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ORIGINAL ARTICLE

Clinical variability of isovaleric acidemia in a genetically homogeneous population

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Abstract Isovaleric acidemia (IVA) is one of the most common organic acidemias found in South Africa. Since 1983, a significant number of IVA cases have been identified in approximately 20,000 Caucasian patients screened for metabolic defects. IVA is caused by an autosomal recessive deficiency of isovaleryl-CoA dehydrogenase (IVD) resulting in the accumulation of isovaleryl-CoA and its metabolites. In total, 10 IVA patients and three carriers were available for phenotypic and genotypic investigation in this study. All patients were found to be homozygous for a single c.367 G > A (p.G123R) mutation. The amino acid substitution of a glycine to arginine resulted in a markedly reduced steady-state level of the IVD protein, which explains the nearly complete lack of IVD enzyme activity as assessed in fibroblast homogenates. Despite the genetic homogeneity of this South African IVA group, the clinical presentation varied widely, ranging from severe mental handicap and multiple episodes of metabolic derangement to an asymptomatic state. The variation may be due to poor dietary intervention, delayed diagnosis or even epigenetic and polygenetic factors of unknown origin.

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Introduction

Virtually all inherited metabolic diseases are characterized by heterogeneity at the genetic, biochemical and clinical level. This phenomenon is well established, but much remains to be discovered about this complex phenotypic diversity. The aim of this study was to characterize isovaleric acidemia (IVA), prevalent in the Caucasian South African population. Isovaleric acidemia is due to the deficient activity of the enzyme isovaleryl-CoA dehydrogenase (IVD) (E.C.1.3.99.10) and results in the defective catabolism of leucine. IVA and the related enzyme deficiency were identified in 1966, and since then, more than 100 patients have been described. The IVD gene is located on the long arm of chromosome 15(15q14). The IVD enzyme is a tetramer consisting of identical 43 kDa subunits. IVD belongs to the family of acyl-CoA dehydrogenases and relies on the presence of electron transfer flavoprotein (ETF) and ETF dehydrogenase for the transfer of electrons to the mitochondrial respiratory chain (Ikeda et al. 1983). The IVD proteins from different species share 85–90% amino acid sequence identity with human IVD (Mohsen et al. 1998).

A deficiency of IVD leads to the accumulation of isovaleric acid as well as a number of secondary metabolites, for example N-isovalerylglycine, N-isovalerylcarnitine and 3-hydroxyisovaleric acid, derived from isovaleryl-CoA. IVA was initially classified into two groups by Sweetman and Williams (2001). The first IVA group manifests with an acute neonatal presentation and non-specific symptoms, including poor feeding, vomiting, seizures, metabolic acidosis and a decreased level of consciousness within the first 2 weeks of life (Vockley et al. 1991; Sweetman and Williams 2001). A second group of IVA patients presents with a late-onset and intermediate clinical profile, characterized by a variable degree of developmental delay and failure

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to thrive (Tanaka et al. 1966; Sweetman and Williams 2001). In addition to the classical features of IVA, other symptoms may include hyperammonemia, hypoglycemia and hypocalcemia. Some patients suffer from thrombocytopenia, pancytopenia, neutropenia as well as inhibition of bone marrow cell proliferation. They may have a characteristic odour of "sweaty feet" observed during acute stages of metabolic decompensation (Sweetman and Williams 2001).

Variability in the presentation of the disease has resulted in a revised metabolic classification consisting of a "metabolically severe group" and a "metabolically mild or intermediate group" (Vockley and Ensenauer 2006). The metabolically mild patient group includes the patients identified by newborn screening having moderately elevated levels of N-isovalerylcarnitine in their dried blood spots (Ensenauer et al. 2004). The clinical spectrum of the metabolically severe patients ranges from asymptomatic to serious neurometabolic disease. These patients can clinically be classified as severe (extensive neurological symptoms and marked developmental delay), moderate (some developmental delay and moderate neurological dysfunction which may or may not improve in time) and mild (absence of significant neurological disease and developmental delay) cases.

