration of 6% and the samples were maintained at 4°C for at least 3 h. The mixture was centrifuged at 2,000 rpm for 5 min and the supernatant was subjected to Folch partitioning. The aqueous phase was removed for scintillation counting.

Other Methods

Immunoblotting was performed as described previously using ECL Plus Western blotting detection reagent (Kurisu et al. 2003). The protein concentration was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as the standard.

Case Report

We report a 65-year-old male patient. His first reported symptom had been gait difficulty, which first began when he was 40 years old. An original spasticity of the lower extremities increased very slowly. At the age of 60, the patient developed a urinary excretion disturbance. At the age of 65, neurological examination revealed a spasticity of the lower extremities with increased deep tendon reflexes and a positive Babinski sign, with no sensory symptoms. He was still able to walk with a cane. Neuropsychological examination found normal cognitive function. His family history revealed that his nephew had been diagnosed with childhood cerebral ALD at the age of 7. He has suffered from adrenal insufficiency and has been received adrenal hormone therapy for more than 20 years. In addition, his mother and sister had developed spastic paraplegia. They had been diagnosed as being symptomatic female heterozygotes for X-ALD, due to the presence of increased VLCFA levels in the serum.

Routine laboratory studies were normal. The plasma concentrations of ACTH and cortisol were normal and tests for antinuclear antibodies were negative. Serology for syphilis, HTLV-I, or HIV was negative. The VLCFA ratios of C24:0/C22:0, C25:0/C22:0, and C26:0/C22:0 were markedly elevated in plasma from the patient (1.24, 0.044 and 0.046) compared to the control (1.05 ± 0.16, 0.024 ± 0.006 and 0.012 ± 0.005), respectively. Motor and sensory nerve conduction studies revealed a slightly decreased conduction velocity in the posterior tibial nerves on both sides. Somatosensory evoked potentials recorded after median and posterior tibial nerve stimulation displayed a prolonged central sensory conduction time bilaterally. Magnetic resonance imaging (MRI) of the brain revealed mild cerebellar atrophy. On T2-weighted images, no white matter abnormalities were found (Fig. 1). An MRI of the spinal cord revealed mild atrophy in the upper thoracic spinal cord.

Results and Discussion

Analysis of *ABCD1* Gene in the Patient

Genomic DNA was extracted from the patient's white blood cells. DNA sequence analysis demonstrated two novel,

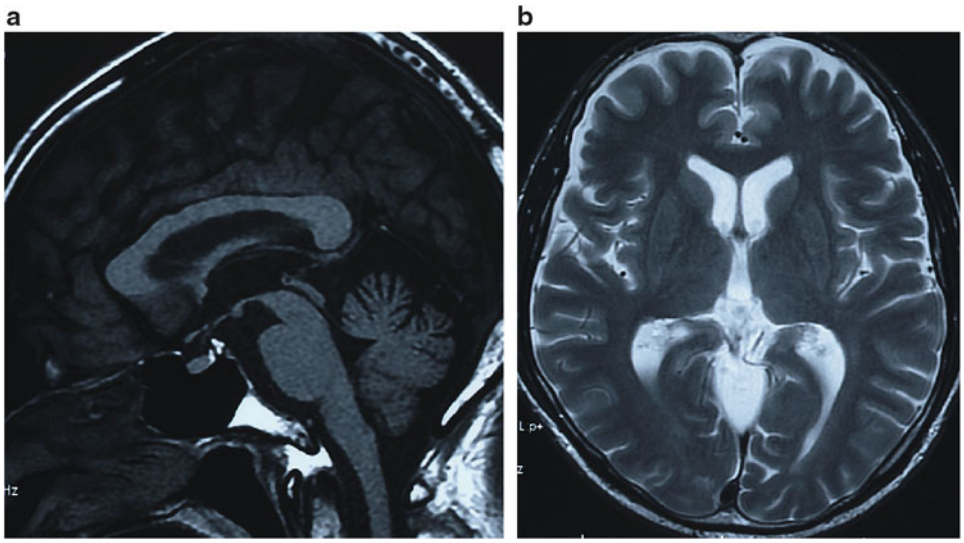

Fig. 1 An MRI image in the X-ALD patient. (**a**) A T1-weighted midsagittal image shows mild atrophy of the cerebellum. (**b**) A T2-weighted axial image shows there are no definite signal intensity changes in the cerebral white matter

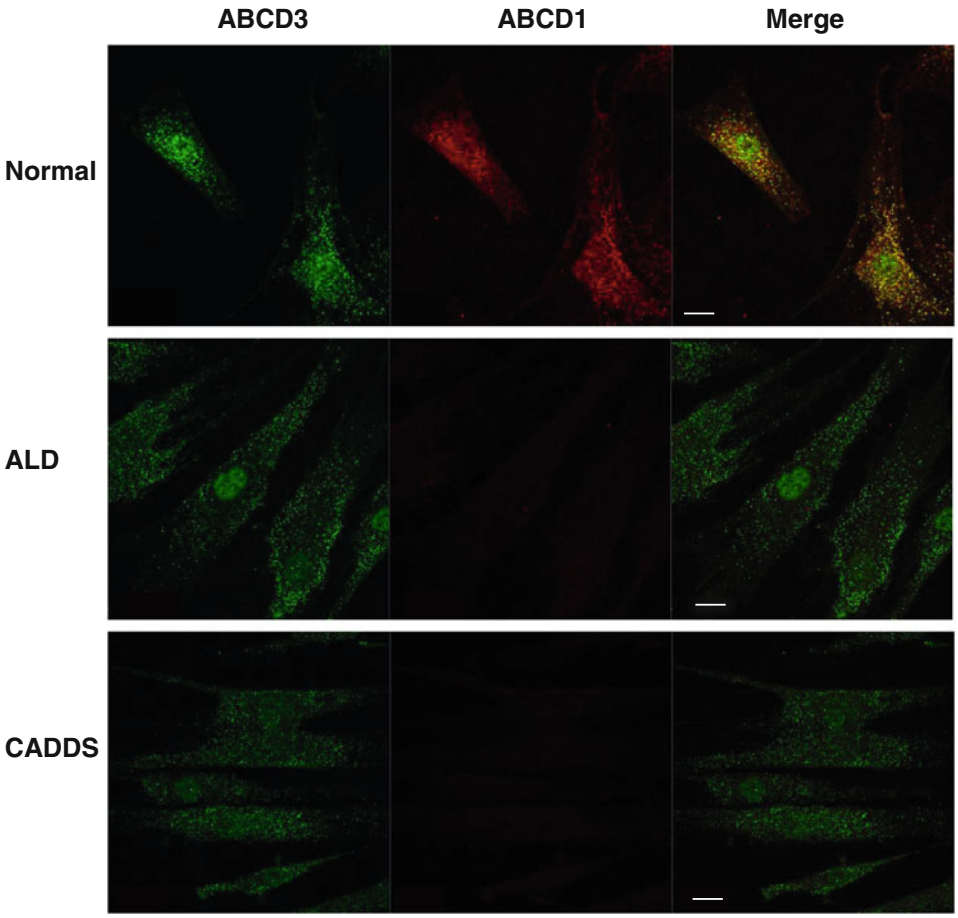

Fig. 2 Immunofluorescence analysis of ABCD1 expression in normal, X-ALD, and CADDS fibroblasts. Expression of mutant ABCD1 proteins in fibroblasts was analyzed by immunofluorescence analysis. Normal, X-ALD, and CADDS fibroblasts were stained with anti-ABCD3 and anti-ABCD1 antibodies followed by secondary antibodies conjugated with the dye reagents. Fluorescent dots were observed under confocal microscopy. Bar = 20 μm. ABCD1 protein was not detected in X-ALD and CADDS fibroblasts

non-synonymous variants in the *ABCD1* gene (c.284C>A, c.290A>T, leading to p.A95D, p.H97L changes, respectively). Another missense mutation (p.H97P) has been reported in amino acid residue in the X-ALD database, but the mutation in c.284C>A (p.A95D) and c.290A>T (p.H97L) has not been previously reported. p. A95D and p.H97L are located at a distance of only one amino acid residue and are located in TMD1, as deduced from a hydrophobicity plot.

Expression of the ABCD1 Proteins and VLCFA β-Oxidation in Fibroblasts from the X-ALD Patient

We first analyzed the expression of the mutant ABCD1 protein in skin fibroblasts from the patient by immunofluorescence analysis. Peroxisomes were stained with an anti-ABCD3 antibody that recognized the ABCD3 protein, which is a main peroxisomal membrane protein. In this experiment, CADDS fibroblasts were used as negative control because they lack the *ABCD1* gene. As shown in Fig. 2, normal, X-ALD, and CADDS fibroblasts exhibited ABCD3-positive immunofluorescent dots (green), suggesting the presence of intact peroxisomes in these cells. In normal fibroblasts, ABCD1-positive dots were detected and superimposed with peroxisomes. In contrast, no immunofluorescent dots were observed in X-ALD fibroblasts or CADDS fibroblasts. In the X-ALD fibroblasts, the C24:0 β-oxidation activity (0.18 ± 0.02 nmol/mg/h) was less than 20% of the normal fibroblasts (1.07 ± 0.05 nmol/mg/h), and this activity was similar to that of CADDS fibroblasts (0.19 ± 0.03 nmol/mg/h) (Fig. 3a), while the ABCD1 protein in the X-ALD fibroblasts was scarcely detected by immunoblot analysis. Taken together, the mutant ABCD1 protein with the double mutation (p.A95D and p.H97L) was not functional, because the mutant ABCD1 protein was degraded in the cytosol, resulting in a reduction of peroxisomal fatty acid β-oxidation.

To investigate in detail which of the missense mutations is a cause of X-ALD phenotype, we investigated the expression level, subcellular localization, and restoration of defected β-oxidation activity by ABCD1 (p.A95D), ABCD1 (pH97L), and ABCD1 (p.A95D/p.H97L). CHO/GFP-SKL cells are useful for the analysis of peroxisomal localization of an expressed protein. On the other hand, CADDS fibroblasts lacking the ABCD1 protein are beneficial to examine whether mutant ABCD1 is functional by transfection with the corresponding mutant *ABCD1* gene.

