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ORIGINAL ARTICLE

Genotype and phenotype characterization in a Spanish cohort with isovaleric acidemia

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Isovaleric acidemia (IVA) is a rare disorder of leucine metabolism. We carried out a multicenter study of IVA patients diagnosed by newborn screening (NBS) or symptoms clinics over a period of 28 years in Spain. Evaluated at diagnosis, data included age, detection method, levels of C5 and IVG, enzymatic studies, clinical presentation parameters and genotype in 16 patients. Follow-up data included C5 levels, intellectual quotient and correlation genotype–phenotype. IVA was detected by NBS in 8 patients (prevalence of 1/326 629). Except 1, all the 8 patients identified by NBS were asymptomatic at diagnosis and had isovalerylcarnitine (C5) levels of 1.6–6.4 μM and isovalerylglycine (IVG) levels <1100 mmol per mol creatinine; they remained asymptomatic with a natural protein intake $\geqslant 1.5\ g\ kg^{-1}$ per day. Symptomatic patients with chronic intermittent or acute neonatal IVA had C5 levels of 3.9–16.3 μM and IVG levels >3400 mmol per mol creatinine. The percentage of isovalerate incorporation in fibroblasts was 64–80% in patients detected by NBS and 4.9–13% in symptomatic patients. Cognitive function was within normal ranges in all patients but was negatively correlated with IVG at detection (–0.592; $P<0.05$). The genetic analysis revealed nine novel mutations. The clinical/biochemical phenotype correlated fairly well with the phenotype predicted by the mutations found. In conclusion, although blood C5 levels have traditionally been considered the prognostic marker of choice, urine IVG levels would appear to be a better predictor, as they correlated well with severity of mutations and were associated with a lower incorporation rate of IVA in fibroblasts and a less favorable clinical course.
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INTRODUCTION

Isovaleric acidemia (IVA, OMIM 243500) is a rare autosomal-recessive metabolic disorder characterized by abnormal leucine metabolism owing to isovaleryl-CoA dehydrogenase (IVD) deficiency. IVD catalyzes the α,β-dehydrogenation of isovaleryl-CoA resulting in 3-methylcrotonyl-CoA and transfers the reducing equivalents to the electron transfer flavoprotein.[1] This enzyme deficiency results in the accumulation of isovaleryl-CoA derivatives that are detected in urine and blood as the conjugates isovalerylglycine (IVG) and isovalerylcarnitine (C5).[2] In Western populations, the estimated prevalence of IVA is 1:526 000 (0.19 cases per 100 000 population) based on clinical detection rates[3] and 1:62 500–1:362 000 based on screening detection rates.[4–6]

The clinical manifestations of IVA are highly variable and range from a complete absence of symptoms to severe involvement. Three characteristic phenotypes have been identified: asymptomatic presentation, a chronic intermittent form with onset in infancy or childhood, and an acute neonatal form with early onset of metabolic decompensation that can lead to coma and death. Chronic intermittent IVA is frequently associated with developmental delay and/or failure to thrive and optic nerve atrophy. A distinctive sweaty feet odor is a presenting symptom in almost all patients at diagnosis.[2,7–10]

The main goal of treatment is to achieve a state of anabolism to reduce the formation of isovaleryl-CoA from leucine catabolism. Long-term treatment strategies comprise restricted intake of protein or leucine combined with L-carnitine and/or glycine supplementation to enhance the conversion of potentially neurotoxic-free isovaleric acid to non-toxic conjugates excreted in urine.[11,12]

The *IVD* gene is located on chromosome 15q14–15, which consists of 12 exons that span ~15 kb of genomic DNA.[13] To date,

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>70 disease-causing mutations have been detected (Human Gene Mutation Database http://www.hgmd.cf.ac.uk). The mutations are highly heterogeneous and genotype–phenotype correlations described to date have generally been weak.[9,14–21] Nonetheless, almost half of the mutant *IVD* alleles sequenced from infants with IVA detected by newborn screening (NBS) have been found to contain the milder c.932C>T mutation associated with a potentially asymptomatic phenotype.[22]

Expanded NBS with tandem mass spectrometry (MS/MS) and calculation of the ratio of C5 to octanoylcarnitine, butyrylcarnitine and propionylcarnitine in whole blood is now used to detect presymptomatic IVA.[6] IVA detected by NBS appears to be associated with a mostly favorable clinical course, although long-term prognosis has yet to be established.[21] We conducted this study to determine genotype, phenotype and follow-up data for a cohort of children with IVA diagnosed in Spain by NBS or clinical symptoms and identify predictors of prognosis. We also describe nine novel mutations and their clinical relevance.