Over 35 mutations in the IVD gene have been documented (http://www.hgmd.cf.ac.uk). The most common mutation identified in IVA patients, mainly picked up by newborn screening, resides in exon 9: c.932 C > T (p.A282V) with ~19% residual IVD activity. The literature indicates that 47–52% of IVA cases have this particular mutation and that this mutation is associated with an attenuated clinical phenotype (Mohsen et al. 1998; Ensenauer et al. 2004). Some mutant alleles are associated with an abnormal splicing of the IVD mRNA resulting in the lack of IVD protein and no residual activity (Vockley et al. 1991; Vockley et al. 2000). Patients carrying such mutations may show either the severe or mild/intermediate form of the disease. In general the mutations and their effect on the IVD protein and bioprocessing of the protein have been well studied and understood but the genotype-phenotype correlation is still ill-defined. In this study we describe the clinical, biochemical, enzymological and molecular findings in 10 homozygous IVA patients, with a diverse phenotypic presentation.

Materials and methods

Materials

All chemicals were supplied by Sigma-Aldrich unless otherwise specified in the "Methods" section.

Methods

Collection of information and samples of IVA patient group and family members

All patients were initially referred to the Laboratory for Inherited Metabolic Defects in Potchefstroom, South Africa by clinicians, when a metabolic defect was suspected. All patients belong to families in which at least one child was admitted because of a serious metabolic decompensation. None of the patients died during the metabolic crisis. The clinical phenotype was evaluated, as described in the "Introduction", and four patients were classified as severe and six patients were classified as mild (Table 1).

An appropriate informative session was conveyed with all IVA families in which all aspects of the investigations were clearly discussed. Informed consent was given by all participating individuals or their legal guardians. Biological samples for enzyme and genetic testing were collected from IVA patients and unaffected siblings. Non-fasting urine and serum specimens were collected on the same day, in a controlled environment, where the IVA patients were served a low protein diet. None of the patients suffered from any metabolic decompensation at the time of sample collection. Treated metabolite levels were determined in these selected samples. The information on the 10 IVA patients is summarized in Tables 1 and 2.

GC-MS and ESI-MS-MS analysis of urinary IVA biomarkers

Urinary organic acids and glycine conjugates were extracted, derivatized and analyzed by GC-MS as previously described by Erasmus et al. (1985) and further refined by Reinecke et al. (2011). Automated Mass Spectral Deconvolution and Identification System software (AMDIS, version 2.66 from the National Institute for Standards and Technology) was used to perform component peak identification and spectral deconvolution. Urine acylcarnitine analysis was done by ESI-MS-MS according to Pitt et al. (2002).

Fibroblast culture

The cell lines were cultured in DMEM medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Bio-Whittaker, Walkersville, MD), 100 U/ml penicillin, 100 mg/ml streptomycin, and 25 mM HEPES buffer with L-glutamine in a humidified atmosphere of 5% CO_2 at 30°C and 37°C.

IVD Mutation analysis

Genomic DNA was isolated from the skin fibroblasts using the NucleoSpin Tissue genomic DNA purification kit

(Macherey-Nagel, Germany, Düren) according to the manufacturer's manual. All 12 exons of the IVD gene were amplified by PCR, including part of the flanking intron sequences. All forward and reverse IVD-specific primers (supplied by Biolegio, Nijmegen, Netherlands) were tagged with a −21 M13 (5′-TGTAAAACGACGGCCAGT-3′) sequence or a M13-Rev (5′-CAGGAAACAGCTATGACC-3′) sequence respectively. PCR fragments were sequenced in two directions using '−21 M13' and 'M13rev' primers by means of BigDye Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, USA) and analyzed on an Applied Biosystems 377A automated DNA sequencer, following the manufacturer's protocol. The IVD sequence data were compared to the reference IVD sequence (GenBank accession number: NM_002225.3) with nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

