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2-Ethylhydracrylic Aciduria in Short/Branched-Chain Acyl-CoA Dehydrogenase Deficiency: Application to Diagnosis and Implications for the *R*-Pathway of Isoleucine Oxidation

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Background: **Isolated excretion of 2-methylbutyrylglycine (2-MBG) is the hallmark of short/branched-chain acyl-CoA dehydrogenase deficiency (SBCADD), a recently identified defect in the proximal pathway of L-isoleucine oxidation. SBCADD might be underdiagnosed because detection and recognition of urine acylglycines is problematic. Excretion of 2-ethylhydracrylic acid (2-EHA), an intermediate formed in the normally minor *R*-pathway of L-isoleucine oxidation, has not previously been described in SBCADD.**

Methods: **Samples from four patients with 2-MBG excretion were analyzed by gas chromatography–mass spectrometry for urine organic acids, quantification of 2-MBG, and chiral determination of 2-methylbutyric acid. Blood-spot acylcarnitines were measured by electrospray–tandem mass spectrometry. Mutations in the *ACADSB* gene encoding SBCAD were identified by direct sequencing.**

Results: **SBCADD was confirmed in each patient by demonstration of different *ACADSB* gene mutations. In multiple urine samples, organic acid analysis revealed a prominent 2-EHA peak usually exceeding the size of the 2-MBG peak. Approximately 40–46% of total 2-methylbutyric acid conjugates were in the form of the *R*-isomer, indicating significant metabolism via the *R*-pathway.**

Conclusions: **If, as generally believed, SBCAD is responsible for *R*-2-MBG dehydrogenation in the *R*-pathway, 2-EHA would not be produced in SBCADD. Our observation of 2-ethylhydracrylic aciduria in SBCADD implies that a different or alternative enzyme serves this function. Increased flux through the *R*-pathway may act as a safety valve for overflow of accumulating *S*-pathway metabolites and thereby mitigate the severity of SBCADD. Awareness of 2-ethylhydracrylic aciduria as a diagnostic marker could lead to increased detection of SBCADD and improved definition of its clinical phenotype.**

Catabolism of the branched-chain amino acid L-isoleucine occurs predominantly via the *S*-pathway, but also via the minor *R*-pathway (Fig. 1). Short/branched-chain acyl-CoA dehydrogenase deficiency (SBCADD;[5] OMIM +600301) is an autosomal recessively inherited defect in isoleucine catabolism. The first identified patient with SBCADD was reported only in the year 2000 *(1)*. An additional patient and his affected asymptomatic mother

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[5] Nonstandard abbreviations: SBCADD, short/branched-chain acyl-CoA dehydrogenase deficiency; 2-MBG, 2-methylbutyrylglycine; 2-EHA, 2-ethylhydracrylic acid; TMS, trimethylsilyl; and GC-MS, gas chromatography–mass spectrometry.