MATERIALS AND METHODS

Study population

The study population comprised 16 patients (9 males) from 13 unrelated families who had been diagnosed with IVA following detection by NBS, family evaluation or clinical findings in 11 hospitals in Spain (which are reference for such diseases) over a period of 28 years (January 1987–December 2015). All patients included in the study had genetic study; it was completed in 3 patients who did not previously. The study protocol was approved by the Research Ethics Committee of Santiago-Lugo (2016/470). Informed consent was obtained from all parents.

Evaluation of patients

The following variables were evaluated at diagnosis: age, sex, consanguinity, detection method, presence or absence of clinical symptoms, blood acylcarnitines, urine organic acid levels, and acylglycine profile. Additional characterization tests included the incorporation of radiolabel from [^{14}C] isovaleric acid in macromolecules in cultured fibroblasts and mutation analysis of the *IVD* gene.

On confirmation of the diagnosis, treatment was administered according to Spanish recommendations on IVA management.[23] This included restricted leucine intake adapted to age, tolerance and disease severity, combined in some cases with carnitine and glycine supplementation. Intercurrent infections were treated aggressively by withdrawing or reducing protein intake to 50% for ⩽24 h, depending on the severity of symptoms, while providing high energy intake with a 20% increase in calories through carbohydrates, lipids and a double dose of carnitine.

Follow-up

Clinical course was monitored throughout follow-up. Biochemical follow-up included periodic monitoring of blood-free carnitine and C5 levels. Anthropometric evaluation included measurement of body weight, body length and head circumference, expressed as percentiles with respect to the reference population.

Cognitive function was assessed by Psychomotor Development Index (PDI) or Intellectual Quotient (IQ) using the Wechsler Intelligence Scale (WISC R) for school-age children, the McCarthy Scales of Children's Abilities for preschool-age children and the Brunet–Lèzine Scale in infants. The overall index score of PDI or IQ is considered in the normal range when it is >85.

Biochemical tests

Acylcarnitines, including C5, were analyzed by MS/MS as butyl ester derivatives in dried blood spots taken on the third day of life as a part of the NBS programs. Quantitative acylcarnitine analysis in plasma was performed as described in Ferrer *et al.*[24] Urine organic acids, including IVG, were analyzed by MS/MS in the case of dried spots, as previously published[25] and by gas chromatography/MS in the case of liquid samples. The incorporation of radiolabel from [^{14}C] isovaleric acid in macromolecules in cultured fibroblasts was performed as described in Kleijer *et al.*[26]

Molecular tests

DNA was isolated and sequenced using standard procedures for whole blood or fibroblasts from all patients and their parents, except in the case of one patient who had been conceived by *in vitro* fertilization with oocyte donation. Mutation analysis of *IVD* was performed by conventional Sanger sequencing using standard procedures. Primers were designed to overlap the coding sequences and their flanking regions (sequences available on request). In the past 3 years, mutation analysis was performed using next-generation sequencing (Nextera Rapid Capture Custom Enrichment Kit and Miseq, Illumina, San Diego, CA, USA), which detected a genomic rearrangement encompassing the first exons of the gene. The pathogenicity of novel variants was evaluated using Alamut Visual Interactive Biosoftware, Mutalyzer 2.0.14 (Alamut, Rouen, France), which predicts the possible functional impact of mutations.

Statistical analysis

Statistical analysis was performed using R version 3.2.3 (R Core Team (2015), R Foundation for Statistical Computing, Vienna, Austria). In order to determine the significance of the associations and/or differences between measured variables, data were analyzed using the Wilcoxon signed-rank test, when one of the variables was quantitative and the other one was qualitative, and the Spearman's rank correlation coefficient, when both variables were qualitative. Statistical significance was set at the P-value<0.05 level.

RESULTS

Clinical and biochemical diagnosis

We evaluated 16 patients with IVA, including 3 pairs of siblings. Eight patients (p1–p8) were diagnosed by NBS, with a mean time to sample collection of 2.7 days (range, 2–5 days). Mean C5 concentration in dried blood spot samples was 4.2 μM (range, 1.4–7.91; cutoff, ⩽0.57 μM), and the mean age at diagnosis was 16 days (range, 6–30 days). Mean levels of C5 and IVG at diagnosis for 7 of the 8 patients were 3.7 μM (range, 1.6–6.4 μM) and 561 mmol per mol creatinine, respectively (Table 1). The eighth patient (p1), who had clinical symptoms (poor feeding and a sweaty feet odor) and hyperammonemia, had a C5 level of 39.8 μmol l^{-1} at diagnosis. After recovering stable condition, C5 levels decreased to 9.8 μM, maintaining an average of 10.8 μM in the first follow-up months with good clinical evolution.