Isovaleryl-CoA dehydrogenase (IVD) activity

Fibroblast pellets, stored at −80°C, were homogenized in a medium containing 50 μM FAD in phosphate-buffered saline (PBS) and sonicated twice (40 Joule) on ice with intervals of at least 30 s between bursts. The protein content of samples was determined according to the BCA protocol (Smith et al. 1985). The final composition of the reaction mixture was: 200 mM Tris buffer (pH =8.0), 0.4 mM FAD, 0.05 mM ferricenium hexafluorophosphate, 0.2 mM isovaleryl-CoA, 0.025 mM butyryl-CoA and 0.2 mg protein/ml in a total volume of 100 μl. Reactions were stopped with 10 μl of a 2 M HCl solution after 30 min incubation at 37°C. The samples were put on ice, neutralized with 10 μl of a 2 M KOH/0.6 M MES solution, whereafter 10 μl of a 10 mM L-cysteine solution was added to reduce excess ferricenium hexafluorophosphate. All enzyme assays of each patient were done in triplicate. Product formation (the sum of 3-methylcrotonyl-CoA and 3-hydroxyisovaleryl-CoA) was measured by HPLC following the addition of 30 μl methanol and centrifugation at 4°C and 20,000*g* for 5 min. Each sample was analyzed with a UV-HPLC system, consisting of a Perkin Elmer Series 200 pump with a Shimadzu SPD-detector, at a wavelength of 260 nm. Separation of substrates and products was achieved by means of a linear gradient of methanol in 50 mM potassium phosphate (pH =5.3) buffer, on a Supelcosil SPLC-18-DB column (supplied by Supelco, St. Louis, MO). Enzyme testing was performed on samples of 10 IVA patients, 3 IVA carriers and 10 controls. The assay was originally designed as a combined assay, validated for the simultaneous measurement of short-chain acyl-CoA dehydrogenase (SCAD) and IVD in one sample.

Immunoblot analysis

Fibroblast homogenates (50 μg of protein) were subjected to electrophoresis on a 10% (w/v) SDS polyacrylamide gel and transferred to a nitrocellulose sheet. After blocking with 50 g/L non-fat dried milk (Protifar) and 10 g/L Bovine Serum Albumin (BSA) in 1 g/L Tween-20/PBS for 1 h, the blot was incubated for 2 h with rabbit polyclonal IVD antibodies (diluted 1:3,000 in 3 g/L BSA) as a primary antibody (the IVD antibody was kindly supplied by Dr. J. Vockley, Pittsburgh, PA, USA). After being washed five times with PBS/0.1% Tween-20, the blots were incubated for 1 h with goat anti–rabbit IgG alkaline phosphatase (supplied by Biorad, Hercules, CA) in 5% Protifar in PBS/0.1% Tween-20 (Ikeda et al. 1985; Mohsen et al. 1998). The blots were then washed five times with PBS/0.1% Tween-20 and stained with the NBT/BCIP according to the instruction of the manufacturer (supplied by Pierce, Rockford, IL).

Results

Clinical description of IVA patient group

The biochemical classification of the IVA patient group was done according to the proposed classification system of Vockley and Ensenauer (2006). All 10 patients investigated in this study presented with a severe metabolic phenotype based on the occurrence of at least one episode of metabolic decompensation in the respective families. In addition, they invariably displayed the characteristic odour of "sweaty feet" at a given moment, indicative of a failure of N-isovalerylglycine or N-isovalerylcarnitine formation. Table 1 summarizes the relevant features and illustrates the clinical classification based on the neurometabolic character of the disease in the particular patients. The age of diagnosis ranged from 6 days to 4 years.

Patients 1 and 2 are sisters. Until now, the younger sister has not presented with metabolic decompensation or mental disability but she did have the offensive body odour. Both sibs are clinically classified as mild. Patients 3 and 4 are a brother and younger sister, of whom the latter was diagnosed first. Both siblings had severe mental retardation; accordingly they had the severe clinical presentation. Patient 5 and 6 are also brother and sister. Again, the younger sister was diagnosed before her older brother. Both had several episodes of metabolic acidosis, mild developmental delay consistent with the severe clinical picture, and the dietician and parents had difficulty in enforcing the treatment regime. Patients 7, 8, 9 and 10 are unrelated