Impact of the p.A95D or p.H97L Substitution on the Stability of ABCD1 Protein

To determine which missense mutation causes the ABCD1 protein instability, we expressed each mutant ABCD1 protein

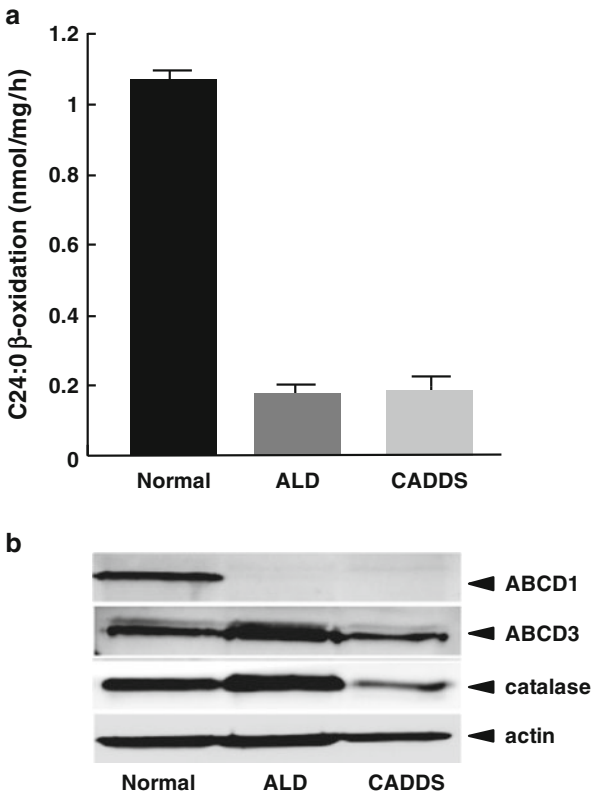

Fig. 3 VLCFA β-oxidation activity and ABCD1 protein expression in X-ALD fibroblasts. VLCFA β-oxidation activity in the normal, ALD, βand CADDS fibroblasts was measured using [1-^{14}C] C24:0 as substrate (**a**). Results are the means ± S.D.; $n = 3$. Expression of ABCD1 proteins in each of the cells was analyzed by immunoblot analysis (**b**). The C24:0 β-oxidation was less than 20% of the normal fibroblasts and the ABCD1 protein was not detected in the X-ALD fibroblasts

with the p.A95D, p.H97L, or p.A95D/p.H97L mutation in CHO/GFP-SKL cells. The cells express GFP in fusion with the serine-lysine-leucine (SKL) sequence at the C-terminal, which is a targeting signal for peroxisomal localization. As shown in Fig. 4, peroxisomes were detected as green fluorescent dots in the CHO/GFP-SKL cells. Three mutant ABCD1 proteins were transiently expressed in the CHO/GFP-SKL cells and subjected to immunofluorescence analysis with an anti-human ABCD1 antibody. When wild type (WT) ABCD1 was expressed, ABCD1-positive dots (indicated by the red dots) were detected and superimposed with green fluorescent dots, suggesting that WT ABCD1 correctly localized to peroxisomes. Like the WT ABCD1, the mutant ABCD1 (p.H97L) also localized to peroxisomes. In contrast, when the mutants ABCD1 (p.A95D) or ABCD1 (p.A95D/p.H97L) were expressed in CHO/GFP-SKL cells, fluorescent dots were not formed, and the fluorescence was diffuse in the cytosol. Since the mutants ABCD1 (p.A95D) and ABCD1 (p.A95D/p.H97L) were scarcely detected by immunoblot analysis in fibroblasts (as shown in Figs. 3b and 5b), it is likely that these diffused fluorescence signals were

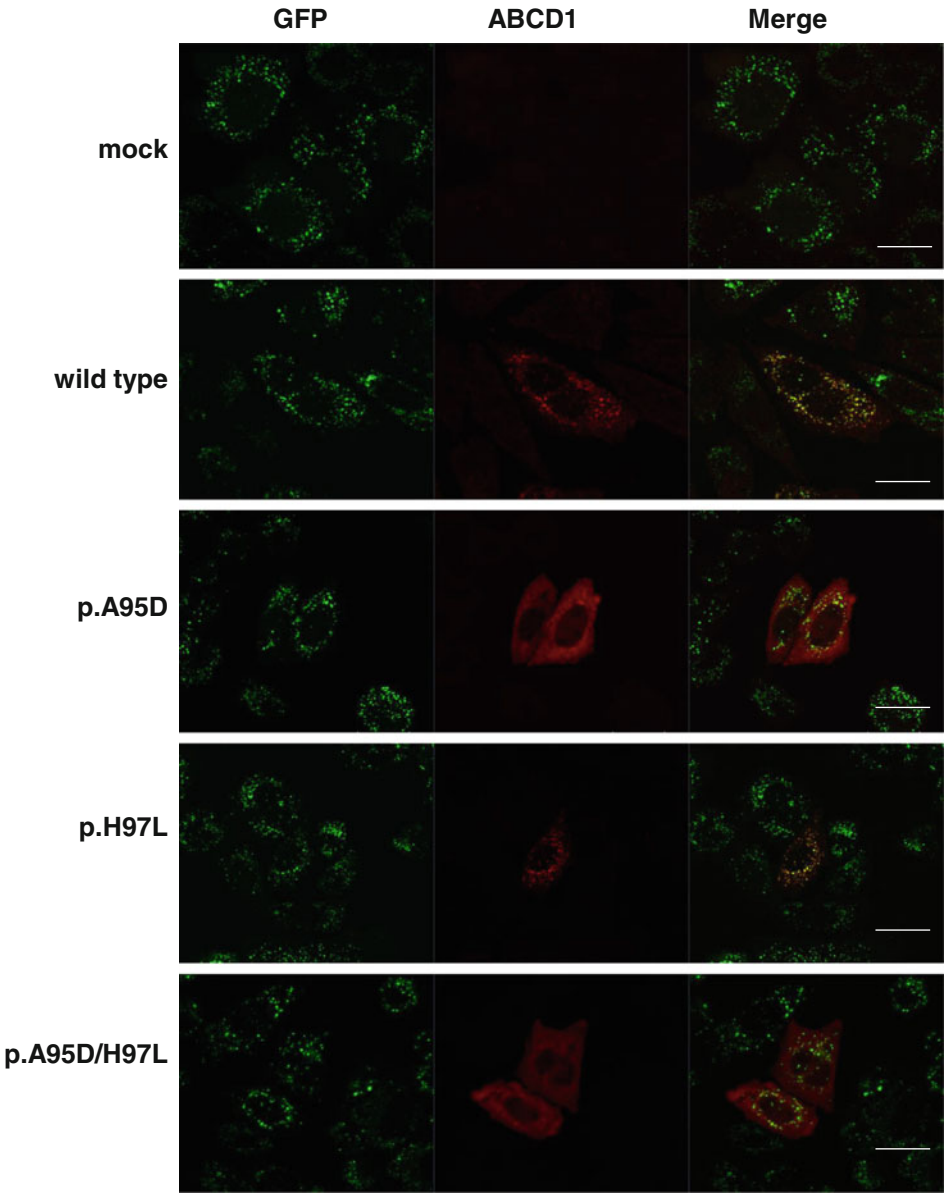

Fig. 4 Expression of mutant ABCD1 proteins in CHO/GFP-SKL cells. CHO/GFP-SKL cells were transfected with empty vector, pcDNA4/WT ABCD1 (wild type), or pcDNA4/mutant ABCD1 (p.A95D, p.H97L, or p.A95D/p.H97L). After the transfection, cells on the coverslips were fixed and subjected to immunofluorescence analysis. The expressed GFP-SKL proteins that localized in the peroxisomes were detected as green fluorescent dots. The expressed ABCD1 proteins were detected using an anti-human ABCD1 antibody followed by secondary antibodies conjugated with the dye reagent. Bar = 20 μm. The mutant ABCD1 (p.H97L) was localized to peroxisomes, but the mutants ABCD1 (p.A95D) and ABCD1 (p.A95D/p.H97L) were diffuse in the cytosol

the result of the non-degraded mutant ABCD1 proteins due to the overexpression in CHO/GFP-SKL cells. Taken together, the instability of the mutant ABCD1 with the double mutation is caused by the missense mutation at c.284C>A (p.A95D) but not c.290A>T (p.H97L).

Impact of the p.A95D or p.H97L Substitution on VLCFA β-Oxidation Activity

In contrast to the mutant ABCD1 (p.A95D), which was degraded in cytosol, the mutant ABCD1 (p.H97L) was correctly localized in peroxisomes. To determine whether the mutant ABCD1 (p.H97L) is functional, we next expressed the mutant ABCD1 proteins in the CADDS fibroblasts lacking the ABCD1 protein. In the CADDS fibroblasts, the C24:0 β-oxidation activity was approximately 0.2 nmol/mg/h, as shown in Figs. 2 and 5a. When WT ABCD1 was expressed in the CADDS fibroblasts, the C24:0 β-oxidation activity was increased to approximately 0.35 nmol/mg/h (Fig. 5a). Similar results were observed by expressing mutant ABCD1 (p.H97L). As expected, no increase was observed by expressing mutant ABCD1

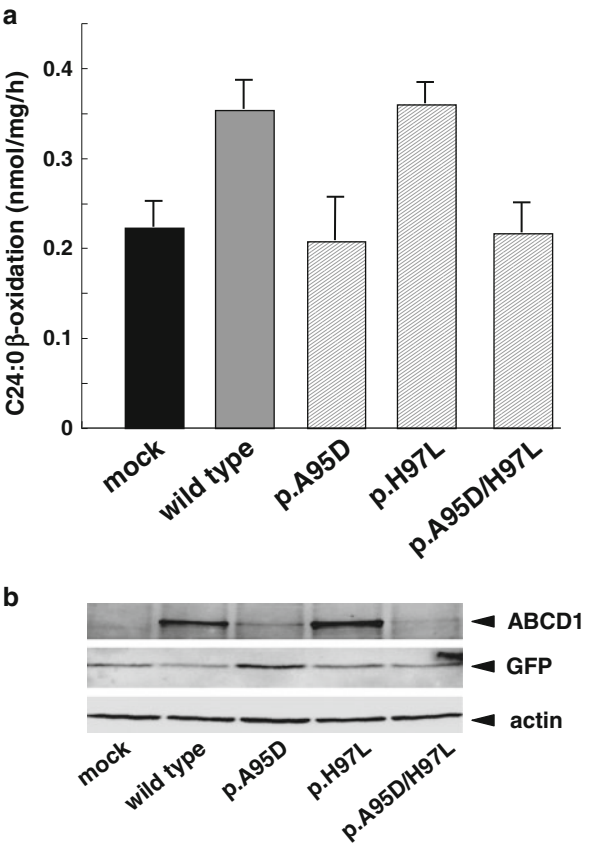

Fig. 5 C24:0 β-oxidation activity in CADDS fibroblasts expressing mutant ABCD1 proteins. C24:0 β-oxidation activity in the CADDS fibroblasts expressing mutant ABCD1s was analyzed as in Fig. 3. CADDS fibroblasts were transfected with empty vector, pcDNA4/WT ABCD1 (wild type) or each pcDNA4/mutant ABCD1 (p.A95D, p.H97L, p.A95D/p.H97L). After 3-day transfection, the cells were harvested for determination of the C24:0 β-oxidation activities (**a**) and immunoblotting (**b**). Results are means ± S.D.; $n = 3$. The mutant ABCD1 (p.H97L) was expressed in CADDS fibroblasts and the C24:0 β-oxidation activity was recovered as wild type ABCD1. In contrast, the mutants ABCD1 (p.A95D) and ABCD1 (p.A95D/p.H97L) were scarcely expressed and the C24:0 β-oxidation activities were not recovered

(p.A95D) or ABCD1 (p.A95D/p.H97L). These results are in good agreement with the results that WT ABCD1 and mutant ABCD1 (p.H97L) were clearly detected by immunoblot analysis, but mutant ABCD1 (p.A95D) and ABCD1 (p.A95D/p.H97L) were only faintly detected (Fig. 5b). The transfection efficiencies for the ABCD1 variants were 20–30%. Taken together, we conclude that mutation at c.284C>A (p.A95D) is the cause of the X-ALD phenotype, but the mutation at c.290A>T (p.H97L) is a genetic polymorphism.