Two cases of IVA were detected based on family history and both patients (p9 and p10) remained asymptomatic with treatment. Six patients (p11–p16) were detected on clinical grounds. The symptoms included poor feeding in all cases and vomiting/dehydration and lethargy in 5 cases (83.3%). Three had a sweaty feet odor and hyperammonemia. IVA was detected during the neonatal period in the more severe cases and during infancy in the more moderate cases. Mean levels of C5 in plasma and IVG in urine were 8.1 μM (range, 3.9–16.3 μM) and 6683 mmol per mol creatinine at diagnosis (Table 1).

Incorporation of isovalerate into fibroblasts was measured in 10 patients. In all cases, the percentage of incorporation seemed to correlate with clinical phenotype (Table 2), as higher rates were observed in patients diagnosed by NBS (64–80% of control values) than in symptomatic patients (4.9–13%).

Follow-up

Mean follow-up for 15 of the 16 patients was 9 years and 10 months (range, 6 months–22 years). The other patient died 2 days after detection of IVA.

Table 1 Characteristics of 16 patients with isovaleric acidemia at diagnosis and during follow-up

						C5 and IVG levels at the start of treatment		*Clinical and biochemical features at diagnosis*							*Treatment*			*Evolution of C5 levels throughout life (μmol l⁻¹)*			
Patient's	*gender*	*Detection method/ clinical phenotype*[a]	*C5 (DBS) μmol l⁻¹ at detection*	*Age at diagnosis (days/ months/ years)*	*IVA incorp (%)*[b]	*C5 (DBS) μmol l⁻¹*	*IVG in urine mmol permol creatinine*	*Poor feeding*	*Vomit/ dehydration*	*Lethargy/ coma*	*Sweaty feet odor*	*Ammonium (μmol l⁻¹)*	*pH*	*Current age (months/ years)*	*Protein intake (g kg⁻¹ per day)*	*Carnitine*	*Glycine*	*1 year*	*2–5 years*	*6–20 years*	*PDI/IQ in follow-up*
1[d]	M	NBS A	7.9	6 days		39.8	19 069	+	–	–	+	191↑	7.45	6 months	1.2	Yes	Yes	10.8			85
2	M	NBS C	2.0	30 days		2.4	1105	–	–	–	–	37	7.34	2 years 3 months	1.5	No	No	1.3	1.2		89
3	M	NBS C	1.8	10 days		2.3		–	–	–	–	36	7.35	1 year	1.5	No	No	1.21			⩾85
4[A]	F	NBS C	4.0	26 days	80	4.0	242	–	–	–	–	31	7.37	3 years 1 month	1.5	Yes	No	1.74	2.38		92
5[A]	M	NBS C	7.9	10 days	64	6.0	955	–	–	–	–	65	7.41	11 months	1.5	Yes	No	2.6			⩾85
6	F	NBS C	2.9	10 days		3.1	230	–	–	–	–	56	7.37	4 years 5 months	1.5	No	No	1.3			104
7	M	NBS C	1.4	28 days	72	1.6[c]	272	–	–	–	–	51	7.39	2 years 5 months	1.5	No	No	1.3	0.8		93
8[B]	M	NBS C	5.7	11 days		6.4		–	–	–	–	30	7.36	2 years	1.5	No	No	2.35	2.46		108
9[B]	F	FE C		3 years		3.5		–	–	–	–	32	7.34	3 years 1 month	1.8	No	No	1.47			95
10[C]	F	FE B		2 days	7	15.7	4376	–	–	–	–		7.35	11 years 2 months	0.64	Yes	Yes	3.6	5.83		85
11[C]	F	CF B		2 years 3 months	13	—	3488	+	+	+	+	47	7.33	19 years 11 months	1.0	Yes	Yes	6.8			85
12	F	CF A		27 days	10	3.9[c]	7308	+	+	+	–	55	7.35	7 years 10 months	0.95	Yes	Yes	5.53	4.81	2.98	85
13[d]	M	CF A		20 days	5.5	16.3	3352	+	–	+	+	606↑	7.26	9 years	0.8	Yes	Yes	5.3	5.89	8.59	87
14[d]	M	CF B		1 year 9 months	12	4.3[c]	16 594	+	+	+	–	126↑	7.17	24 years 6 months	0.7	Yes	Yes	4.3	5.6	11.5	88
15	F	CF B		6 years 1 month	4.9	7.8[c]	4653	+	+	–	–	28	7.18	15 years 1 month	0.8	Yes	Yes	9.6	8.25	9.49	92
16[d]	M	CF A		6 days	13	—	4706	+	+	+	+	500↑	7.25	8 days†	0.7	Yes	Yes				