Table 1 Phenotypic description of IVA patients of this study

Patient number and familial relations	Age	Gender	Body Odour ("Sweaty feet")	Clinical symptoms	Clinical classification of phenotype	Current diet prescibed and L-carnitine and glycine intake (§)
1(Family 1)	D: 3 years C: 6 years, 6 months	F	+	Asymptomatic, normal development after therapeutic intervention	Clinically mild	0.91 g/kg/day protein (Vegetarian). L-carnitine and glycine supplementation
2 (Family 1)	D: 5 years, 7 months C: 8 years	F	+	Metabolic acidosis, ketosis, repeated episodes of dehydration, vomiting and comas (twice in life time - first episode at 3yr of age). Normal ammonia level, normal liver function, normal development after therapeutic intervention	Clinically mild	0.92 g/kg/day protein (Vegetarian). L-carnitine and glycine supplementation
3 (Family 2)	D: 2.5 months C: 12 years	F	++	Vomiting, failure to thrive, elevated ammonia level, severe mental retardation	Clinically severe	No information on diet. L-carnitine supplementation
4 (Family 2)	D: 4 years C: 16 years	M	+++	Vomiting, failure to thrive, elevated ammonia level severe mental retardation	Clinically severe	No information on diet, L-carnitine supplementation
5 (Family 3)	D: 3 years C: 7 years	M	+++	Adverse effect to protein intake, vomiting, poor bone structure, susceptible to infection, compromised immune system, aggressive behaviour and mood disorder, poor development: not reaching milestones	Clinically severe	1 g/kg/day protein. L-carnitine and glycine supplementation
6 (Family 3)	D: 3 months C: 4 years	F	+++	Compromised immune system (septicemia), poor bone structure, respiratory problems, poor development: not reaching milestones	Clinically severe	1.03 g/kg/day protein. L-carnitine and glycine supplementation
7 (Family 4)	D: 4 years C: 12 years	M	++	Chronic episodes of vomiting, slight developmental delay, normal development after therapeutic intervention	Clinically mild	1.04 mg/kg/day protein. L-carnitine and glycine supplementation
8 (Family 5)	D: 15 days C: 6 years	F	+	Metabolic decomposition in neonatal period, hypotonia and failure to thrive, ketotic episodes, normal development after therapeutic intervention	Clinically mild	No information on diet. L-carnitine and glycine supplementation
9 (Family 6)	D: 11 days C: 2 years	F	+++	Symptoms started at 7 days after birth. Jaundice, blood coagulation problems, symptoms of neurological deterioration, metabolic acidosis, neutropenia and dehydration were initially observed. Normal development after therapeutic intervention	Clinically mild	1.03 g/kg/day protein. L-carnitine and glycine supplementation
10 (Family 7)	D: 6 days C: 24 years	M	+	Metabolic decompensation during infections accompanied by attacks of hyperammonia. Patient was diagnosed early due to affected brother, thus preventing early onset. Normal development after therapeutic intervention.	Clinically mild	No protein restricted diet. L-carnitine and glycine supplementation

*D: Diagnosed

*C: Current age (after treatment)

*M: Male

*F: Female

+: faint; ++: present; +++: offensive

§: Precise amount of L-carnitine intake by individual patients, varied from 20 mg/kg/day to 100 mg/kg/day. Precise amount of glycine intake varied from 10 mg/kg/day to 100 mg/kg/day. Patients 3 and 4 do not take glycine.

IVA cases. Their entirely normal development following the therapeutic intervention puts them in the clinically mild group. Patient 10 was identified following the death of a previous sibling with IVA. He performs well at the intellectual and physical level. Siblings either heterozygous for the c.367 G > A mutation or lacking the mutation altogether, did not present with symptoms.

Biochemical markers of IVA patients

The typical urinary IVA metabolites were identified in all 10 IVA patients. An elevation of N-isovalerylglycine, N-isovalerylcarnitine and 3-hydroxyisovaleric acid accompanied by lactic acidosis and ketosis in the first diagnostic sample was observed in most patients although the actual levels varied between individuals (Table 2). These values are comparable, and in some cases even higher, than the patient values determined by Loots et al. (2005). L-carnitine treatment resulted in an increased N-isovalerylcarnitine excretion. Levels of 3-hydroxyisovaleric acid, lactic acid and ketones normalized upon institution of a low protein diet and additional intake of L-carnitine and glycine.