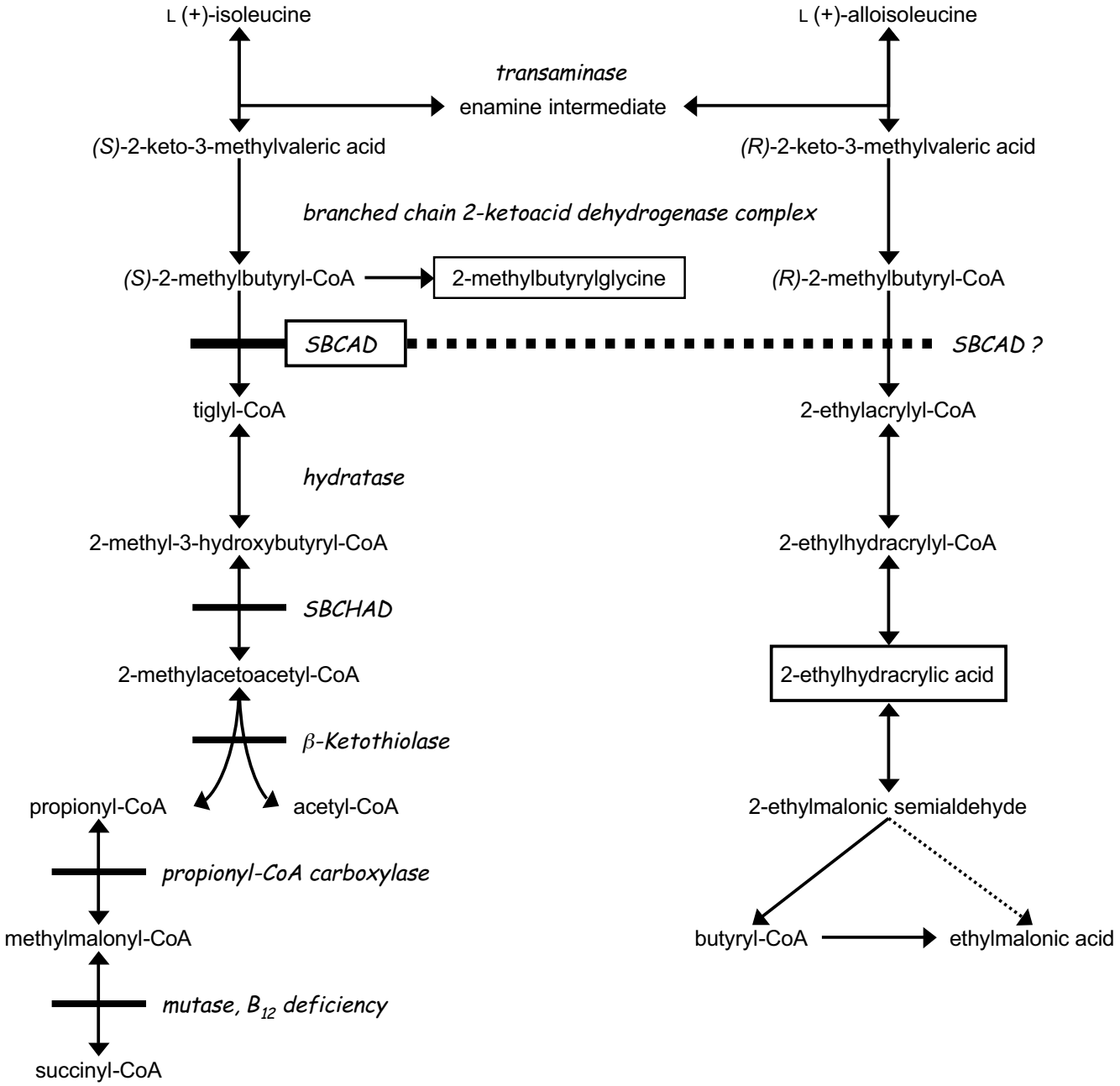

Fig. 1. The *S*- (*left*) and *R*-pathways (*right*) of L-isoleucine catabolism.

Enzyme names are shown in *italics*. *Horizontal solid bars* indicate inborn errors. The deficient enzyme and accumulating metabolites that are the subject of this report are *boxed*.

were reported in the same year *(2)*. The diagnosis in these patients was first suspected after analysis of organic acids in urine revealed an isolated increase in excretion of 2-methylbutyrylglycine (2-MBG). This metabolite is derived from 2-methylbutyryl-CoA, the substrate of SBCAD (Fig. 1). Since the reports of these two initial families, the only other description of patients with this disorder has been in the Hmong communities in Minnesota and Wisconsin *(3)*. In these cases, however, the disorder was identified not by urine organic acid analysis but by detection of increased blood-spot C5 carnitine within the framework of a universal newborn-screening program.

Judging by the number of diagnosed patients, SBCADD would appear to be a rare disorder. Nevertheless, we have diagnosed SBCADD in four unrelated individuals, suggesting that the disorder might be more common than is currently appreciated. The detection of acylglycines in urine is problematic *(4)*, and it is possible that additional cases are being missed because of failure to identify or appreciate the characteristic 2-methylbutyrylglycinuria.

In this report we show that increased excretion of 2-ethylhydracrylic acid (2-EHA) is a prominent and easily recognized abnormality on analysis of organic acids in urine from patients with SBCADD. Awareness of 2-ethylhydracrylic aciduria as an indicator of SBCADD may lead to increasing diagnosis of this disorder and further definition of its phenotype. Furthermore, our observation of the excretion of 2-EHA and other metabolites in SBCADD raises questions regarding the presumed role of SBCAD in the *R*-pathway of isoleucine oxidation.

Patients and Methods

PATIENTS AND GENOTYPING

Patient 1 with SBCADD was identified by newborn screening and is healthy with normal development, whereas patients 2–4 were identified after metabolic studies performed for evaluation of clinical disease. In each case, the diagnosis of SBCADD was confirmed by demonstration of homozygosity or compound heterozygosity for *ACADSB* gene mutations by direct sequence analysis of the entire coding region from the patients and available family members (genotypes summarized in Table 1). PCR amplification of genomic DNA and sequence analysis were performed as described previously *(2)*. Patient 1 *(5)* and patients 3 and 4 *(6)* have been reported previously in abstract format.

ORGANIC ACID, AMINO ACID, AND ACYLCARNITINE ANALYSIS

Organic acids were extracted from random urines by a standard ethyl acetate–diethyl ether procedure, converted to their trimethylsilyl (TMS) derivatives, and analyzed on a Varian/Finnigan™ or an Agilent 5971™ gas chromatography–mass spectrometry (GC-MS) system.

Dried-blood-spot and urine acylcarnitines were measured by electrospray–tandem MS *(7, 8)*, and plasma amino acids were measured by ion-exchange chromatography with postcolumn ninhydrin derivatization.

MEASUREMENT OF 2-MBG BY GC-MS

2-MBG and 2-methylbutyryl-$^2H_2$-glycine ($^2H_2$-2-MBG) were synthesized from 2-methylbutyric acid, glycine, and $^2H_2$-glycine as described previously *(9)*. Briefly, 1 mL of urine was mixed with 100 μL of 100 μmol/L $^2H_2$-2-MBG, acidified with 100 μL of 6 mol/L hydrochloric acid, saturated with sodium chloride, and extracted with 5 mL of ethyl acetate. The organic layer was removed, dried under an air stream, and derivatized with 20 μL of pyridine and 80 μL of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide by heating at 100 °C for 30 min. The GC oven was held at 120 °C for 1 min after injection, followed by ramping at 10 °C/min. Selected-ion monitoring was performed for *m/z* 330 and *m/z* 332, the $[M-C_4H_9]^+$ fragments of the *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide derivatives of 2-MBG and $^2H_2$-2-MBG, respectively.