Abbreviations: CF, clinical findings; F, female; FE, family evaluation; IVA, isovaleric acid; M, male; NBS, newborn screening; PDI/IQ, Psychomotor Development Index/Intellectual Quotient.
Capital letters A, B and C represent pairs of siblings.
[a]A, acute form; B, intermittent form, C, asymptomatic case; C5 (DBS), C5 in dried blood spot, cutoff ⩽0.57 μmol l^{-1}.
[b]% IVA incorporation in fibroblasts.
[c]In plasma, control value <0.2 μmol l^{-1}; IVG in urine, isovalerylglycine in urine, control value <4 mmol per mol creatinine.
[d]Patients with elevated values of ammonium.
†Patient dead.

Table 2 Mutations in 16 patients with isovaleric acidemia

Patient	*Detection method*	*Clinical form*[a]	*Allele 1 nucleotide change (protein change)*	*Allele 2 nucleotide change (protein change)*	*PPM*[b]
1	NBS	A	**c.465+22_519delinsGTTG**	c.465+22_519delinsGTTG	Severe
2	NBS	C	c.941C>T (p.Ala314Val)	c.941C>T (p.Ala314Val)	Mild
3	NBS	C	c.941C>T (p.Ala314Val)	c.941C>T (p.Ala314Val)	Mild
4	NBS	C	**c.1214T>C (p.Ile405Thr)**	c. 500T>C (p.Met167Thr)	—
5	NBS	C	c.1214T>C (p.Ile405Thr)	c. 500T>C (p.Met167Thr)	—
6	NBS	C	c.158G>A (p.Arg53His)	c.941C>T (p.Ala314Val)	Mild
7	NBS	C	**c.706G>A (p.Gly236Ser)**	c.706G>A (p.Gly236Ser)	Mild
8	NBS	C	**c.941C>G (p.Ala314Gly)**	c.941C>G (p.Ala314Gly)	Mild
9	FE	C	c.941C>G (p.Ala314Gly)	c.941C>G (p.Ala314Gly)	Mild
10	FE	B	**c.884T>C (p.Leu295Pro)**	**c.1211A>G (p.Glu404Gly)**	
11	CF	B	c.884T>C (p.Leu295Pro)	c.1211A>G (p.Glu404Gly)	
12	CF	A	**c.111-112delCG (p.Val38Glyfs*20**	c.111-112delCG (p.Val38Glyfs*20)	Severe
13	CF	A	c.158G>C (p.Arg53Pro)	c.158G>C (p.Arg53Pro)	Severe
14	CF	B	**c.1-?_c.295+? (Delexons 1–3)**	c.1184G>A (p.Arg395His)	Severe
15	CF	B	**c.1099T>C (p.Ser367Pro)**	c.1-?_c.295+? (Delexons 1–3)	Severe
16	CF	A	c.367G>A (p.Gly123Arg)	c.367G>A (p.Gly123Arg)	Severe

Abbreviations: CF, clinical findings; FE, family evaluation; NBS, newborn screening; PPM, phenotype predicted by mutation.
Nucleotide changes are numbered according to GenBank entry NM_002225.3 and the corresponding amino-acid numbers are designated according to NP_002216.2, both in accordance with HGMD nomenclature; ?, deletion with uncharacterized breakpoint.
[a]A, acute form; B, intermittent form; C, asymptomatic case.
[b]Phenotype predicted by mutation in homozygous or hemizygous patients. New mutations are indicated in bold.

Patients diagnosed following NBS or on the basis of family history were placed on a mild leucine restriction diet, combined with carnitine and glycine supplementation in two cases (p1, p10). In this group, C5 levels remained below 6 μM in all patients except p1 and p6, who showed levels up to 22 and 7.8 μM, respectively (Table 1).

Only two patients, p14 and p15, required admission to hospital outside the detection period. The total hospitalization period was 46 days and the patients had plasma C5 levels >14 μM and IVG levels $>12\,000$ mmol per mol creatinine.

PDI/IQ scores were average in all patients, although the mean ± s.d. score was slightly though not significantly higher in patients with IVA detected by NBS (92.6 ± 8.9 vs 87.4 ± 2.9). Our series of patients is small, there was a significant negative correlation between PDI/IQ and IVG levels at diagnosis (−0.592; $P<0{,}05$) but not significant between PDI/IQ and the changes in C5 levels over time (−0.408; $P=0.13$). Growth was within the established percentiles (p10–p90) in all patients.

Based on the biochemical and clinical data, eight patients had mild/asymptomatic IVA (p2–p9), four had chronic intermittent IVA (p10, p11, p14, p15) and four had acute neonatal IVA (p1, p12, p13, p16).