Genotyping of IVA patients and carriers

Sequence analysis of the IVD gene revealed a missense mutation in exon 4 (c.367 G > A) which leads to a glycine to arginine substitution at position 123 (p.G123R). The c.367 G > A (p.G123R) mutation was present as a homozygous mutation in all 10 IVA patients. Alignment of the IVD proteins from different species revealed that only a glycine or an alanine residue is present at position 123 (Fig. 1). The c.367 G > A mutation was also present on a single allele in the IVD gene of 3 IVA carriers in our investigation. The

Table 2 Urine concentrations of diagnostic and secondary metabolites of 10 IVA patients

		Diagnostic and secondary markers in urine (mmol/mol creat)						
		N-Isovaleryl-glycine ¥	N-Isovaleryl-carnitine ‡	3-Hydroxy-isovaleric acid ¥	Lactic acid ¥*	3-Hydroxy-butyric acid ¥ *	L-Carnitine ‡	Glycine ¥
Patient 1	Diagnosed	1508	2.9	2.0	17.4	n.d	11.6	426.5
	Treated	1439	51.1	2.9	6.2	n.d	33.9	201.0
Patient 2	Diagnosed	1313	5.1	60.7	37.3	39.2	1.7	27.3
	Treated	1702	41.1	1.8	3.0	n.d	12.6	135.2
Patient 3	Diagnosed	897	4.5	3.5	12.0	n.d	1.3	311.0
	Treated	1072	191.1	2.3	8.0	n.d	21.6	91.9
Patient 4	Diagnosed	1199	8.2	9.4	355.3	10.2	0.4	298.0
	Treated	743	20.5	2.4	12.5	n.d	6.0	94.1
Patient 5	Diagnosed	304	1.0	279.4	84.2	102.6	14.0	331.1
	Treated	1076	77.8	5.3	26.0	n.d	14.7	100.2
Patient 6	Diagnosed	1345	2.1	629.6	593.4	370.5	5.3	314.5
	Treated	1872	63.7	3.4	8.4	n.d	35.8	445.0
Patient 7	Diagnosed	479	1.6	465.7	368.4	346.2	5.2	131.4
	Treated	662	46.9	3.7	13.7	n.d	38.3	134.8
Patient 8	Diagnosed	1345	3.9	852.0	351.5	314.0	5.7	54.1
	Treated	1327	131.4	3.8	0.9	1.5	28.9	314.0
Patient 9	Diagnosed	1540	4.9	429.5	127.2	n.d	2.1	486.0
	Treated	1233	128.1	41.8	53.5	0.6	67.0	360.0
Patient 10	Diagnosed	406	3.1	11.7	10.4	n.d	1.0	170.0
	Treated	828	65.9	2.0	10.5	n.d	66.6	299.8
Ref Values		n.d†	0.24–0.38 §	0.02–31.70†	25–86†	0.7–3.60†	3.77–13.93§	43–1097†

‡: Concentration determined by MS-MS analysis

¥: Concentration determined by GC-MS analysis

* Secondary metabolite indicating metabolic status of IVA patients

†: GC-MS ref values (mean of all age groups): Hoffmann and Feyh 2002 and Shih 2002