Concerning multiple mutations of ABCD1, some of the features have been characterized in two cases: ABCD1 (p.N13T/p.K217E) (Dvorakova et al. 2001) and ABCD1 (p.R236H/p.G512S) (Guimaraes et al. 2002). In the case of ABCD1 (p.N13T/p.K217E), p.K217E was suggested to be the disease-causing mutation and p.N13T a polymorphism, since p.K217E, but not p.N13T, failed to restore defective VLCFA β-oxidation in X-ALD fibroblasts. However, the expression level of p.K217E was not examined. In the case of ABCD1 (p.R236H/p.G512S), p.G512S has already described as being associated with X-ALD, but it has not yet been determined whether p.R236H is a disease-causing mutation (Guimaraes et al. 2002). No data was reported on the expression of p.R236H, p.G512S, or p.R236H/p.G512S.

With regard to ABCD1 mutation, there is a cluster of mutations associated with X-ALD in TMD1: p.A95D (in this study), p.H97P, p.S98P, p.S98L, p.S98W, p.A99D (the X-ALD mutation database listed at http://www.x-ald.nl). In contrast, p.H97L in this study was shown to be a polymorphism. It has been suggested that post-translated ABCD1 associates with Pex19p in the cytosol targets the peroxisomal membranes and is inserted into the membranes through Pex3p. The NH_2-terminal Pex19p-binding region (aa.68-82) and TMD1 are involved in the targeting of the peroxisomal membranes (Landgraf et al. 2003; Halbach et al. 2005). It is of interest to determine whether and how each mutation in the cluster in TMD1 disturbs the targeting.

In any event, for the determination of the disease-causing mutations of ABCD1 an extended familial screening of the probands is needed for effective bone marrow transplantation at the early stages of the cerebral symptoms. In addition, polymorphism analysis is required for the determination of the genetic status of at-risk individuals. The protocol presented here is useful for identifying the genetic polymorphisms and improving the quality of genetic counseling in the prenatal diagnosis.

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CASE REPORT

Long-Term Follow-up of a Successfully Treated Case of Congenital Pyridoxine-Dependent Epilepsy

Malcolm Proudfoot · Philip Jardine · Agne Straukiene · Rupert Noad · Andrew Parrish · Sian Ellard · Stuart Weatherby

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Abstract Autosomal recessive disorders affecting pyridoxine (vitamin B6) metabolism are a rare but well-recognized cause of neonatal seizures. Antiquitin deficiency, caused by mutations in *ALDH7A1*, is a disorder of the lysine degradation pathway causing accumulation of an intermediate that complexes with pyridoxal phosphate. Reports of long-term follow-up of neonatal pyridoxine-dependent seizures (PDS) remain scarce and prognostic information is varied. We report a case of PDS in a 47-year-old lady who originally presented shortly after birth in 1964. Pyridoxine replacement was successful and diagnostic confirmation was obtained later in life, initially by biochemical analysis of serum pipecolic acid. Subsequently we organized genetic analysis of *ALDH7A1*, which revealed compound heterozygous mutations. To our knowledge, this represents the longest duration of follow-up published to date.

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M. Proudfoot (✉)
Dept of Neurology, Derriford Hospital, Plymouth, UK
e-mail: m.proudfoot@nhs.net

P. Jardine
Dept of Neurology, Bristol Royal Hospital for Children, Bristol, UK

A. Straukiene
Dept of Neurology, Derriford Hospital, Plymouth, UK

R. Noad
Dept of Clinical Neuropsychology, Derriford Hospital, Plymouth, UK

A. Parrish
Dept of Molecular Genetics, Royal Devon & Exeter NHS Foundation Trust, Exeter, UK

S. Ellard
Dept of Molecular Genetics, Royal Devon & Exeter NHS Foundation Trust, Exeter, UK

S. Weatherby
Dept of Neurology, Derriford Hospital, Plymouth, UK

Case

Clinical History

Our patient was born full term weighing 3.6 kg; hours after delivery, generalized convulsions began. No cause was immediately apparent for the continuous seizures. Her sibling had died shortly after birth with uncontrollable seizures of unknown aetiology. After 24 h of unsuccessful treatment with anti-convulsants and sedatives, pyridoxine was prescribed. Convulsions ceased 5 min after administration of 75 mg IV. Maintenance therapy with oral pyridoxine, 20 mg 8 hourly, was commenced. EEG demonstrated excess generalized theta.

Confirmatory testing of PDS was performed aged 8 months. After 48 h of pyridoxine withdrawal, there were frank convulsions and recurrence of encephalopathy. Recovery after reinstitution of pyridoxine was prompt. Oral replacement therapy was continued long term.

Developmental assessment aged 6 years revealed mild developmental delay despite freedom from seizures. At 11 years, a further EEG was performed. An excess of theta and relative paucity of rhythmic alpha activity was noted. She attended a school for children with learning difficulties and has relatively poor literacy.

Aged 20 years, she was reviewed in the adult neurology clinic, querying the necessity of continued pyridoxine therapy. Pyridoxine, then 50 mg three times daily, was withdrawn for 2 weeks. Seizure recurred and pyridoxine recommenced. A subsequent EEG revealed minimal epileptiform activity.

Current Status

She returned for review at 42 years. She lives independently, works as a pre-school assistant and has one healthy

daughter. She enjoys ongoing seizure freedom but is troubled by migraine with aura. Examination was normal and nerve conduction studies excluded peripheral neuropathy. MRI brain revealed mild ventriculomegaly, prominent cisterna magna and no evidence of parenchymal abnormalities. No change was noted on 2-year interval scanning.

Neuropsychological assessments have been undertaken, after initial interview in November 2010. She declined cognitive testing in March 2011 but consented in August 2012.

She was assessed on the Wechsler Adult Intelligence Scale – Version IV. Her full scale IQ was calculated as 75, which equates to the 5th percentile. In terms of profile of abilities, there was a significant discrepancy between her verbal and non-verbal abilities ($p<0.05$). Her verbal intellectual abilities were an area of relative weakness (2nd percentile). Her non-verbal intellectual abilities were a relative strength (34th percentile), and she performed particularly well on block design (high average range). She scored at the 13th percentile on tests of working memory and 4th percentile for processing speed.

In summary, her overall intellectual ability would be categorized in the 'borderline' range with relative strengths in non-verbal as opposed to verbal abilities.

Investigations

Metabolic confirmation of PDS was provided by raised serum pipecolic acid (PPA) at 7.4 μmol/l (<2.6 μmol/l) and a significant mass spectrometry peak of α-amino adipic semialdehyde (AASA) in urine (Bok et al. 2007). Sequence analysis of the *ALDH7A1* gene (RefSeq NM_001182.3) revealed compound heterozygosity for a missense mutation c.1279G>C (p.E427Q) in exon 14 and a cryptic splicing mutation c.834G>A (p.V250V) in exon 9. Both mutations have been reported previously (Salomons et al. 2007). Further detail is provided in a supplementary footnote.

Comment

PDS, although rare as a cause of neonatal seizures, may have a higher incidence than early estimates of 1 in 730,000 (Baxter 1999; Been et al. 2005). Classically seizures occur within a few days of birth and may perhaps be detected as abnormal intra-uterine movements (Bejsovec et al. 1967). Antenatal treatment may improve cognitive outcomes (Bok et al. 2012). The seizures may be accompanied by EEG changes and both focal and diffuse inter-ictal dysfunction is recognized, but paroxysmal movements may be unaccompanied by EEG discharges (Schmitt et al. 2010).

Our case was typical in having complete freedom from seizures on pyridoxine monotherapy, plus withdrawal of pyridoxine led to a recurrence of seizures. Relative normalization of the EEG following therapy provides supportive evidence. Atypical cases described include those with an initially favourable response to anti-epileptic drugs, those with a later age of onset (Goutières and Aicardi 1985) and those in whom later withdrawal of pyridoxine is tolerated. Some such cases do not have currently identifiable genetic mutations (Bennett et al. 2009).

Diagnosis of Pyridoxine-Dependent Seizure

Confirmatory testing of suspected PDS cases has evolved in line with improved molecular and genetic analysis techniques (Mills et al. 2006; Stockler et al. 2011).

Neurochemical abnormalities in PDS include reduced CSF GABA and elevated glutamic acid. More specifically, significant elevation of pipecolic acid occurs in both plasma and CSF, and may increase further during pyridoxine withdrawal (Plecko et al. 2000). Various mutations in *ALDH7A1*, situated on chromosome 5q31, are now recognized as a major cause of PDS (Mills et al. 2006). This gene encodes an enzyme in the cerebral lysine degradation pathway, AASA dehydrogenase (antiquitin). Diagnosis can thus be made both antenatally and after initiation of pyridoxine (Segal et al. 2011) without risking potentially harmful withdrawal of therapy (Plecko et al. 2007).

Treatment, Prognosis and Risks of Therapy

Pyridoxine toxicity in high doses, typically beyond 200 mg/day, is well established and peripheral neuropathy is reported to be dose dependent in severity (Berger et al. 1992). Nonetheless, reports of iatrogenic neuropathy in PDS are rare (McLachlan and Brown 1995) and reassuringly, nerve conduction studies performed on our patient at 44 years of age, returned normal motor and sensory responses.

MRI studies of PDS patients have revealed varying degrees of abnormality; typically atrophy (Gospe and Hecht 1998) and in keeping with this, our patient demonstrated mild ventriculomegaly with prominent cisterna magna only. Dysplasia of the corpus callosum has been noted in some series (Mills et al. 2010) as have white matter abnormalities, but these findings are aetiologically non-specific and correlate poorly with functional outcomes.