Mutation analysis

The mutations found in the 16 patients from 13 unrelated families are summarized in Table 2. Nine patients were homozygous for one mutation and seven were compound heterozygotes. In total, 15 mutations were detected in 26 mutant alleles. Six of the mutations have been previously described—p.Arg53His, p.Arg53Pro, p.Gly123Arg, p.Met167Thr, p.Ala314Val, and p.Arg395His—and nine are novel. Of the novel mutations, six are presumably missense mutations (p.Gly236Ser, p.Leu295Pro, p.Ala314Gly, p.Ser367Pro, p.Glu404Gly and p.Ile405Thr). In addition, there was a small deletion mutation (c.111-112delGC), a deletion/insertion mutation (c.465+22_519 delinsGTTG) and a large genomic rearrangement encompassing exons 1–3 (c.1-?_c.295+?; '?' means deletion with uncharacterized breakpoint). Analysis of the pathogenic potential of the novel missense mutations in Alamut Visual showed that they were all potentially disease causing but that three of them (p.Ala314Gly and p.Ser367Pro) were likely to be benign (Table 3). The genomic rearrangement was not characterized by long PCR amplification because it involved exon 1 (data not shown). The nucleotide changes detected in p15 and p16 were detected in homozygosity in mRNA.

DISCUSSION

In this study of clinical, biochemical and genetic data from a heterogeneous group of Spanish patients with IVA characterized by different phenotypes (eight asymptomatic, four chronic intermittent and four acute neonatal) and detection methods (NBS, family evaluation and/or clinical symptoms), we sought to identify potential factors that might have an influence on disease outcome. We also assessed possible associations between detection method, C5 levels, clinical findings and cognitive development. Our study comprised 16 patients from 11 hospitals, with the highest proportion of IVA cases in Spain. At the end of the study period (December 2015), 10 patients were ⩽4 years. The other patients, all diagnosed on the basis of clinical manifestations, were older.

In Spain, 1 306 518 newborns were screened for IVA between January 2001 and December 2013. The detection of 8 cases by NBS in our series corresponds to a prevalence of 1/326 629, which is similar to rates reported elsewhere.[5,27]

Newborns with metabolically mild IVA (that is, with dried blood spot C5 concentrations of 0.8–6 μmol ml^{-1}) have been found to remain asymptomatic during episodes of febrile illness, even without dietary therapy.[2] Our data are consistent with this observation, as most patients who were screened at birth had C5 levels <6 μM and low IVG in urine levels and remained asymptomatic with a protein intake of at least 1.5 g kg^{-1} per day. IVG in urine at diagnosis is a good biochemical marker of prognosis, and in our series, all the patients with chronic intermittent or acute neonatal forms of IVA had an IVG level >3000 mmol per mol creatinine on detection of the disease.

Incorporation of isovaleric acid into protein, as an indirect measure of IVD activity, also appears to be a good predictor of disease severity. All the patients diagnosed on the grounds of their symptoms had a low incorporation rate ($<13\%$ of control values), while the three patients with mild disease detected by NBS had rates close to normal. It is unlikely that these patients with such considerable values become

Table 3 New missense mutations identified in the *IVD* gene

		Prediction (alamut visual software application)									
Mutation	*Exon*	*PhyloP*	*GVGD*	*SIFT*[e]	*Polyphen2*[f]	*Mutation taster*[g]	*Predictor Provean*[a]	*Predictor mutation assessor*	*MAF*[b]	*EVS*[c]	*ExAc*[d]
c.1214T>C (p.Ile405Thr)	12	4.81 (−14.1; 6.4)	C65 (GV: 0.00–GD: 89.28)	Deleterious score: 0	Probably damaging score of 0.999	Disease causing	Deleterious—4.952	High (4.855)			0.00082%
c.706G>A (p.Gly236Ser)	7	6.18 (−14.1; 6.4)	C55 (GV: 0.00–GD: 55.27)	Deleterious score: 0	Probably damaging score of 1.000	Disease causing	Deleterious—5.783	High (4.28)			
c.941C>G (p.Ala314Gly)	9	6.02 (−14.1; 6.4)	C0 (GV: 90.76–GD: 28.37)	Tolerated score: 0.07	Benign score of 0.391	Disease causing	Neutral—1.698	Medium (3.255)			
c.884T>C (p.Leu295Pro)	8	4.81 (−14.1; 6.4)	C25 (GV: 30.92–GD: 68.57)	Deleterious score: 0.01	Probably damaging score of 0.994	Disease causing	Deleterious—4.952	High (3.82)			
c.1211A>G p.Glu404Gly	12	4.81 (−14.1; 6.4)	C65 (GV: 0.00–GD: 97.85)	Deleterious score: 0	Probably damaging score of 1.000	Disease causing	Deleterious—6.933	High (3.695)			
c.1099T>C p.Ser367Pro	11	−0.36 (−14.1; 6.4)	C0 (GV: 205.53–GD: 0.00)	Tolerated score: 0.16	Benign score of 0.433	Disease causing	Deleterious—2.512	Medium (2.855)			