§: MS-MS ref values (mean of all age groups): Mueller et al. 2003

n.d: not detectable

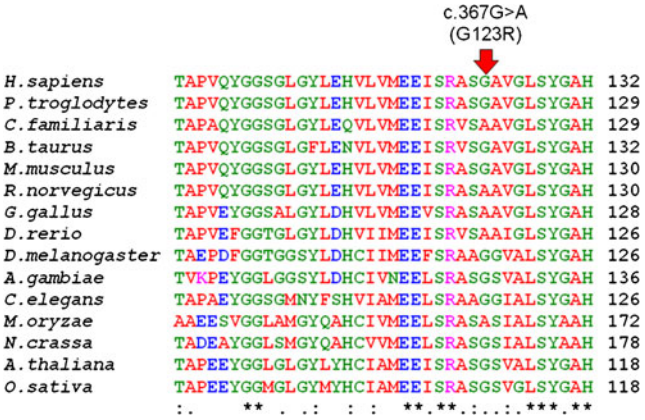

Fig. 1 Comparison of different IVD orthologues. This is a multiple sequence alignment of the region containing the G123 (indicated by an arrow), which is mutated in the IVA patients in this study. (The symbols can be explained as follows: An **"*"** (*asterisk*) indicates positions which have a single, fully conserved residue; a **":"** (*colon*) indicates conservation between groups of strongly similar properties - scoring >0.5 in the Gonnet PAM 250 matrix; a **"."** (*period*) indicates conservation between groups of weakly similar properties - scoring = or <0.5 in the Gonnet PAM 250 matrix)

carriers consisted of two siblings and one parent. This mutation was previously reported in a compound heterozygous form, in an American patient, by Ensenauer et al. (2004) and was known as the c.358 G > A, p.G91R mutation (communication with R. Ensenauer confirmed the origin of the patient).

Isovaleryl-CoA dehydrogenase activity

IVD enzyme activity measurements in fibroblasts of all 10 IVA patients revealed a virtually complete lack of activity, with levels below the quantification level (LOQ) of the validated assay. The IVD activity of three genetically proven carriers was also investigated and found to be between 0.78 and 0.99 nmol/min.mg, which corresponds to 30 –38% of control subjects. Culturing the fibroblast at 30°C did not result in an appreciable elevation of the residual IVD activity, expressed as a percentage of controls (Table 3).

Table 3 IVD enzyme activity in fibroblasts from control individuals, homozygous IVA patients and IVA carriers

Cell culture description	IVD activity in nmol/min.mg protein*	Percentage of control activity
Controls (37°C) *n*=10	2.61 ± 0.05	
Controls (30°C) *n*=3	2.02 ± 0.34	
IVA patients (37°C) *n*=10	not detectable #	<5%
IVA patients (30°C) *n*=3	not detectable #	<5%
IVD carrier group (37°C) *n*=3	0.91 ± 0.09	35%

* mean ± standard deviation (nmol/min.mg)

Limit of quantification (LOQ) = 0.13nmol/min.mg

n= number of patients (Each patient sample was done in triplicate)

Immunoblot analysis

In order to study the effect of the p.G123R amino acid substitution on the IVD protein stability, immunoblot analysis was performed. The results depicted in Fig. 2, show that the IVD protein is clearly expressed in control fibroblasts cultured at 37°C. However, markedly reduced IVD protein levels were detected in fibroblasts (cultured at 37°C) of the IVA patients, homozygous for the c.367 G > A mutation. Lowering of the culture temperature to 30°C had little effect on residual IVD protein levels (Fig 2) as well as the IVD activity (Table 3).

Discussion

The present cohort of isovaleric acidemia patients had a homozygous c.367 G > A nucleotide change in exon 4, which resulted in the replacement of the amino acid glycine (a neutral amino acid) by an arginine (a positively charged residue). The identification of a single specific mutation in all 10 IVA patients suggests a founder effect. The genetic background of our group of patients consisted of descendants of the original rather small group of German, Dutch, French and, later on, English pioneers who colonized South Africa in the 17th –18th century. As such the population is still rather homogeneous, although less so than closed societies such as the Pennsylvanian Amish (Strauss and Puffenberger 2009).

Immunoblot analysis revealed reduced steady-state levels of IVD protein in patient's fibroblasts, suggesting that the c.367 G > A (p.G123R) mutation renders the protein unstable. Some missense mutations have a temperature sensitive effect on protein folding as for example in mevalonate kinase deficiency (Houten et al. 2002). The phenotype of such a temperature sensitive mutant protein can be modified by external factors. Subsequent immunoblot studies and enzyme measurements in our IVA patient's fibroblasts, cultured at 30°C, showed virtually no increase in steady-state levels of IVD protein or IVD activity. Therefore, we conclude that the p.G123R mutation has a debilitating effect on the folding and stability of the protein as well as the IVD residual activity.