Other long-term case reports of treated PDS have remarked upon discrepancy between verbal and performance IQ scores in the context of mild developmental delay (Baynes et al. 2003), but cognitive profiles may be globally blunted (Rankin et al. 2007). A broad range of cognitive outcomes are described in the literature, with a trend to higher function in those treated earlier in life (Bok et al. 2012). Our adult subject demonstrates psychometric function slightly above previously averaged IQ values of children with PDS, in keeping with her early and continued therapy. It seems likely that preservation of intellect is

dependent upon adequate pyridoxine replacement (Baxter et al. 1996), but may vary with differing genotypes (Striano et al. 2009; Scharer et al. 2010) or unknown environmental influences (Alfadhel et al. 2012). Further restoration of higher functions may prove possible with additional dietary modification such as lysine restriction (van Karnebeek et al. 2012), emphasizing the complex pathogenesis of antiquitin defects beyond that of central pyridoxine deficiency.

Conclusion

At the time of our patient's birth, only 12 cases of PDS had been described worldwide. To our knowledge, the duration of follow-up in this case is the longest yet reported and descriptions of cognitive outcomes in treated adults remain scarce. Our patient achieved complete seizure freedom on long-term pyridoxine replacement without any apparent significant side effects. We hope that this case will provide encouragement to patients and their families, and remind clinicians of the sustained beneficial impact from early PDS diagnosis.

Synopsis

This report demonstrates an instance of long-term successful treatment of pyridoxine-dependent seizures, which was achieved without serious adverse effects.

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Supplementary Information

Mutation numbering has been updated to reflect the mitochondrial leader sequence. The missense mutation p.E427Q (RefSeq NM_001182.3) is the same mutation previously reported as E399Q (NM_001182.2) and the cryptic splicing mutation c.834G>A (NM_001182.3) was previously reported as c.750G>A (NM_001182.2). We initially failed to identify this as pathogenic in part due to this nomenclature change and also since only one of the four splicing prediction tools within Alamut (www.interactive-biosoftware.com) predicted an effect.

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CASE REPORT

Pyruvate Dehydrogenase-E1α Deficiency Presenting as Recurrent Demyelination: An Unusual Presentation and a Novel Mutation

Pratibha Singhi · Linda De Meirleir · Willy Lissens · Sunit Singhi · Arushi Gahlot Saini

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Abstract The nucleus-encoded mitochondrial pyruvate dehydrogenase enzyme complex plays key roles in cellular energy metabolism and acid-base equilibrium. Pyruvate dehydrogenase complex deficiency is due to loss-of-function mutation in one of the five component enzymes, most commonly E1α-subunit. The common clinical presentation ranges from fatal infantile lactic acidosis in newborns to chronic neurological dysfunction. We describe here an unusual presentation of E1α-subunit deficiency presenting as recurrent demyelination, Guillain-Barré syndrome-like demyelinating polyneuropathy at the onset, and ophthalmoplegia in a young infant. The clinical phenotype of the mutation in the patient was unique as compared to the previous reported cases of pyruvate dehydrogenase deficiency. The mother was found to be a mosaic carrier of the mutation. This phenotypic variability of pyruvate dehydrogenase complex deficiency and early suspicion of its unusual neurological manifestations is highlighted. Thiamine and ketogenic diet can be helpful.

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P. Singhi · S. Singhi · A.G. Saini
Department of Pediatrics, Postgraduate Institute of Medical Education and Research, Chandigarh, India

L. De Meirleir · W. Lissens
Center for Medical Genetics, UZ Brussel and Vrije Universiteit Brussel, Brussel, Belgium

P. Singhi (✉)
Advanced Pediatrics Centre, PGIMER, Chandigarh 160012, India
e-mail: pratibhasinghi@yahoo.com; doctorpratibhasinghi@gmail.com

Introduction

The nucleus-encoded mitochondrial pyruvate dehydrogenase (PDH) complex plays key roles in cellular energy metabolism and acid-base equilibrium. PDH complex consists of three catalytic enzymes, pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2), dihydrolipoamide dehydrogenase (E3); an additional protein known as E3-binding protein (E3BP) or protein X; and two regulatory enzymes, pyruvate dehydrogenase kinase and phosphatase. The E1-subunit is encoded by the X-linked *PDHA1* gene and irreversibly oxidizes pyruvate to acetyl-CoA in presence of thiamine pyrophosphate. PDH deficiencies are largely due to defects in this E1-subunit that result in clinically diverse mitochondrial disorders ranging from fatal infantile lactic acidosis in newborns to chronic neurological dysfunction (Dahl et al. 1992). However, a clinical picture suggestive of recurrent demyelination, as seen in our patient, is an unusual presentation in infancy. The genetic analysis revealed a mutation of *PDHA1* gene and the mother was found to be mosaic carrier. The need for identifying unusual neurological presentations of this rare disorder is emphasized.

Case Report

An 18-month-old male child was admitted to our hospital with progressive weakness, loss of previous milestones, ophthalmoplegia, excessive crying, and difficulty in feeding and speaking. There were no seizures or fever. He was born to non-consanguineous parents by emergency caesarian section due to umbilical artery stenosis with compromise of fetomaternal circulation. Antenatal period was normal. His birth weight was 3.1 kg. Subsequently, he was noticed to have developmental delay; he attained social smile at 3 months, neck control at

Table 1 Electrophysiological data of the patient at the time of initial presentation

	Patient	cut-off values for age (mean-2.5SD)
Motor NCV (m/s)		
Median	13.51	26.22
Peroneal	18.81	25.4
Tibial	5.12	24.75
F-wave latency (peroneal)	17.62	21
Sensory NCV (m/s)		
Sural	14.05	20.52

5 months, cooing at 6 months, reaching out for objects at 7 months, and sitting with support at 8 months. However, when the child was brought to us during his acute illness, he was unable to sit or reach out. Examination showed generalized hypotonia, absence of deep tendon reflexes, mute plantars, and external ophthalmoplegia. He was conscious but irritable and crying excessively. Gag reflex was absent along with difficulty in feeding. He received a 5-day course of pulse methylprednisolone followed by oral steroids in view of a demyelinating illness. The child had an episode of aspiration pneumonia requiring mechanical ventilation and prolonged ICU care for shock and sepsis.

The child had significant past history. At 8 months of age, the baby had upper respiratory infection with mild fever followed by progressive weakness of lower and upper limbs, generalized hypotonia, areflexia, and ophthalmoplegia. He was admitted in a local hospital. Magnetic resonance imaging (MRI) brain was normal. Cerebrospinal fluid (CSF) examination revealed proteins 29 mg/dL, sugar 71 mg/dL (blood sugar 119 mg/dL), cells: 2 lymphocytes/mm^3, gram stains, and cultures sterile. Nerve conduction velocity (NCV) was suggestive of motor sensory demyelinating neuropathy (Table 1). Electromyography was suggestive of neurogenic changes with decreased motor units. Repetitive nerve stimulation test was normal. A diagnosis of autoimmune demyelinating polyneuropathy (AIDP) was considered, and he received intravenous immunoglobulin at 2 g/kg after which the weakness gradually improved.

At 15 months of age, the child was again admitted in a local hospital with generalized weakness, ptosis, and regression of milestones. MRI brain showed T2/FLAIR hyperintensities involving bilateral periventricular areas and the hilus of the dentate nucleus in the cerebellum suggestive of demyelination (Fig. 1a and b). NCV showed no evidence of conduction blocks or dispersion. Conduction velocities of all motor and sensory nerves were altered, consistent with sequelae of earlier attack of AIDP. CSF examination revealed no cells and proteins 31 mg%. CSF-PAGE was positive for oligoclonal bands. The child received a 5-day course of pulse methylprednisolone in view of acute demyelinating encephalomyelitis followed by oral steroids. He showed rapid recovery but again deteriorated and was then brought to our hospital.

In the current admission at 18 months of age, laboratory investigations showed pH 7.29, serum ammonia 64 μmol/L (9–33 μmol/L), and lactate 7.7 mmol/L (0.7–2.1 mmol/L). CSF lactate was 25.02 mmol/L. Fundus examination was normal. MRI brain revealed non-enhancing subtle T2/FLAIR hyperintensities in bilateral periventricular parietooccipital white matter (Fig. 1c and d). The mutational analysis for mitochondrial disease was negative. In view of recurrent peripheral and central demyelination, ophthalmoplegia and waxing-waning course, genetic analysis for PDH deficiency was sent which showed hemizygosity for the c.412C>T, p. Leu138Phe mutation in exon 4 of the *PDHA1* gene. His mother was found to be mosaic for this mutation. The patient was started on oral thiamine and ketogenic diet. Supportive care was continued in form of physiotherapy and tracheostomy tube care. Follow-up MRI brain 1 year after the illness showed symmetrical gliotic changes in bilateral periventricular white matter and globus pallidi with communicating hydrocephalus, thinned-out corpus callosum, and cerebral atrophy (Fig. 1e and f).

Discussion

A subset of PDH-deficient patients present with an atypical course with intermittent neurological symptoms often triggered by infectious illnesses, like intermittent ataxia (Debray et al. 2008), recurrent acute dystonia (Head et al. 2004), extrapyramidal movement disorders, and episodic peripheral weakness mimicking Guillain-Barré syndrome (GBS) (Strassburg et al. 2006; Debray et al. 2006). Such an early initial presentation of PDH deficiency clinically mimicking GBS is quite rare. Remarkably, the first episode in our patient occurred at the age of 8 months, while classic GBS is unusual in this age group (Hughes and Cornblath 2005). This suggests that acute "GBS-like" weakness at the outset is a potentially reversible and probably under-recognized manifestation of PDH deficiency and should be diagnosed early by nerve conduction studies. Defects in cellular energy metabolism lead to a reduced synthesis of adenosine triphosphate and pathological accumulation of lactate, H^+, and free radicals which possibly impair myelination and other vital functions of nervous tissue (Strassburg et al. 2006). External ophthalmoplegia, considered the hallmark of mitochondrial disorders in adults, was also an important clue in our patient (Jackson et al. 1995).