Abbreviations: IVD, isovaleryl-CoA dehydrogenase; MAF, minor allele frequency; EVS, Exome Variant Server.
Genomic coordinates are given in hg19/GRCh37. Nomenclature of the mutations according to Human Genome Variation Society (HGVS) and checked using Mutalyzer (https://mutalyzer.nl).
[a]http://provean.jcvi.org/.
[b]http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/.
[c]http://evs.gs.washington.edu/EVS/.
[d]http://exac.broadinstitute.org/.
[e]http://sift.jcvi.org/.
[f]http://genetics.bwh.harvard.edu/pph2/.
[g]http://www.mutationtaster.org/.

symptomatic. It was not possible to perform this test in the only symptomatic patient diagnosed following NBS, but his C5 and IVG levels at diagnosis suggest a more severe form of disease and a need for stricter protein restriction and clinical control than the other patients who underwent NBS. Although our findings are based on a small sample of patients, they suggest that mild forms detected at screening might not progress later in life.

It is striking that, of the six patients detected on the basis of clinical manifestations, just two (p14, p15) had acidosis (pH<7.25) and four (p1, p13, p14, p16) had hyperammonemia at diagnosis. IVA appears to be exceptional within the classic organic acidurias in that it is associated with mild rather than severe neuropathological changes.[7,28] All the patients in our series had PDI/IQ scores within the normal range during follow-up, although the mean score was >5 points lower in patients diagnosed on the basis of their symptoms than those diagnosed following NBS (86.8 vs 92.6). Neurological status at the time of disease detection might explain this difference, although the transient period of encephalopathy in IVA is generally believed to be fully reversible and not responsible for global or focal ischemic brain damage.[7,10] None of the three patients with severe catabolic episodes at diagnosis (p1, p14, p15) showed a diminished IQ. However, we did observe slightly higher C5 concentrations over time in patients with a lower IQ, suggesting that chronic rather than acute damage might be the predominant factor determining the extent of neurological damage in IVA patients, as has been previously proposed.[7] Oxidative damage may also, at least in part, be involved in the neuropathology of IVA.[29] The above findings and observations reinforce the importance of NBS for IVA.

Our cohort of patients is genetically heterogeneous. We identified 16 mutations, including 9 novel ones, thereby adding to current knowledge on pathogenic mutations in the *IVD* gene. Three of the novel mutations can be considered to be disease causing because they are loss-of-function mutations (c.465+22_519delinsGTTG, c.111-112delCG and c.1-?_c.295+?). Of the six single nucleotide changes, four were predicted to be potentially harmful by the Alamut Visual software application. No effect on the splicing process was detected for the two mutations predicted to be benign: p.Ser367Pro and p.Ala314Gly. Nevertheless, the results of the bioinformatics analysis need to be confirmed by functional analysis.

Some other studies have demonstrated variable clinical presentations for common genotypes,[16,30] but, although this is a small cohort of IVD patients, their clinical/biochemical findings correlated fairly well with the phenotype predicted by the mutations found. It is noteworthy that several patients diagnosed by NBS were homozygous for the previously described mild mutation p.Ala314Val[22] or for the novel predicted benign variant change p.Ala314Gly located in the same

codon, suggesting that p.Ala314Gly is probably a very mild mutation or a functional polymorphism.[1] Other patients were compound heterozygous for mutations already described in mild forms of the disease, namely p.Met167Thr and p.Arg53His.[20,22] These are probably also thus mild mutations, together with the novel change p.Gly236Ser, which was detected in homozygosity in a patient with a probably asymptomatic form of IVA. Three of the symptomatic patients had mutations previously described in symptomatic IVA: p.Arg53Pro, p.Arg395His, and p.Gly123Arg.[14,16,31] These mutations can probably be classified as severe, together with p.Ser367Pro, which was detected in a patient with a chronic intermittent form of the disease in hemizygosity.

This study has several limitations, principally its retrospective design and the fact that NBS data are only available for 2011 onwards, which is when NBS for IVA was introduced in most regions in Spain. Nevertheless, the cases presented are a good reflection of IVA in Spain at the present time and interestingly reflects IVG urine as the best biomarker to predict the prognostic of IVA patients.