Despite the genetic and enzymatic homogeneity of the metabolically severe IVA group, a marked variability in the phenotypic presentation was found (Table 1). Siblings 3 and 4 as well as 5 and 6 were severely affected with regular attacks of metabolic acidosis and had developmental retardation, thus classified as clinically severe cases. All other patients (six) had normal psychomotor development following therapeutic intervention and were accordingly classified as clinically mild. Patient 7 was originally classified as moderate, based on his slight developmental delay, but

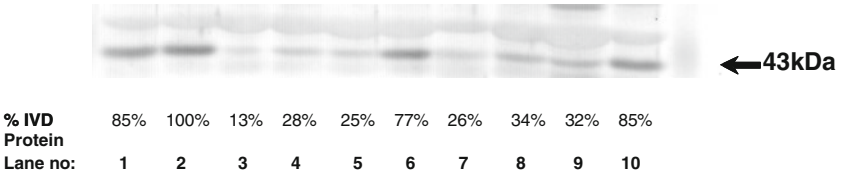

Fig. 2 Immunoblot analysis of the IVD protein in control and IVA patients. Fibroblast homogenate of controls, cultured at 37°C (lanes 1, 2 and 10) and homogenate of a control, cultured at 30°C (lane 6). Fibroblast homogenates of IVA patients homozygous for p.G123R, cultured at 37°C (lane 3, 4 and 5) and homogenate of IVA patients cultured at 30°C (lanes 7, 8 and 9). Quantification of the intensity of the bands was done with "Odyssey imager software". The control in lane 2 was set to 100%. Equal amounts of protein (50 μg) were applied to the gel and blotted

reassuringly showed a normal development following the start of treatment. Two patients (8 and 9) had an acute neonatal presentation in the first 2 weeks of life but ended up as clinically mild cases, thereby confirming the statement of Vockley and Ensenauer (2006) that patients can fall anywhere in the spectrum of mild to severe clinical presentation independent of the seemingly predictive value of the initial presentation.

As expected, the biochemical parameters clearly indicate an organic acid profile associated with isovaleric acidemia. In most of the first time diagnostic cases in our study, a metabolic crisis was present and lactic acidosis with secondary (ω-1)-hydroxylation was evident. Symptomatic- and metabolic presentation generally improved when glycine and L-carnitine were added to a low protein diet. The formation of the detoxification markers suggested a stabilization of the metabolic condition of the patient. The latter was paralleled by the decline in ketones and lactic acid levels. The normalization of 3-hydroxyisovaleric acid in patients, recovering from an episode of metabolic decompensation indicates diminishing secondary (ω-1)-hydroxylation of isovaleric acid. The tendency of metabolic "recovery" was observed in most patients, but the excretion values varied within the patient group as shown in Table 2.

The initial urinary N-isovalerylcarnitine levels were quite low, but did increase in L-carnitine treated patients. Urinary free carnitine was likewise low in most patients at the time of diagnosis (metabolic decompensation) but normalized with L-carnitine supplementation. It has been documented by Chalmers et al. (1984) that the availability of L-carnitine is insufficient in undiagnosed patients with these types of organic acidemias and this is evident in our study. The reason for carnitine depletion in tissues in various organic acidemias has been explained by Stumpf et al. (1985) who postulated that carnitine acts as a "buffer," trapping acyl compounds, e.g. isovaleryl-CoA, during mitochondrial failure. L-carnitine supplementation facilitates excretion of such acyl groups in the urine. Future monitoring of effective detoxification may lie in the determination of free carnitine levels in plasma as suggested by Ensenauer and Vockley (2006).