CHIRAL DETERMINATION OF 2-METHYLBUTYRIC ACID BY GC-MS

2-Methylbutyric acid was released from glycine and carnitine conjugates by alkaline hydrolysis, and the *R*- and *S*-isomers were separated by GC-MS after preparation of L-valine/TMS derivatives. We mixed 1 mL of urine with 100 μL of 1 mmol/L (*R,S*)-2-methylvaleric acid (internal standard) and 900 μL of 6 mol/L sodium hydroxide and heated the mixture at 100 °C for 60 min. The mixture was acidified with 1 mL of 6 mol/L hydrochloric acid, saturated with sodium chloride, and extracted with 2 mL of *n*-hexane. The *n*-hexane layer was removed, mixed with 75 μL of thionyl chloride, and heated at 60 °C for 1 h. After cooling on ice, 1 mL of 0.67 mol/L L-valine in 3 mol/L sodium hydroxide was added with vortex-mixing.

Table 1. Genotypes and metabolite concentrations in four SBCAD-deficient patients.

	Results[a]				Reference interval
	Patient 1	Patient 2	Patient 3	Patient 4	
Genotype	1165A>G/848A>G[b]	908G>C/908G>C[c]	443C>T/443C>T	1102T>C/1102T>C	
Dried-blood-spot C5 carnitine, μmol/L	1.2; 2.5; 1.1	0.97	1.75	0.5; 0.55	<0.46
Plasma alloisoleucine, μmol/L	2.6	1.5	ND[d]	ND	<2.1
Urine C5 carnitine, μmol/mmol creatinine	84.4; 58.1; 64.8	35.3; 80.6	2.7	ND	<3.0
Urine 2-EHA, arbitrary units[e]	36; 31; 22	116; 132	32; 8; 15	152; 19; 43	<5
Urine 2-MBG, μmol/mmol creatinine	36.3; 29.3; 35.2	20.2; 36.7	14.5	ND	<2.3
Urine 2-methylbutyric acid conjugates as *R*-isomer, %	45; 40	45; 43	46	ND	43–56[f]

[a] Values separated by semicolons indicate results of analyses of samples collected at different times.
[b] Compound heterozygote.
[c] This patient was also homozygous for a neutral polymorphism, 38G>A.
[d] ND, not done because suitable samples were not available for assay.
[e] Ratio of the peak areas for 2-EHA and the internal standard, corrected for creatinine concentration.
[f] n = 5.

The mixture was left on ice for 20 min, then at room temperature for 20 min with occasional vortex-mixing. The mixture was acidified with 500 μL of 6 mol/L hydrochloric acid, saturated with sodium chloride, and extracted twice with 3 mL of ethyl acetate. The extracts were combined, dried under an air stream at 60 °C, and derivatized with 20 μL pyridine and 80 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 10 mL/L trimethylchlorosilane by heating at 80 °C for 30 min. The GC oven was held at 100 °C for 1 min after injection, followed by ramping at 5 °C/min. Selected-ion monitoring was performed for *m/z* 258 and *m/z* 272, the $[M-CH_3]^+$ fragments of the TMS/valine derivatives of 2-methylbutyric and 2-methylvaleric acids, respectively. By running standards of (*R,S*)-2-methylbutyric acid and (*S*)-2-methylbutyric acid, we determined that the derivatives of the *S*- and *R*-isomers of 2-methylbutyric acid eluted at 14.19 and 14.31 min, respectively. The derivatives of (*R,S*)-2-methylvaleric acid eluted at 15.86 and 16.04 min.

Results

Organic acids were analyzed in 12 separate urine samples from the four patients. Illustrative total-ion chromatograms from patients 2, 3, and 4 are presented in Fig. 2 (panels A, B, and C, respectively), with a chromatogram from a healthy control for comparison (panel D). In all samples there was increased excretion not only of 2-MBG, as expected in SBCADD, but also of 2-EHA. The metabolite concentrations are summarized in Table 1. Quantification of urine 2-MBG by stable-isotope-dilution GC-MS showed concentrations in the range 14.5–36.7 μmol/mmol creatinine compared with a reference interval of <2.3 μmol/mmol creatinine. 2-EHA could not be quantified because there is no commercially available standard and attempts to synthesize it were unrewarding. 2-EHA is therefore expressed qualitatively as arbitrary units reflecting the ratio of the area under the curve of its peak to that of the internal standard, corrected for creatinine concentration.

In the healthy individual (Fig. 2D), only a trace of 2-EHA is visible on the chromatogram and no 2-MBG peak is detectable. To gauge the relative prominence of 2-EHA and 2-MBG excretion and their consequent ease of detection by visual inspection of the chromatograms, we compared their peak heights. In the samples from the SBCADD patients, the height of the 2-EHA peak always exceeded that of the 2-MBG peak, with the exception of patient 4 (Fig. 2C). The mean ratio of 2-EHA to 2-MBG peak heights for nine samples was 3.8, with a range of 0.5–10.8. Whereas the 2-EHA peak was clearly increased in all SBCADD samples, the 2-MBG peak was sometimes relatively small and might easily be overlooked (Fig. 2B).