In summary, this study has provided information that extends the existing knowledge of IVA. The identification of novel *IVD* mutations and their linking to clinical presentation may be useful for the future implementation of NBS based on mutation detection by massive parallel sequencing, although such findings must be confirmed by biochemical analysis prior to treatment. Early diagnosis and treatment contribute to favorable outcomes in patients with IVA detected in the neonatal period.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Tanaka, K., Budd, M. A., Efron, M. L. & Isselbacher, K. J. Isovaleric acidemia: a new genetic defect of leucine metabolism. *Proc. Natl Acad. Sci. USA* **56**, 236–242 (1966).
2 Vockley, J. & Ensenauer, R. Isovaleric acidemia: new aspects of genetic and phenotypic heterogeneity. *Am. J. Med. Genet. C Semin. Med. Genet.* **142C**, 95–103 (2006).
3 Moorthie, S., Cameron, L., Sagoo, G. S., Bonham, J. R. & Burton, H. Systematic review and meta-analysis to estimate the birth prevalence of five inherited metabolic diseases. *J. Inherit. Metab. Dis.* **37**, 889–898 (2014).
4 Schulze, A., Lindner, M., Kohlmuller, D., Olgemöller, K., Mayatepek, E. & Hoffmann, G. F. Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: results, outcome, and implications. *Pediatrics* **111**, 1399–1406 (2003).
5 Wilcken, B., Wiley, V., Hammond, J. & Carpenter, K. Screening newborns for inborn errors of metabolism by tandem mass spectrometry. *N. Engl. J. Med.* **348**, 2304–2312 (2003).
6 Ensenauer, R., Fingerhut, R., Maier, E. M., Polanetz, R., Olgemöller, B., Röschinger, W. *et al.* Newborn screening for isovaleric acidemia using tandem mass spectrometry: data from 1.6 million newborns. *Clin. Chem.* **57**, 623–626 (2011).
7 Grünert, S. C., Wendel, U., Lindner, M., Leichsenring, M., Schwab, K. O., Vockley, J. *et al.* Clinical and neurocognitive outcome in symptomatic isovaleric academia. *Orphanet. J. Rare Dis.* **7**, 9 (2012).
8 Cho, J. M., Lee, B. H., Kim., G. H., Kim, Y. M., Choi, J. H. & Yoo, H. W. Chronic intermittent form of isovaleric aciduria in a 2-year-old boy. *Korean. J. Pediatr.* **56**, 351–354 (2013).
9 Ozgul, R. K., Karaca, M., Kilic, M., Kucuk, O., Yucel-Yilmaz, D., Unal, O. *et al.* Phenotypic and genotypic spectrum of Turkish patients with isovaleric academia. *Eur. J. Med. Genet.* **57**, 596–601 (2014).
10 Kölker, S., Valayannopoulos, V., Burlina, A. B., Sykut-Cegielska, J., Wijburg, F. A., Teles, E. L. *et al.* The phenotypic spectrum of organic acidurias and urea cycle disorders. Part 2: the evolving clinical phenotype. *J. Inherit. Metab. Dis.* **38**, 1059–1074 (2015).
11 Roe, C. R., Millington, D. S., Maltby, D. A., Kahler, S. G. & Bohan, T. P. L-carnitine therapy in isovaleric acidemia. *J. Clin. Invest.* **74**, 2290–2295 (1984).
12 Yudkoff, M., Cohn, R. M., Puschak, R., Rothman, R. & Segal, S. Glycine therapy in isovaleric academia. *J. Pediatr.* **92**, 813–817 (1978).
13 Parimoo, B. & Tanaka, K. Structural organization of the human isovaleryl-CoA dehydrogenase gene. *Genomics* **15**, 582–590 (1993).
14 Mohsen, A. W., Anderson, B. D., Volchenboum, S. L., Battaile, K. P., Tiffany, K., Roberts, D. *et al.* Characterization of molecular defects in isovaleryl-CoA dehydrogenase in patients with isovaleric acidemia. *Biochemistry.* **37**, 10325–10335 (1998).
15 Lee, Y. W., Lee, D. H., Vockley, J., Kim, N. D., Lee, Y. K. & Ki, C. S. Different spectrum of mutations of isovaleryl-CoA dehydrogenase (IVD) gene in Korean patients with isovaleric academia. *Mol. Genet. Metab.* **92**, 71–77 (2007).
16 Dercksen, M., Duran, M., Ijlst, L., Mienie, L. J., Reinecke, C. J., Ruiter, J. P. *et al.* Clinical variability of isovaleric acidemia in a genetically homogeneous population. *J. Inherit. Metab. Dis.* **35**, 1021–1029 (2012).