The presence of N-isovalerylglycine (IVG) indicates the importance of this metabolite as a biological detoxification marker in the initial diagnostic and treated IVA samples. Krieger and Tanaka (1976) showed that glycine intake in IVA patients decreases the number of episodes of vomiting and acidosis and facilitates better growth rates, due to the tolerance of a low, but essential protein diet in developing infants. The IVG levels before and after treatment did not correlate directly with the glycine treatment regimes in our study. Similar variations have been reported in numerous case studies. Duran et al. (1979) and De Sousa et al. (1986) described patients who were prone to hyperglycinemic attacks during continuous glycine treatment. Their IVG levels before and after treatment did not change significantly. In contrast to these findings, Yudkoff et al. (1978) successfully applied long-term glycine treatment in a 3½year old girl and IVG levels raised three fold during a leucine loading test, without any adverse effects. These described reports, support the variation in IVG levels observed in our study, a potential complicating factor in the development of biochemical parameters for treatment monitoring.

There are several other factors which may help explain the upwards or downwards variation in the IVG level in general. Shigematsu et al. (1982) indicated that the capacity of glycine conjugation is challenged as leucine levels suddenly exceed normal levels. Even the elevation of other substrates for glycine N-acylase, such as benzoic acid and salicylic acid, may hinder effective glycine conjugation with isovaleric acid and result in lowered IVG production. Krieger and Tanaka (1976) also suggested that the intracellular pH may be another factor influencing the glycine N-acylase activity. A lowered pH during ketoacidosis is not favourable for glycine conjugation as the glycine N-acylase activity is optimal at a higher pH. Thus, a slight or sudden change in the cellular homeostasis can limit glycine conjugation. L-carnitine and glycine supplementation, together with a low protein diet, optimize the body's detoxification mechanism and each patient may be expected to use a selected detoxification pathway. The tolerance of the supplementation must be seen as an individual based observation and the capacity and activity of glycine N-acylase as well as the endogenous glycine pool are important factors to consider.

With all these aspects taken into account, it must be emphasized that the discussed markers only indicate the metabolic status of the patient and not the efficacy of the over all treatment regime through time. Vockley and Ensenauer (2006) indicated that no reliable metabolic marker has been identified with regard to the monitoring of prescribed treatment in patients during periods of (relatively) well-being. This illustrates the need for an evidence-based guideline study. Assessing physical features, such as weight gain may probably be the best indicator of efficacy of treatment for the time being, but the necessity for biochemical monitoring to evaluate therapeutic intervention is needed (Sweetman and Williams 2001).

The wide phenotypic variation of isovaleric acidemia in this semi-isolated society is in sharp contrast to the homogeneous clinical profile of other metabolic disorders in true isolates such as SUCLA2-deficiency in the Faroe Islands (Morava et al. 2009) and glutaric acidemia type 1 in the old-order Amish population (Strauss and Puffenberger. 2009). In general, the neurological involvement of IVA is not extremely significant (Sweetman and Williams 2001), hence it cannot be excluded with certainty that the profound mental retardation observed in patients 3 and 4, who were siblings, arose from another genetic or environmental event. Isovaleric acidemia with malformations and severe mental retardation has been found to coincide before (Duran et al. 1982). Phenotypic variation was evident in the clinical background assessment of the patients and it is speculated to be due to delayed diagnosis, poor dietary control or secondary factors not yet established.

Our investigation suggests that inconsistencies in treatment regimes may have contributed to a deviating phenotype. This may also be true for other populations in which a common genotype and a variable clinical presentation have been identified as was recently demonstrated in the Thai population by Vatanavicharn et al. (2011). We want to stress the importance of personalized medicine in patients when prescribing L-carnitine and glycine supplementation. It is our experience that some of the patients were prone to hyperglycinemic attacks. Moreover, social problems have arisen from fish-like body odour due to high intake of L-carnitine. Indeed, biochemical monitoring of optimal dosages of glycine and L-carnitine for detoxification must be part of a future perspective in the monitoring of patients. If optimization of the dietary and drug treatment does not result in a decrease of the phenotypic variability, advanced investigations such as whole-genome sequencing, may shed some light on possible secondary genetic variations as contributing factors to the outcome of isovaleric acidemia screening. The favorable outcome of patient 10, who was diagnosed as a symptom-free neonate, is good evidence of the potential benefits of newborn screening of this condition.

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