Urine C5 carnitine (Table 1) was significantly increased in patients 1 and 2 but within the reference interval in patient 3. The C5 carnitine measurement, however, includes both 2-methylbutyrylcarnitine and isovalerylcarnitine. It is therefore possible that a slight increase in 2-methylbutyrylcarnitine in this patient could be masked by background concentrations of isovalerylcarnitine within the reference interval. C5 carnitine concentrations in dried-blood-spot samples were mildly to moderately increased in all individuals. Again, the actual increase in 2-methybutyrylcarnitine may be underestimated because of masking of 2-methylbutyrylcarnitine by isovalerylcarnitine, which also contributes to this signal. It is noteworthy that the blood-spot C5 carnitine concentration was within the reference interval in one of the first reported SBCADD patients *(2)*.

Chiral analysis of 2-methylbutyric acid released from urine conjugates in patients 1–3 showed that 40–46% was in the form of the *R*-isomer, similar to that measured in controls albeit at much higher absolute concentrations. Plasma alloisoleucine was slightly increased in patient 1 (although this was against a background of slight increases in other branched-chain amino acids) and within the reference interval in patient 2.

Discussion

Deficiency of SBCAD leads to accumulation within the mitochondrion of its substrate, 2-methylbutyryl-CoA. This is transesterified with glycine by the mitochondrial enzyme acyl-CoA:glycine-*N*-acyltransferase (glycine-*N*-acylase) to form 2-MBG *(9)*. Detection of abnormal excretion of 2-MBG in the urine led to the discovery of SBCADD in the first patients identified with this disorder.

2-MBG and other biologically important acylglycines can be detected in urine by routine organic acid analysis, based on the GC retention times and mass spectra of their TMS derivatives *(10, 11)*. However, inadequate sensitivity of acylglycine detection (because of variable extraction, chromatographic instability, or failure of spectrum recognition) is a major cause for missed diagnosis of fatty acid oxidation and organic acid defects *(4)*. To overcome this problem, techniques have been developed for the specific determination of acylglycines based on stable-isotope dilution with chemical *(12)* or negative chemical ionization *(13)* GC-MS with selected-ion monitoring, and electrospray–tandem MS *(14)*. Because these techniques are not available or practicable for widespread routine use, the isolated 2-methylbutyrylglycinuria of SBCADD may be easily overlooked.

In this context, our observation of prominent 2-ethylhydracrylic aciduria in SBCADD may have important practical implications in the diagnosis of this disorder. Unlike 2-MBG, 2-EHA is easily detected on routine GC-MS examination of urine organic acids. It is present in small amounts in the urine of healthy individuals. Excessive 2-EHA excretion may thus serve as an important marker for SBCADD. Although 2-ethylhydracrylic aciduria was not mentioned in the previous reports of this disorder, it is unlikely that this phenomenon is peculiar to our patients because they were genetically heterogeneous.