Treatment of most patients with PDH deficiency has been disappointing and no intervention specific to this disease has

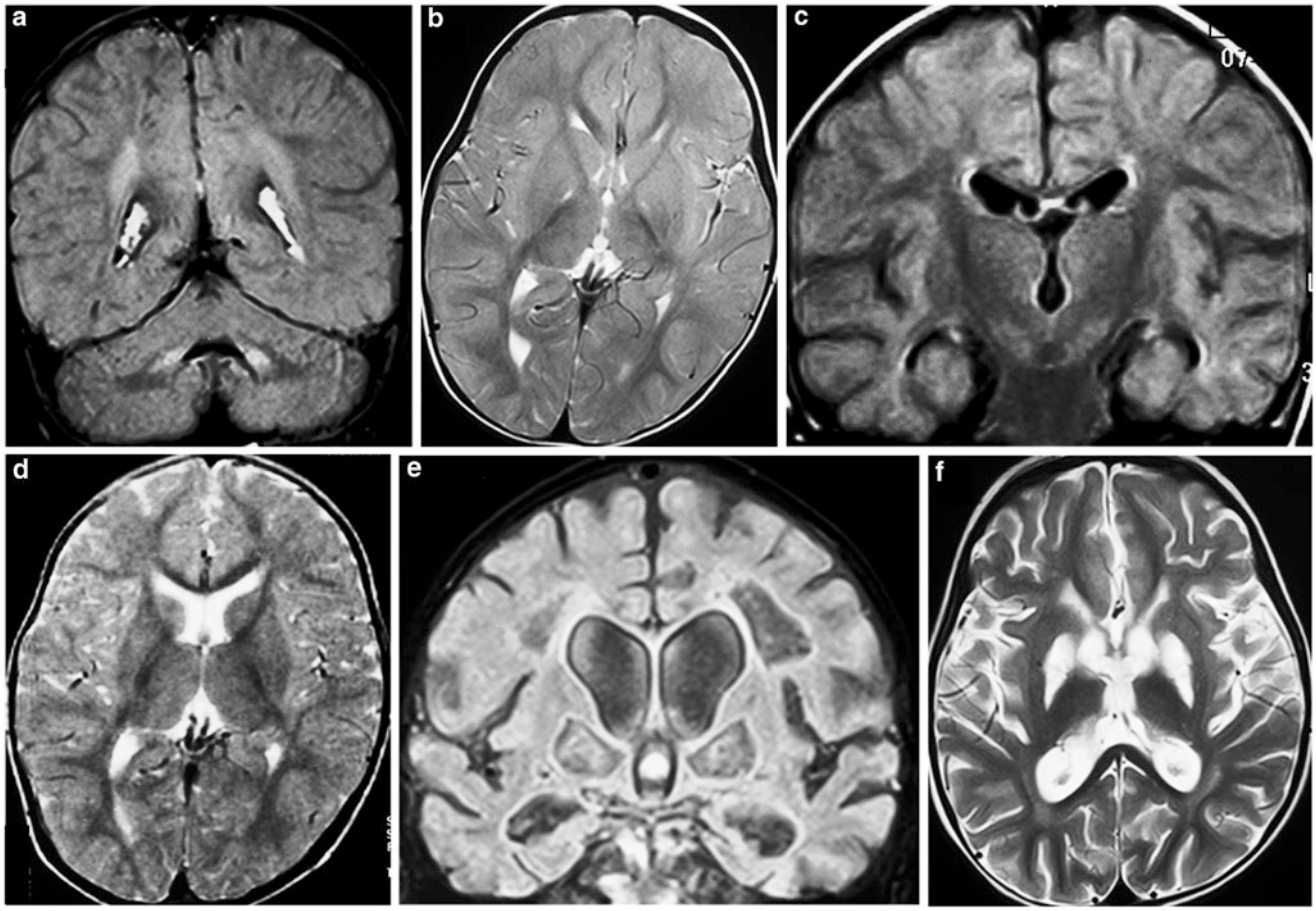

Fig. 1 (**a** and **b**): MRI brain (FLAIR and T2) at the age of 15 months (second deterioration) shows hyperintense signal involving bilateral hilum of dentate nucleus, superior and middle cerebellar peduncles, and periventricular white matter suggestive of demyelination. These areas were hypo- to isointense on T1 images and showed no restriction on diffusion weighted or any enhancement post-contrast. Rest of the supratentorial brain parenchyma is normal in signal intensity and gray-white matter differentiation. Bilateral basal ganglia, thalami, and corpus callosum are normal. (**c** and **d**): MRI brain (FLAIR and T2) at the age of 18 months (first presentation to us) shows subtle T2/FLAIR hyperintensities in bilateral periventricular parietooccipital white matter. These showed no evidence of diffusion restriction or post-contrast enhancement. Bilateral basal ganglia, thalami, and corpus callosum are normal. (**e** and **f**): MRI brain (FLAIR and T2) at the age of 22 months (follow-up at 1 year after the illness) shows bilateral symmetrical linear areas of gliotic change in bilateral periventricular white matter, centrum semiovale, and globus pallidi. No diffusion restriction was seen. Corpus callosum is thinned out in body portion. Ventricular system is dilated with dilated 3rd and 4th ventricles. Bilateral thalami appear normal

been evaluated in randomized controlled trials (Patel et al. 2012). As the symptoms are due to energy depletion from inefficient ATP production and lactate accumulation, ketogenic diet helps by providing the brain with an alternate "ketone" fuel and minimizing lactate accumulation by limiting carbohydrate intake. By the same mechanism, PDH deficiency usually responds to thiamine and ketogenic diet (Naito et al. 2002). A possible additional benefit of the diet is increased brain insulin-like growth factor receptor expression and neuronal glucose transporter expression (Cheng et al. 2003). Reports of a few children with PDH deficiency whose clinical course improved dramatically while following a high-fat diet are consistent with this postulate and have resulted in the incorporation of such diets in the routine care of many patients (Wexler et al. 1997; Kossoff et al. 2009). Nutritional mixtures of various cofactors and vitamins have been tried, usually, with limited biochemical rationale. Recommendations of the International Ketogenic Diet Study Group (Kossoff et al. 2009) states that the diet is the treatment of choice for two distinct disorders of brain energy metabolism: GLUT-1 deficiency syndrome and PDH deficiency. Unfortunately, despite this, our patient deteriorated. This could have been, perhaps, due to the initiation of the diet at a later age when the child presented to us at 18 months.

The metabolic causes of demyelinating polyneuropathy in young children are limited such as lysosomal disorders (metachromatic leukodystrophy, Krabbe disease), beta-mannosidosis, sialidosis, Gaucher disease, Niemann-Pick C, Refsum and other peroxisomal disorders, mitochondrial disorders like MNGIE, or rarely acute liver or renal failure. Acute polyneuropathies mimicking GBS have been

described in acute attacks of porphyrias (Sedel et al. 2007), tyrosinemia type I (Mitchell et al. 1990), and in PDH deficiency (Debray et al. 2006). Treatable causes of metabolic neuropathies in children worth remembering are biotinidase deficiency, homocysteine remethylation defects, ornithine aminotransferase deficiency, vitamin E deficiency, serine deficiency, Wilson disease, and cerebrotendinous xanthomatosis (Garcia-Cazorla et al. 2009) though these are not exclusively demyelinating type of neuropathies.

The diagnosis in our patient was confirmed after mutation analysis which revealed hemizygosity for the c.412C>T mutation in exon 4 of the *PDHA1* gene causing substitution of Leucine for 138 Phenylalanine (L138F). The mosaicism for the mutation in the mother can be explained by the fact that a subset of heterozygous females carries an E1-subunit mutation but have mild or no symptoms at all, presumably because of an X-inactivation pattern favoring expression of the normal X chromosome and resulting in a mosaic population of cells (Brown et al. 1994). The mutation has been described before in a 20-year-old male with a different clinical presentation of Leigh's disease, absence of lactic acidemia, and basal ganglia changes on neuroimaging (Cameron et al. 2004). Our patient was unique to have recurrent demyelination as a manifestation of this mutation in PDH deficiency at such an early age.

Conclusion

PDH deficiency should be considered in patients with unexplained recurrent acute neurological symptoms with lactic acidemia. Long-term prognosis and outcome remain uncertain though thiamine and ketogenic diet are useful.

Acknowledgments None

Take-Home Message

PDH deficiency should be considered in patients with unexplained recurrent acute neurological symptoms with lactic acidemia.

Author Contribution

Pratibha Singhi is clinician-in-charge, reviewed neuroradiology and electrophysiological data, and also reviewed the manuscript; Linda De Meirleir helped in the diagnosis and genetic studies; Willy Lissens helped in mutational analysis; Sunit Singhi is clinician-in-charge of pediatric intensive care; and Arushi Gahlot Saini helped in patient management, draft of manuscript, and literature search.

Author Guarantor

Dr P Singhi (corresponding author)

Conflict of Interest

None

Financial Disclosure

None

Ethical Approval

Manuscript is a retrospective case report that does not require ethics committee approval at the institution.

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CASE REPORT

The Management of Pregnancy in Maple Syrup Urine Disease: Experience with Two Patients

Michel Tchan · M. Westbrook · G. Wilcox · R. Cutler · N. Smith · R. Penman · B.J.G. Strauss · B. Wilcken

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Abstract We describe the management and outcomes of pregnancy in two women affected with Maple syrup urine disease (MSUD). Both patients had classical disease diagnosed in the newborn period and were managed with low-protein diets and supplements, although compliance was moderately poor throughout life. Both pregnancies were complicated by poor compliance and one patient had a metabolic decompensation, which included seizures and profound encephalopathy, at the end of the first trimester. Peri-partum management required a coordinated team approach including a high-calorie and low-protein diet. Both patients had elevated leucine levels in the post-partum period – one due to mastitis and the other due to poor dietary and supplement compliance combined with uterine involution. On later review, leucine had returned to pre-pregnancy levels. Both infants were unaffected and have made normal developmental progress in the subsequent 1 to 2 years.

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Competing interests: None declared

M. Tchan (✉) · M. Westbrook
Department of Genetic Medicine, Westmead Hospital, PO Box 533, Westmead, NSW 2145, Australia
e-mail: michel.tchan@health.nsw.gov.au

M. Tchan · M. Westbrook
Discipline of Genetic Medicine, Sydney Medical School, University of Sydney, Sydney, Australia

B. Wilcken
Western Sydney Genetics Program, The Children's Hospital at Westmead, Sydney, Australia

B. Wilcken
Disciplines of Paediatrics and Child Health and Genetic Medicine, Sydney Medical School, University of Sydney, Sydney, Australia

G. Wilcox · R. Penman · B.J.G. Strauss
Monash University Department of Medicine, Southern Clinical School, Monash University, Clayton, VIC, Australia

R. Cutler · N. Smith
Clinical Nutrition & Metabolism Unit, Monash Medical Centre, Clayton, VIC, Australia

Introduction

MSUD is an autosomal recessive disorder caused by mutations in one of three of the four genes encoding subunits of branched-chain keto-acid dehydrogenase (BCKAD) (Zhang et al. 1989a, b; Herring et al. 1991; Nobukuni et al. 1991) and presents classically in the newborn period with a maple syrup odour in cerumen and urine, irritability and poor feeding by day 3, progressive neurological signs, encephalopathy, coma and death if untreated (Strauss et al. 2009). Treatment is by institution of a very low-protein diet, branched-chain amino acid–free supplement, and provision of calorie support at times of decompensation. Successful treatment means that patients are surviving into adulthood. Nevertheless, there have been very few reports of pregnancy in MSUD.