17 Vockley, J., Parimoo, B. & Tanaka, K. Molecular characterization of four different classes of mutations in the isovaleryl-CoA dehydrogenase gene responsible for isovaleric academia. *Am. J. Hum. Genet.* **40**, 147–157 (1991).
18 Vockley, J., Rogan, P. K., Anderson, B. D., Willard, J., Seelan, R. S., Smith, D. I. *et al.* Exon skipping in IVD RNA processing in isovaleric acidemia caused by point mutations in the coding region of the IVD gene. *Am. J. Hum. Genet.* **66**, 356–367 (2000).
19 Lin, W. D., Wang, C. H., Lee, C. C., Lai, C. C., Tsai, Y. & Tsai, F. J. Genetic mutation profile of isovalericacidemia patients in Taiwan. *Mol. Genet. Metab.* **90**, 134–139 (2007).
20 Sakamoto, O., Arai-Ichinoi, N., Mitsubuchi, H., Chinen, Y., Haruna, H., Maruyama, H. *et al.* Phenotypic variability and newly identified mutations of the IVD gene in Japanese patients with isovaleric acidemia. *Tohoku. J. Exp. Med.* **236**, 103–106 (2015).
21 Lee, Y. W., Lee, D. H., Vockley, J., Kim, N. D., Lee, Y. K. & Ki, C. S. Different spectrum of mutations of isovaleryl-CoA dehydrogenase (IVD) gene in Korean patients with isovaleric academia. *Mol. Genet. Metab.* **92**, 71–77 (2007).
22 Ensenauer, R., Vockley, J., Willard, J. M., Huey, J. C., Sass, J. O., Edland, S. D. *et al.* A common mutation is associated with a mild, potentially asymptomatic phenotype in patients with isovaleric acidemia diagnosed by newborn screening. *Am. J. Hum. Genet.* **75**, 136–1142 (2004).
23 Campistol, J., Bóveda, M. D., Couce, M. L., Lluch, M. D. & Merinero, B. Protocolo de diagnóstico y tratamiento de las acidemias propiónica, metilmalónica e isovalérica. *An. Esp. Pediatr.* **89**, S9–S15 (1997).
24 Ferrer, I., Ruiz-Sala, P., Vicente, Y., Merinero, B., Pérez-Cerda, C. & Ugarte, M. Separation and identification of plasma short-chain acylcarnitine isomers by HPLC/MS/MS for the differential diagnosis of fatty acid oxidation defects and organic acidemias. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **860**, 121–126 (2007).
25 Rebollido-Fernández, M. M., Castiñeiras, D. E., Bóveda, M. D., Couce, M. L., Cocho, J. A. & Fraga, J. M. Development of electrospray ionization tandem mass spectrometry methods for the study of a high number of urine markers of inborn errors of metabolism. *Rapid. Commun. Mass. Spectrom.* **26**, 2131–2144 (2012).
26 Kleijer, W. J., van der Kraan, M., Huijmans, J. G., van den Heuvel, C. M. & Jakobs, C. Prenatal diagnosis of isovaleric acidaemia by enzyme and metabolite assay in the first and second trimesters. *Prenat. Diagn.* **15**, 527–533 (1995).
27 Feuchtbaum, L., Lorey, F., Faulkner, L., Sherwin, J., Currier, R., Bhandal, A. *et al.* California's experience implementing a pilot newborn supplemental screening program using tandem mass spectrometry. *Pediatrics* **117**, S261–S269 (2006).
28 Duran, M., van Sprang, F. J., Drewes, J. G., Bruinvis, L., Ketting, D. & Wadman, S. K. Two sisters with isovaleric acidaemia, multiple attacks of ketoacidosis and normal development. *Eur. J. Pediatr.* **131**, 205–211 (1979).
29 Solano, A. F., Leipnitz, G., De Bortoli, G. M., Seminotti, B., Amaral, A. U., Fernandes, C. G. *et al.* Induction of oxidative stress by the metabolites accumulating in isovaleric acidemia in brain cortex of young rats. *Free. Radic. Res.* **42**, 707–715 (2008).
30 Vatanavicharn, N., Liammongkolkul, S., Sakamoto, O., Sathienkijkanchai, A. & Wasant, P. Phenotypic and mutation spectrums of Thai patients with isovaleric acidemia. *Pediatr. Int.* **53**, 990–994 (2011).
31 Hertecant, J. L., Ben-Rebeh, I., Marah, M. A., Abbas, T., Ayadi., L., Ben, S. *et al.* Clinical and molecular analysis of isovaleric acidemia patients in the United Arab Emirates reveals remarkable phenotypes and four novel mutations in the IVD gene. *Eur. J. Med. Genet.* **55**, 671–676 (2012).