2-EHA was first identified in the urine of a patient with

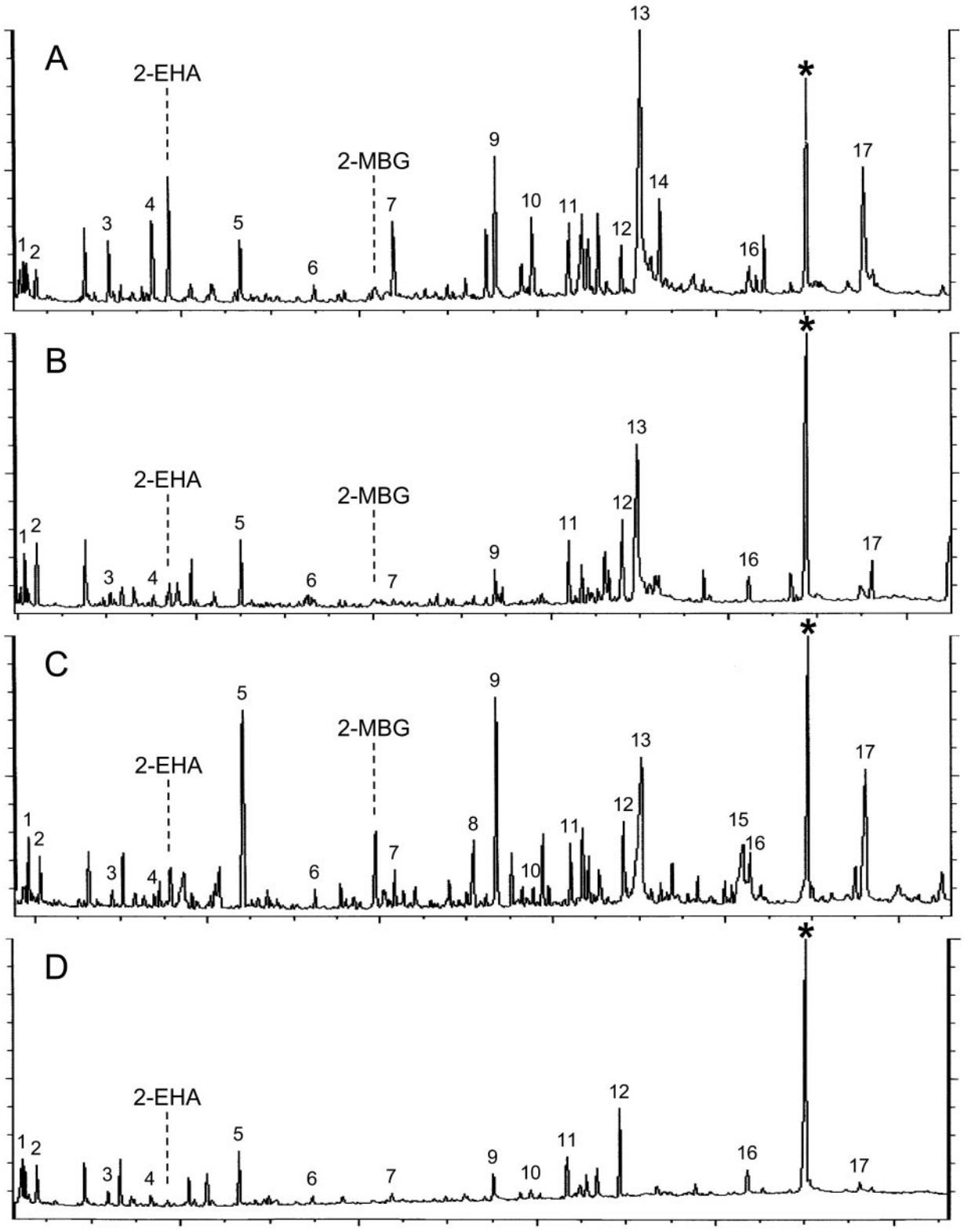

Fig. 2. Total-ion chromatograms of urine organic acids analyzed by GC-MS.

Shown are samples from patients 2 (*A*), 3 (*B*), and 4 (*C*) and a healthy control (*D*); a chromatogram from patient 1 is not presented because the samples were analyzed on a different GC-MS system and are therefore not directly comparable. The peaks for 2-EHA and 2-MBG are indicated. * denotes the internal standard C17:0 (margaric acid). For orientation, additional peaks without diagnostic significance are labeled as follows: *peak 1*, lactic acid; *peak 2*, glycolic acid; *peak 3*, 3-hydroxybutyric and 3-hydroxyisobutyric acids; *peak 4*, 3-hydroxyisovaleric acid; *peak 5*, succinic acid; *peak 6*, glutaric acid; *peak 7*, adipic acid; *peak 8*, α-ketoglutaric acid; *peak 9*, 4-hydroxyphenylacetic acid; *peak 10*, suberic acid; *peak 11*, aconitic acid; *peak 12*, citric acid; *peak 13*, hippuric acid; *peak 14*, sebacic acid; *peak 15*, phenobarbitone metabolite; *peak 16*, palmitic acid; *peak 17*, 4-hydroxyhippuric acid.

β-ketothiolase (2-methylacetoacetyl-CoA thiolase) deficiency *(15)*. This led to the realization that it is an intermediate in a previously unrecognized minor pathway of isoleucine oxidation, the *R*-pathway *(16)*. Because both the second and third carbons of isoleucine are chiral centers, it has four possible enantiomers. The predominant enantiomer is L-(+)-isoleucine (2*S*-amino-3*S*-methylpentanoic acid). Transamination of L-(+)-isoleucine produces (*S*)-2-keto-3-methylvalerate, which is subsequently oxidized via the *S*-pathway (Fig. 1, left pathway). The next most common enantiomer is L-(+)-alloisoleucine (2*S*-amino-3*R*-methylpentanoic acid), which is oxidized via the minor *R*-pathway (Fig. 2, right pathway). Loading studies have demonstrated interconversion between the *S*- and *R*-pathways *(16)*. This was presumed until recently to occur by keto-enol tautomeric racemization of the *S*- and *R*-enantiomers of 2-keto-3-methylvaleric acid. It is now believed that the interconversion actually occurs by tautomerization of an enamine intermediate formed during the transamination of L-(+)-isoleucine *(17–19)*.

This interconversion between the *S*- and *R*-pathways is the basis for the observation that excretion of 2-EHA, an *R*-pathway intermediate, is increased when the *S*-pathway for isoleucine oxidation is either overloaded or interrupted. In such situations, the flux through the *R*-pathway increases, leading to increased formation and excretion of 2-EHA. Intuitively, one might expect the increased flux through the *R*-pathway to lead to increased excretion of ethylmalonic acid, the terminal metabolite in the *R*-pathway, rather than the intermediate metabolite 2-EHA. However, loading studies with L-(+)-alloisoleucine and *R*-pathway metabolites have clearly shown that 2-EHA, and not ethylmalonic acid, is the predominant metabolite excreted *(16)*. It would appear that the catabolic steps beyond 2-EHA are attenuated relative to those that precede it and to the corresponding steps in the *S*-pathway.