We report the successful outcome of pregnancy in two women affected with MSUD. A significant episode of metabolic decompensation at the end of the first trimester for one patient did not appear to have any effect on the health of the infant when assessed at 4 and 20 months of age, thus suggesting that maternal MSUD does not have a detrimental effect on the developing fetus.

Patient 1

The patient was born following an uneventful pregnancy and was diagnosed with classical MSUD by urine metabolic screen after she presented with seizures at 10 days

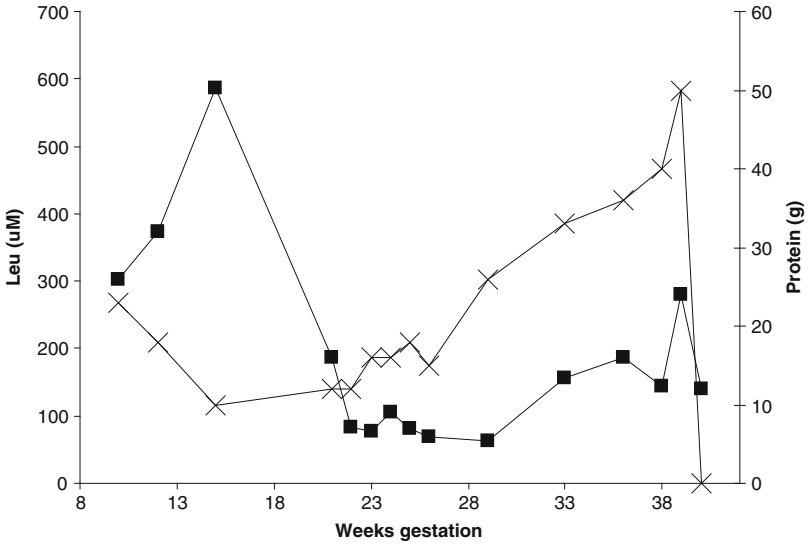

Fig. 1 Leucine levels (■ – μmol/L, left axis) and natural protein intake (x – g, right axis) plotted against gestational age. The peak at week 15 corresponds to her leucine level upon return from overseas

of age. She was managed with a low-protein diet and branched-chain amino acid–free supplements (MSUD Maxamum and MSUD Express). She had a number of significant decompensations during the first few years of life and was left with some neurological impairment and very mild intellectual impairment. She adhered loosely to a low-protein diet supplemented with MSUD Express Coolers (Vitaflo Australia). Her usual intake of natural protein was 15–35 g daily with 60 g protein from MSUD Express Coolers. Her leucine levels were persistently 500–1000 μM; unfortunately, improved compliance was difficult to achieve. Her past medical history included panic attacks, which were managed with fluvoxamine, and psoriasis. Up until her pregnancy, she worked full time at a department store.

She presented to our clinic at 10 weeks gestation. Plasma leucine levels were elevated (708 μM) and a low-protein diet (23 g natural protein equivalent and 60 g MSUD Coolers) was commenced, with the intention of regular monitoring of branched-chain amino acid levels via dried blood spot analysis.

Our patient planned to travel overseas to visit her non-consanguineous husband's family. She travelled with her dietary supplements and explicit letters of instruction for any episodes of illness. Unfortunately, she experienced an episode of fever and gastroenteritis after 1 week (14 weeks gestation). She could not maintain oral intake and progressively deteriorated, had a number of seizures and was said to have been unconscious for up to 24 h. She was treated with IV fluids and nasogastric administration of MSUD Express Coolers at a hospital in Pakistan. She recovered sufficiently to return to Australia, and when reviewed shortly after her arrival she was quite well with no neurological sequelae. Her leucine level at that time was 586 μM. There were no further episodes of decompensation during the pregnancy.

Dietary management for the remainder of the pregnancy included a low-protein diet with increases in allowances over the duration of the pregnancy according to amino acid levels (Figs. 1, 2). MSUD Express Cooler intake was increased from 60 g to 75 g at 15 weeks gestation. Monitoring of protein intake was by patient estimates of intake provided by phone and email, as she lived remote from the clinic. Pregnancy weight gain was around 15 kg and there was not significant vomiting at any stage.

A management plan for the peri-partum period was instituted and distributed to involved members of the obstetric, medical, nursing and dietician staff (Table 1). She went into spontaneous labour at 40 weeks gestation and successfully gave birth by vaginal delivery to a baby boy following a 7-h labour. Natural protein intake was zero on day 1 post partum and increased by 5 g increments to her usual pre-pregnancy intake of approximately 20 g over 1 week. The post-partum period was uneventful and she was discharged on day 8. She subsequently presented to her local hospital on day 12 post partum and was diagnosed with mastitis on the basis of fever and a painful breast. Her leucine level peaked at 549 μM. This episode was treated with antibiotics, increased calorie intake and a reduction in natural protein intake with a quick resolution over 3 days.

The baby's Apgar scores were 9 and 9 at 1 and 5 min. The birth weight was 3336 g, head circumference 34 cm and length 51 cm. Initial testing demonstrated normal levels of branched-chain amino acids, but elevated alloisoleucine that subsequently decreased (at birth = 15 μM, at 24 h = 2 μM, and 48 h = 1 μM). His newborn screen did

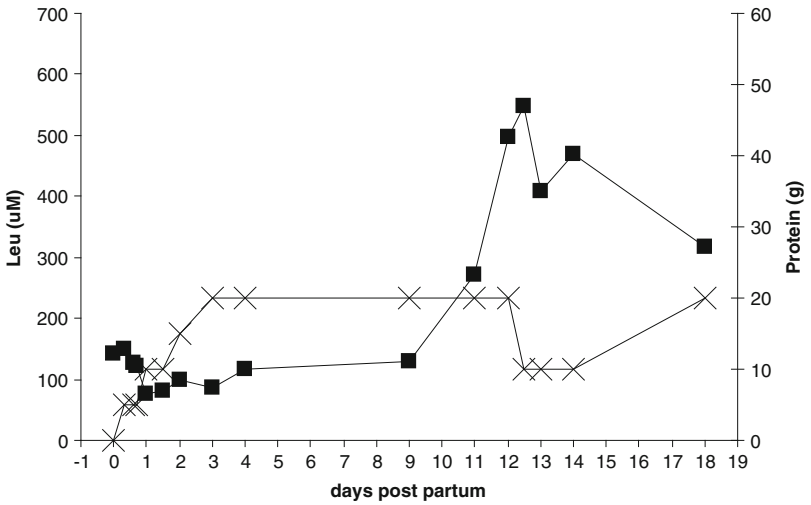

Fig. 2 Leucine levels (■ – μmol/L, left axis) and natural protein intake (x – g, right axis) plotted against days post partum. An episode of mastitis was diagnosed on day 12

Table 1 Management plan for the peri-partum period of patient 1

During labour and for 24 h post partum
- a. EUC and blood glucose taken at every 6 h
- b. Amino acids taken daily

Early labour
- a. Insertion of adequate venous access by anaesthetic team
- b. Food as usual
- c. High-energy (no protein) supplements – Energivit 30 % aiming for 1 L per 24 h
- d. MSUD Express Cooler supplements (5 × 130 mL per 24 h = 75 g protein)

Established labour or early labour and not tolerating oral intake
- a. IV 4 % dextrose in 1/5 normal saline (+/− additional K^+) at 100 mL/h
- b. Intralipid 20 % (3 g/kg/day)
- c. More intensive glucose + insulin therapy if required

First 24 h post partum if tolerating oral intake
- a. Protein-free food (0 g natural protein)
- b. High-energy (no protein) supplements – Energivit 30 % aiming for 1 L per 24 h
- c. MSUD Express Cooler supplements (5 × 130 mLper 24 h = 75 g protein)

First 24 h post partum: if not tolerating oral intake, then TPN given over 24 h containing
- a. 1 L 10 % dextrose
- b. 500 mL 20 % Intralipid
- c. Standard electrolytes
- d. Leucine-isoleucine-valine-free amino acids (60 g/day)

Day 2 and thereafter post partum
- a. Gradual increase in dietary protein as directed by the metabolic team/dieticians
- b. High-energy (no protein) supplements – Energivit 30 % aiming for 1 L per 24 h
- c. MSUD Express Cooler supplements (4 × 130 mL per 24 h)

not show evidence of MSUD. We interpreted the initially elevated alloisoleucine as being maternal in origin. On assessment at 4 months and then 20 months, he was thriving and showed appropriate development for his age.

Patient 2

The patient initially presented at 3 days of age becoming unwell with vomiting and increasing lethargy, progressing to seizures and loss of consciousness at 9 days of age, and was diagnosed with classical MSUD by urine metabolic screen on day 15. Her childhood course of disease was characterised by frequent recurrent metabolic decompensation, with over 70 hospitalisations by age 12 years. She had evidence of significantly delayed development. Formally, neuropsychological testing was consistent with moderate intellectual disability, presumed secondary to MSUD.

She adhered to an unmeasured low-protein diet supplemented with MSUD Express Coolers (Vitaflo Australia) and valine. By age 20 years, her usual intake of natural protein was estimated to be 30–50 g daily with 20–30 g protein from MSUD Express Coolers. Her compliance was suboptimal and leucine levels were typically 700–1300 μM. Her past medical history included panic attacks, exacerbated by poor metabolic control.