Whereas the origin of the 2-ethylhydracrylic aciduria in distal defects of the *S*-pathway (such as β-ketothiolase deficiency) is readily apparent, this is not the case for SBCADD, a proximal defect of the *S*-pathway. It is believed that SBCAD functions not only in the *S*-pathway to dehydrogenate (*S*)-2-methylbutyryl-CoA, but also in the *R*-pathway to dehydrogenate (*R*)-2-methylbutyryl-CoA *(17, 20)*. Indeed, SBCAD is active in vitro against both enantiomers, the relative activity of SBCAD for the *R*-enantiomer compared with the *S*-enantiomer being 22% for the purified enzyme from rat liver *(21)*, 61% for the rat enzyme expressed in *Escherichia coli (22)*, and 64% for the expressed human enzyme *(23)*. This is in contrast to the situation for human very long-chain, long-chain, and medium-chain acyl-CoA dehydrogenases, which are stereospecific for the *S*-enantiomers only of their methyl branched-chain substrates *(24)*.

Further evidence for the role of the *R*-pathway was provided by the chiral analysis of 2-methylbutyric acid released from urine conjugates. This showed that a substantial proportion (40–46%) was present as the *R*-isomer. Plasma alloisoleucine, the first intermediate of the *R*-pathway, was also slightly increased in one patient, but this was an inconsistent finding because the concentration was within the reference interval in another patient.

If SBCAD were indeed the enzyme responsible for the dehydrogenation of (*R*)-2-methylbutyryl-CoA to 2-ethylhydracryl-CoA in the *R*-pathway, then 2-EHA would not be produced in SBCADD. Our observation that 2-EHA excretion is actually increased in SBCADD implies either that a different enzyme is responsible for the dehydrogenation of (*R*)-2-methylbutyryl-CoA or that an alternative enzyme performs this function when SBCAD is deficient. There are several enzyme systems with the potential to fill this role, such as isobutyryl-CoA dehydrogenase in the valine pathway, or the omega hydroxylation system. This being the case, the *R*-pathway remains functional in SBCADD and may therefore act as a safety valve for overflow of accumulating toxic *S*-pathway intermediates. Indeed, SBCADD has been contrasted with isovaleric acidemia, in which the analogous enzyme in the leucine catabolism pathway (isovaleryl-CoA dehydrogenase) is deficient. Isovaleric acidemia is characterized by a more severe clinical course and higher concentrations of metabolites, whereas a substantial proportion of SBCAD-deficient patients appear to be asymptomatic *(1–3, 25, 26)*. It has been speculated that alternative pathways might account for the milder clinical and biochemical phenotype of SBCADD *(1)*. Our results support this notion, indicating that the severity of the disorder may be modulated by the *R*-pathway. In this context, it is noteworthy that the only case in which the 2-MBG peak exceeded the 2-EHA peak (suggesting less efficient clearance of toxic metabolites via the *R*-pathway) was from patient 4, who presented neonatally with acute metabolic decompensation and neurologic dysfunction.

Interestingly, 2-EHA was not listed in a recent review of abnormalities of urinary organic acid excretion *(27)*. Nevertheless, it is an important metabolite of diagnostic significance in several disorders, all of which can be differentiated from SBCADD by identification of additional diagnostic metabolites. Increased 2-EHA excretion may be observed when the *S*-pathway is overloaded during ketosis, isoleucine being a ketogenic amino acid *(28)*. None of our patients was ketotic when samples were taken. 2-Ethylhydracrylic aciduria has also been reported in several defects at distal steps in the *S*-pathway, including β-ketothiolase deficiency *(15)*, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency *(29)*, propionic acidemia *(30)*, and methylmalonic acidemia attributable to deficiency of mutase or its cofactor vitamin B_{12}.

2-Ethylhydracrylic aciduria may also be observed in two disorders outside the *S*-pathway. Ethylmalonic encephalopathy is a rare infantile metabolic disorder with a distinctive clinical presentation characterized by hypotonia, developmental delay and regression, orthostatic acrocyanosis, relapsing petechiae, chronic diarrhea, and

progressive pyramidal and extrapyramidal signs *(31)*. As in SBCADD, patients with ethylmalonic encephalopathy have increased urinary excretion of 2-EHA and 2-MBG. Unlike SBCAD-deficient individuals, however, they also excrete ethylmalonic acid in large amounts, as well as isobutyrylglycine. L-Isoleucine loading studies suggested an abnormality in the pathway of isoleucine metabolism *(32)*, although more recent studies have indicated that methionine rather than isoleucine is the precursor of ethylmalonic acid in this disorder *(33)*. However, additional findings of lactic acidemia and muscle cytochrome *c* oxidase deficiency implied a mitochondrial origin for this disorder. Indeed, it was recently shown to be caused by mutations in the *ETHE1* gene, which encodes a mitochondrial matrix protein with a central but as yet defined role in mitochondrial homeostasis and energy metabolism *(34)*.