At 21 years of age, she presented to the paediatric metabolic clinic at 27 weeks gestation, based on ultrasound diagnosis of pregnancy 5 weeks earlier, and transferred to adult services. One week later, she presented with an acute febrile illness and threatened premature labour. Diagnosed with a urinary tract infection, she was also found to be mineral, vitamin and protein deficient. Subsequently, she failed to attend most scheduled antenatal and metabolic clinic visits. However, where amino acids were performed

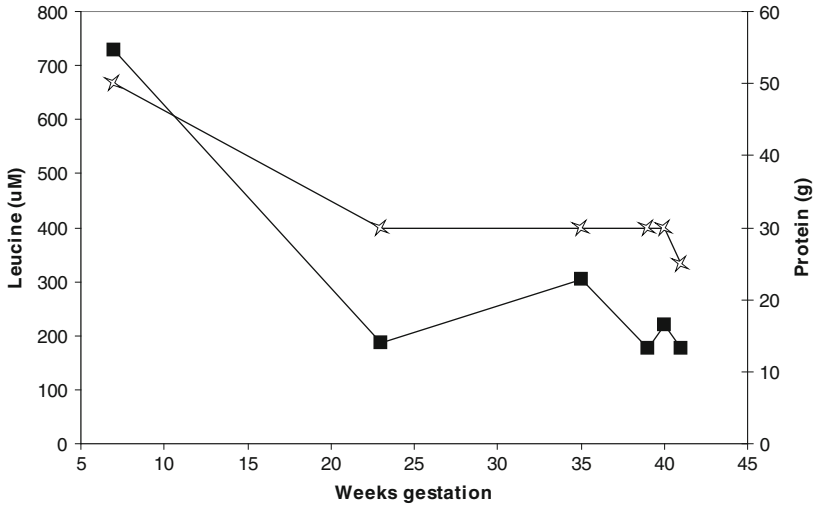

Fig. 3 Leucine levels (■ – μmol/L, left axis) and natural protein intake (x – g, right axis) plotted against gestational age

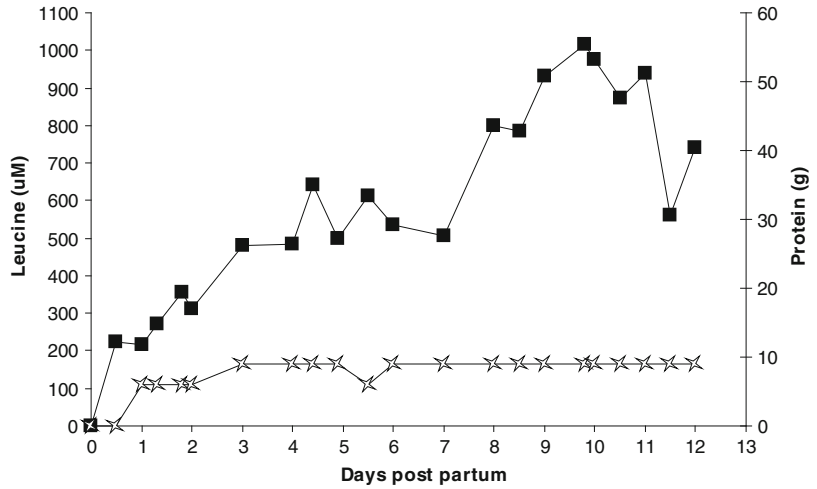

Fig. 4 Leucine levels (■ – μmol/L, left axis) and natural protein intake (x – g, right axis) plotted against days post partum

during the second and third trimesters of pregnancy, none was elevated with respect to leucine (Figs.3, 4). Pregnancy weight gain was around 14 kg and there was not significant vomiting at any stage.

She was admitted at 41 weeks gestation with suspected rupture of membranes but had failure to progress after 12 h which necessitated delivery by emergency lower segment caesarean section. Natural protein intake was zero on day 1 and increased to 9 g daily over 3 days. The patient self-discharged against medical advice on day 12 post partum despite elevated leucine levels, although she was not clinically encephalopathic at that time. There was considerable staff concern related to her social situation and capacity to care for the child in the setting of her intellectual disability. Implantable contraception was instituted prior to discharge.

The baby's Apgar scores were 9 and 9 at 1 and 5 min. The birth weight was 3405 g, head circumference 34 cm and length 50.5 cm. The newborn screen did not show evidence of MSUD. Apart from obesity and reflux in the setting of overfeeding, at 14 months of age the child has met expected developmental milestones.

Discussion

There are three pregnancies in women with classical MSUD documented in the literature, two of which ended without incident (Van Calcar et al. 1992; Grunewald et al. 1998) and the third of which reported maternal death at day 51 post partum (Yoshida and Tanaka 2003), although this death is more likely to have been due to trauma and

subsequent metabolic decompensation than a pregnancy-related decompensation. We describe here two additional cases demonstrating that pregnancy with a successful outcome can be achieved. Both our patients were classified as having "classical" MSUD, on the grounds that they presented with symptoms in the neonatal period. However in both, the protein tolerance before pregnancy was higher than that of most "classical" patients and suggests a more moderate MSUD phenotype.

We predicted two periods during which management would need careful monitoring and adjustment. Firstly, the increased tolerance of natural protein during the later stages of the pregnancy was predicted as the requirements of the fetus grow considerably during this time and maternal physiology changes to accommodate this. Other endocrine changes during pregnancy promote insulin resistance and an anabolic state (Di Cianni et al. 2003), also allowing increased protein tolerance. Careful monitoring was also required in the post-partum period. The patient described by Grunewald et al. had an increase in plasma leucine which peaked at day 9 post partum (Grunewald et al. 1998). This was thought to be as a result of a release of protein from the involuting uterus (Lee 2006), and thus we monitored our patients closely throughout the first 2 weeks post partum. The episode of decompensation in patient 1 precipitated by mastitis at day 12 post partum may have been exacerbated by the increased protein load of the involuting uterus. The poor metabolic control of patient 2 in the post-partum period was thought to be due to poor dietary and supplement compliance combined with uterine involution.

Both our patients had imperfect metabolic control throughout their pregnancies; however, fetal outcomes were good, despite maternal leucine levels that were grossly elevated at times. This contrasts favourably with the situation for maternal phenylketonuria and indicates that leucine is not a similarly potent teratogen. The apparently normal early development of these two children does not, however, preclude subtle long-term effects; therefore, children of such pregnancies should continue to be followed long term.

Documentation of management and outcome of pregnancies seem important, as there are as yet few reports in this or other inborn errors, with the exception of phenylketonuria. The true risk of an adverse effect on an infant, or an adverse post-partum event in the mother is not yet known for MSUD, and these two further cases help to define the situation.

Author Contributions

Michel Tchan – co-author, analysed data, clinician, guarantor
Mary Westbrook – revised article
Gisela Wilcox – co-author, analysed data, clinician
Rachel Cutler – revised article
Narelle Smith – revised article
Rebecca Penman – revised article
Boyd Strauss – clinician, revised article
Bridget Wilcken – clinician, co-author

Conflict of Interest

The authors declare they have no conflicts of interest.

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CASE REPORT

Fatal and Unanticipated Cardiorespiratory Disease in a Two-Year-Old Child with Hurler Syndrome Following Successful Stem Cell Transplant

Sampada Gupta · Anne O'Meara · Robert Wynn · Michael McDermott

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Abstract A 2-year-old female with Hurler syndrome (mucopolysaccharidosis type 1) died suddenly within 3 months of successful unrelated fully matched cord blood transplant, having received weekly enzyme replacement therapy (ERT) prior to transplant. Though an infectious aetiology was clinically suspected to be the cause of her unanticipated acute deterioration and untimely demise, autopsy findings suggested that a combination of pre-existing but sub-clinical Hurler related cardiopulmonary pathology and superimposed transplant related pulmonary venopathy as the basis of her death. This case highlights the limitations of ERT in ameliorating cardiorespiratory disease and the failure of standard pre-transplant investigations to detect significant abnormality related to her underlying condition. It also reinforces the importance of autopsy in explaining unanticipated events.

Introduction

Mucopolysaccharidosis type I (MPS I) or Hurler syndrome (HS) is an autosomal recessive metabolic disorder caused by the deficiency of α-l-iduronidase, resulting in the accumulation of substrates including heparin and dermatan sulphate. This accretion leads to characteristic facial features, hepatosplenomegaly, skeletal abnormalities, multi-organ dysfunction and progressive mental retardation. Allogeneic haematopoietic stem cell transplantation (HSCT) is the standard of care for patients with this condition (Krivit et al. 2005; Boelens et al. 2007; Prasad et al. 2010) and results in amelioration of many of the symptoms associated with this disorder and stabilisation of neuropsychological status but has limited impact on skeletal and corneal abnormalities (Peters and Steward, 2003; Aldenhoven et al. 2008). There are now robust data to support the contention that transplantation in infancy offers superior outcomes (Muenzer et al. 2009; Boelens et al. 2007), and the possibility of newborn screening programmes is a subject of current debate (Marsden and Levy 2010). HSCT, ideally before 18 months of age, while intellectual function is still preserved, has been recommended by some (Peters and Steward 2003), while a recently published European consensus document favours transplant by 2.5 years (de Ru et al. 2011). Weekly infusions of recombinant enzyme replacement therapy (ERT) have been shown to reduce morbidity, particularly in relation to cardiorespiratory function, prior to transplant (Cox-Brinkman et al. 2006; Wiseman et al. 2012) and is now standard practice in many major centres. This report documents the unexpected course of events following HSCT in a toddler whose peri-transplant course was uneventful.

Case Report

A female infant, born to non-consanguineous parents, displaying many of the features of Hurler syndrome, was eventually diagnosed at 23 months with MPS1, genotype W402X/W402X, a known mutation generating a

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S. Gupta · M. McDermott (✉)
Department of Histopathology, Our Lady's Children's Hospital, Crumlin, Dublin 12, Ireland
e-mail: michael.mcdermott@olchc.ie

A. O'Meara
Department of Oncology, Our Lady's Children's Hospital, Crumlin, Dublin, Ireland

R. Wynn
Department of Haematology, Royal Manchester Children's Hospital, Manchester, UK

premature stop codon and associated with no enzyme production. She was asymptomatic from a cardiorespiratory perspective with serial normal blood pressure measurements and was commenced on weekly ERT while awaiting HSCT. Standard pre-transplant assessment included 2D and M mode echocardiography on three separate occasions, all of which revealed normal left ventricular function and no evidence of valvular incompetence. Respiratory status was also unremarkable. Following 4 months of weekly ERT (100 U/kg) while awaiting transplant, she underwent an uneventful HLA identical (10/10) matched unrelated cord blood transplant (CBT) following busulfan (with pharmacokinetic monitoring), fludarabine and ATG conditioning with cyclosporine and methylprednisolone as GVHD prophylaxis. Her transplant course was uneventful with normal organ function, no significant infection and without graft versus host disease. Complete donor leucocyte engraftment was documented at 28 days post transplant using molecular analysis of donor/recipient discriminatory short tandem repeats. She remained well and asymptomatic on close surveillance following discharge on prophylactic cotrimoxazole, penicillin, acyclovir and itraconazole. On D + 86, she presented with a 5-day history of dry cough; on clinical examination, she was afebrile, O_2 saturation was at 60 % in room air and chest x-ray revealed bilateral pulmonary infiltrates. No conduction abnormality or arrhythmia was documented on cardiac monitoring. She was commenced on broad-spectrum antibiotics but deteriorated rapidly over the succeeding 2–3 h, had a cardiorespiratory arrest and failed to respond to resuscitation. The clinical impression at the time of her death was of a respiratory tract infection, probably viral in origin. Pre-mortem nasopharyngeal aspirate was negative for all respiratory pathogens.