Barth syndrome is an X-linked disorder characterized by dilated cardiomyopathy, growth retardation, and neutropenia *(35)*. Affected patients excrete increased amounts of 3-methylglutaconic and 3-methylglutaric acids (usually metabolites of leucine) as well as 2-EHA *(36)*. In this disorder, the 3-methylglutaconic and 3-methylglutaric aciduria appear to arise not from the metabolism of leucine but rather from isoprenoid metabolism via the methylglutaconate shunt. Mutations in the *taffazin* gene are responsible for this disorder and lead to a deficiency of cardiolipin, a component of the inner mitochondrial membrane necessary for proper functioning of the electron transport chain *(37)*.

In the few cases of SBCADD that have been reported to date, the clinical phenotype seems to be quite heterogeneous, ranging from asymptomatic individuals to patients with various neurologic manifestations and acute metabolic presentations. Individuals with SBCADD are now being increasingly identified through routine newborn-screening programs by detection of increased blood-spot C5 carnitine *(3, 26)*. However, because many such individuals are being prospectively treated with a protein-restricted diet and/or L-carnitine supplementation, the natural history of the untreated disorder might remain unclear. Awareness of 2-EHA excretion as a marker of SBCADD may lead to increasing diagnosis of this disorder during selective screening of symptomatic patients and contribute to the definition of its clinical phenotype.

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References

1. Gibson KM, Burlingame TG, Hogema B, Jakobs C, Schutgens RB, Millington D, et al. 2-Methylbutyryl-coenzyme A dehydrogenase deficiency: a new inborn error of L-isoleucine metabolism. Pediatr Res 2000;47:830–3.
2. Andresen BS, Christensen E, Corydon TJ, Bross P, Pilgaard B, Wanders RJ, et al. Isolated 2-methylbutyrylglycinuria caused by short/branched-chain acyl-CoA dehydrogenase deficiency: identification of a new enzyme defect, resolution of its molecular basis, and evidence for distinct acyl-CoA dehydrogenases in isoleucine and valine metabolism. Am J Hum Genet 2000;67:1095–103.
3. Matern D, He M, Berry SA, Rinaldo P, Whitley CB, Madsen PP, et al. Prospective diagnosis of 2-methylbutyryl-CoA dehydrogenase deficiency in the Hmong population by newborn screening using tandem mass spectrometry. Pediatrics 2003;112:74–8.
4. Downing M, Allen JC, Bonham JR, Edwards RG, Manning NJ, Olpin SE, et al. Problems in the detection of fatty acid oxidation defects: experience of a quality assurance programme for qualitative urinary organic acid analysis. J Inherit Metab Dis 1999;22:289–92.
5. Boneh A, Andresen BS, Pitt JJ. Metabolite studies in a baby with short/branched-chain acyl-CoA dehydrogenase deficiency: evidence for the involvement of the R-pathway of isoleucine catabolism [Abstract]. J Inherit Metab Dis 2004;27(Suppl 1):98.
6. Korman SH, Zeharia A, Barash V, Corydon TJ, Gregersen N, Gutman A, et al. Short/branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency: expanded clinical and molecular spectrum [Abstract]. J Inherit Metab Dis 2001;24(Suppl 1):68.
7. Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamoudi M, et al. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. Clin Chem 1997;43:1129–41.
8. Pitt JJ, Eggington M, Kahler SG. Comprehensive screening of urine samples for inborn errors of metabolism by electrospray tandem mass spectrometry. Clin Chem 2002;48:1970–80.
9. Gregersen N, Kolvraa S, Mortensen PB. Acyl-CoA:glycine *N*-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. Biochem Med Metab Biol 1986;35:210–8.
10. Gregersen N, Keiding K, Kolvraa S. N-Acylglycines: gas chromatographic mass spectrometric identification and determination in urine by selected ion monitoring. Biomed Mass Spectrom 1979; 6:439–43.
11. Tjoa SS, Fennessey PV. Acylglycines. The gas chromatograph/mass spectrometric identification and interpretation of their spectra. Clin Chim Acta 1979;95:35–45.
12. Rinaldo P, O'Shea JJ, Welch RD, Tanaka K. Stable isotope dilution analysis of *n*-hexanoylglycine, 3-phenylpropionylglycine and suberylglycine in human urine using chemical ionization gas chromatography/mass spectrometry selected ion monitoring. Biomed Environ Mass Spectrom 1989;18:471–7.
13. Costa CG, Guerand WS, Struys EA, Holwerda U, ten Brink HJ, Tavares de Almeida I, et al. Quantitative analysis of urinary acylglycines for the diagnosis of β-oxidation defects using GC-NCI-MS. J Pharm Biomed Anal 2000;21:1215–24.
14. Bonafe L, Troxler H, Kuster T, Heizmann CW, Chamoles NA, Burlina AB, et al. Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias. Mol Genet Metab 2000;69:302–11.
15. Mamer OA, Tjoa SS. 2-Ethylhydracrylic acid: a newly described urinary organic acid. Clin Chim Acta 1974;55:199–204.
16. Mamer OA, Tjoa SS, Scriver CR, Klassen GA. Demonstration of a new mammalian isoleucine catabolic pathway yielding an R series of metabolites. Biochem J 1976;160:417–26.
17. Mamer OA. Initial catabolic steps of isoleucine, the R-pathway and the origin of alloisoleucine. J Chromatogr B Biomed Sci Appl 2001;758:49–55.
18. Mamer OA, Reimer ML. On the mechanisms of the formation of