A limited post-mortem examination, confined to the chest at parent's request, identified significant pulmonary and cardiac disease. The lungs showed no evidence of any infectious process on histological examination or in microbiological investigations including both culture and serological testing. The underlying airway architecture was unremarkable but there was a range of vascular pathology. Pulmonary arteries exhibited a patchy but often profound vasculopathy with marked intimal wall thickening without aneurysmal, plexiform or necrotising changes (Fig. 1). There was also evidence of a venopathy with pronounced endothelial swelling and atypia in a majority of pulmonary veins associated with fibroproliferative changes and attempted recanalisation (Fig. 2). Peri-arteriolar foreign body giant cells and foreign body giant cell granulomas without refractile material were also detected and occasional large arteries showed eccentric endothelial cushions with cholesterol clefts present while another showed an almost occlusive thrombo-embolus with abundant cholesterol debris. The lung sections also showed fibrin in some alveoli and there were multiple areas of microscopic acute pulmonary haemorrhage.

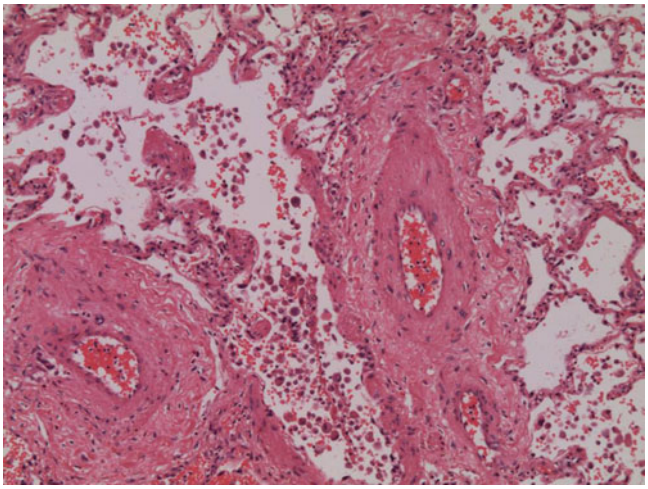

Fig. 1 Section of lung containing pulmonary artery branches with marked myointimal thickening

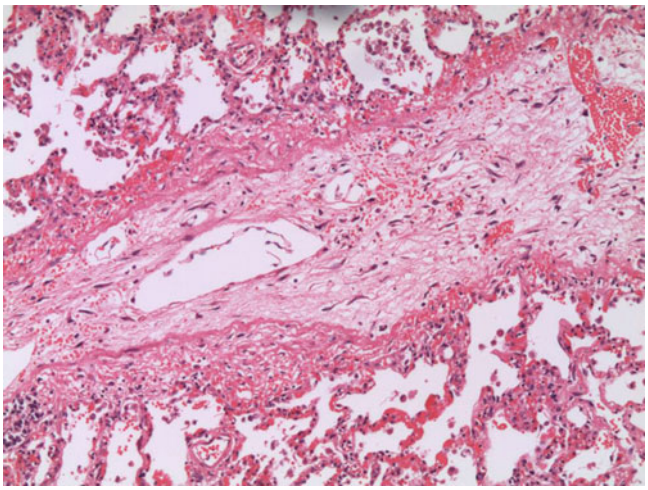

Fig. 2 Section of lung showing pulmonary vein with fibro-obliterative venopathy

Gross examination of the heart showed pericardial, endocardial and valvular thickening (Fig. 3). Histological examination confirmed conspicuous myxoid and fibromyxoid change in atrio-ventricular valve leaflets. There was profound endocardial thickening, particularly in the atrium, and areas of interstitial fibrosis were seen most notably in papillary muscles of the left ventricle. There was a profound coronary arteriopathy with substantial fibro-intimal thickening (Fig. 4). Connective tissue stains confirmed the presence of a vasculopathy and alcian blue histochemistry confirmed the presence of myxoid interstitial infiltrate within the valvular, endocardial and coronary arterial structures.

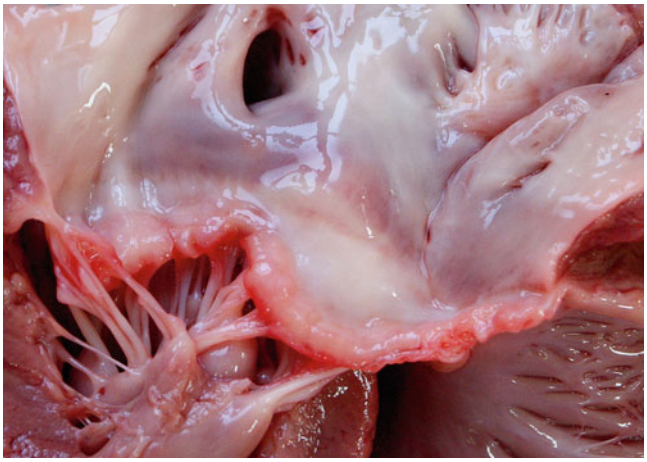

Fig. 3 Left side of the heart at autopsy showing thickened mitral valve leaflets with distinct nodularity of the free margins of the cusps

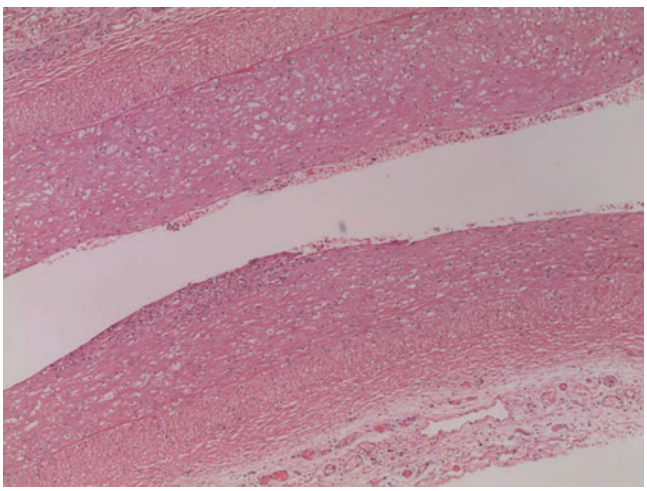

Fig. 4 Section of the proximal left anterior descending coronary artery showing marked intimal thickening

The findings suggested that a combination of pre-existing but sub-clinical Hurler related cardiopulmonary pathology and superimposed transplant related pulmonary venopathy formed the basis of the patient's abrupt clinical deterioration and death.

Discussion

Cardiac disease in MPS 1 has a prevalence and severity believed to be in the range of 60–100 % of those studied and ranges from severe dilated cardiomyopathy presenting within days or weeks of birth to identification of thickened and incompetent cardiac valves in otherwise asymptomatic individuals (Leal et al. 2010; Braunlin et al. 2011; Wiseman et al. 2012). While valvular abnormalities and coronary artery disease are most frequently documented, vascular changes in great vessels and conduction abnormalities have also been described. Transthoracic echocardiography and 12-lead ECG remain the standard methods used to assess cardiac status in these patients, and serial monitoring, following successful transplantation, has proved a reliable indicator of cardiac function in the vast majority of patients. Other more invasive techniques, including transoesophageal echocardiography, cardiac MRI and coronary angiography, are rarely indicated and are not without risk in this unique group of patients and may also underestimate the extent of disease.

Enzyme replacement therapy (ERT), usually commenced once a diagnosis is made and continued until there is evidence of sustained engraftment, has revolutionised the management of dilated cardiomyopathy in infancy (Hirth et al. 2007; Wiseman et al. 2012) with many other centres reporting anecdotal evidence of improvement in shortening fraction and subsequent uneventful HSCT, together with alleviation of upper respiratory symptoms. Valvular thickening, on the other hand, appears irreversible and may even progress (Braunlin et al. 2003; Malm et al. 2008), reflecting the relative avascularity of these structures. While some authors suggest that the status of coronary ostia may be improved following successful transplantation (Braunlin et al. 2001), others have not supported this observation (Yano et al. 2009).

The impact of cardiac disease on survival following bone marrow transplantation is also disputed. In a study carried out in Minnesota comprising 74 patients with MPS 1 undergoing HSCT, the overall survival post transplant was 63 % and 53 % at 1 and 5 years, respectively. An assessment of pre-transplant risk factors found no clear link to cardiac status. However, patients with pulmonary complications were associated with reduced survival. Of the 33 patients who died, the largest proportion died of infection (Orchard et al. 2010).

Pulmonary veno-occlusive disease (PVOD) has been previously documented following HSCT, usually in association with haematological malignancies (Barker et al. 2003; Montani et al. 2010). It is thought that the greatest risk factor for the development of PVOD is endothelial injury from cytotoxic chemotherapy and irradiation. Several reports indicate that patients with MPS 1 may have a higher likelihood of pulmonary complications such as alveolar haemorrhage than patients of similar age transplanted for other diseases (Gassas et al. 2003; Orchard et al. 2010). Hepatic VOD has been well documented following HSCT (Barker et al. 2003; Copell et al. 2010; Corbacioglu et al. 2012), and the risk is increased when busulphan is used as conditioning agent (Vassal et al. 1996; Corbacioglu et al. 2012); PVOD is more difficult to document as lung biopsy is rarely undertaken. It is worth noting that

pharmacokinetic monitoring of intravenous busulphan was well within the therapeutic range in this patient.

In conclusion, the fulminant course experienced by our patient is a sobering reminder that standard pre-transplant evaluation may fail to detect the true extent of cardiorespiratory pathology. This experience raises the question of whether the recently recommended upper age limit of 2.5 years for HSCT in this condition (de Ru et al. 2012) is appropriate for patients with the more severe genotype as was the case in this particular patient. Consideration must also be given to the incorporation of non-busulphan conditioning regimens for this high-risk cohort of patients. This case report also reinforces the role that autopsy can play in explaining unanticipated events for both family and clinical staff, providing information that continues to impact on the shape of future practice.

Take-Home Message

Standard pre-transplant workup may underestimate the extent of cardiorespiratory disease in Hurler syndrome which may be compounded by current conditioning regimens.

Author Contributions

Sampada Gupta: Specialist registrar in histopathology, assisted with the autopsy examination and wrote the initial draft of the manuscript.

Anne O'Meara: Consultant in paediatric oncology, was responsible for the care of the patient from the time of diagnosis of Hurler syndrome and edited the manuscript.

Robert Wynn: Consultant in paediatric haematology & HSCT, was the transplant physician involved in the patient's care.

Michael McDermott: Consultant paediatric pathologist, performed the autopsy examination and edited the manuscript.

Guarantor

Dr McDermott serves as guarantor of the article.

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Ethics and Consent

Ethical approval was not required for this report, but family members have consented to the publication of these findings.

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