L-alloisoleucine and the 2-hydroxy-3-methylvaleric acid stereoisomers from L-isoleucine in maple syrup urine disease patients and in normal humans. J Biol Chem 1992;267:22141–7.
19. Mamer OA, Lepine FL. ^{15}N conservation in the metabolic conversion of isoleucine to alloisoleucine in the rat. J Mass Spectrom 1996;31:1382–8.
20. Sweetman L, Williams JC. Branched chain organic acidurias. In: Scriver CR, Beaudet AL, Sly WS, Valle DT, eds. The metabolic and molecular bases of inherited disease. New York: McGraw-Hill, 2001:2125–63.
21. Ikeda Y, Tanaka K. Purification and characterization of 2-methyl-branched chain acyl coenzyme A dehydrogenase, an enzyme involved in the isoleucine and valine metabolism, from rat liver mitochondria. J Biol Chem 1983;258:9477–87.
22. Willard J, Vicanek C, Battaile KP, Van Veldhoven PP, Fauq AH, Rozen R, et al. Cloning of a cDNA for short/branched chain acyl-coenzyme A dehydrogenase from rat and characterization of its tissue expression and substrate specificity. Arch Biochem Biophys 1996;331:127–33.
23. Vockley J, Mohsen A-WA, Binzak B, Willard J, Fauq A. Mammalian branched-chain acyl-CoA dehydrogenases: molecular cloning and characterization of recombinant enzymes. Methods Enzymol 2000;324:241–58.
24. Battaile KP, McBurney M, Van Veldhoven PP, Vockley J. Human long chain, very long chain and medium chain acyl-CoA dehydrogenases are specific for the S-enantiomer of 2-methylpentadecanoyl-CoA. Biochim Biophys Acta 1998;1390:333–8.
25. Nguyen TV, Andresen BS, Corydon TJ, Ghisla S, Abd-El Razik N, Mohsen A-WA, et al. Identification of isobutyryl-CoA dehydrogenase and its deficiency in humans. Mol Genet Metab 2002;77:68–79.
26. Rhead WJ, White A, Allain D, Lindh H, Hanson K, van Calcar S, et al. Very-long-chain acyl-CoA dehydrogenase (VLCAD), 2-methylbutyryl-AD (2-MBAD), short chain-AD (SCAD) and 3-methylcrotonyl-CoA carboxylase (3-MCC) deficiencies: newborn screening defects clinically benign or very mild cases [Abstract]. Mol Genet Metab 2004;81:186.
27. Kumps A, Duez P, Mardens Y. Metabolic, nutritional, iatrogenic, and artifactual sources of urinary organic acids: a comprehensive table. Clin Chem 2002;48:708–17.
28. Liebich HM, Forst C. Hydroxycarboxylic and oxocarboxylic acids in urine: products from branched-chain amino acid degradation and from ketogenesis. J Chromatogr 1984;309:225–42.
29. Ensenauer R, Niederhoff H, Ruiter JP, Wanders RJ, Schwab KO, Brandis M, et al. Clinical variability in 3-hydroxy-2-methylbutyryl-CoA dehydrogenase deficiency. Ann Neurol 2002;51:656–9.
30. Przyrembel H, Bremer HJ, Duran M, Bruinvis L, Ketting D, Wadman SK, et al. Propionyl-CoA carboxylase deficiency with overflow of metabolites of isoleucine catabolism at all levels. Eur J Pediatr 1979;130:1–14.
31. Burlina AB, Dionisi-Vici C, Bennett MJ, Gibson KM, Servidei S, Bertini E, et al. A new syndrome with ethylmalonic aciduria and normal fatty acid oxidation in fibroblasts. J Pediatr 1994;124:79–86.
32. Nowaczyk MJ, Lehotay DC, Platt BA, Fisher L, Tan R, Phillips H, et al. Ethylmalonic and methylsuccinic aciduria in ethylmalonic encephalopathy arise from abnormal isoleucine metabolism. Metabolism 1998;47:836–9.
33. McGowan KA, Nyhan WL, Barshop BA, Naviaux RK, Yu A, Haas RH, et al. The role of methionine in ethylmalonic encephalopathy with petechiae. Arch Neurol 2004;61:570–4.
34. Tiranti V, D'Adamo P, Briem E, Ferrari G, Mineri R, Lamantea E, et al. Ethylmalonic encephalopathy is caused by mutations in ETHE1, a gene encoding a mitochondrial matrix protein. Am J Hum Genet 2004;74:239–52.
35. Barth PG, Scholte HR, Berden JA, Van der Klei-Van Moorsel JM, Luyt-Houwen IE, Van't Veer-Korthof ET, et al. An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leucocytes. J Neurol Sci 1983;62:327–55.
36. Kelley RI, Cheatham JP, Clark BJ, Nigro MA, Powell BR, Sherwood GW, et al. X-linked dilated cardiomyopathy with neutropenia, growth retardation, and 3-methylglutaconic aciduria. J Pediatr 1991;119:738–47.
37. Barth PG, Valianpour F, Bowen VM, Lam J, Duran M, Vaz FM, et al. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): an update. Am J Med Genet 2004;126A:349–54.