MS/MS-BASED NEWBORN AND FAMILY SCREENING DETECTS ASYMPTOMATIC PATIENTS WITH VERY-LONG-CHAIN ACYL-COA DEHYDROGENASE DEFICIENCY

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Objectives To determine whether asymptomatic persons with biochemical evidence of very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency identified through expanded newborn screening with tandem mass spectometry have confirmed disease.

Study design We characterized 8 asymptomatic VLCAD-deficient individuals by enzyme and/or mutational analysis and compared them with clinically diagnosed, symptomatic patients with regard to mutations, enzyme activity, phenotype, and age of disease onset.

Results VLCAD molecular analyses in 6 unrelated patients revealed the previously reported V243A mutation, associated with hepatic or myopathic phenotypes, on 7/12 alleles. All other mutations were also missense mutations. Residual VLCAD activities of 6% to 11% of normal were consistent with milder phenotypes. In these identified individuals treated prospectively with dietary modification as preventive measures, clinical symptoms did not develop during follow-up.

Conclusions MS/MS-based newborn screening correctly identifies VLCAD-deficient individuals. Based on mutational and enzymatic findings, these infants probably are at risk of future disease. Because life-threatening metabolic derangement can occur even in otherwise mild phenotypes, we advocate universal newborn screening programs for VLCAD deficiency to detect affected patients and prevent development of metabolic crises. Longer-term follow-up is essential to define outcomes, the definite risk of future disease, and appropriate treatment recommendations. *(J Pediatr 2003;143:335-42)*

Very long-chain acyl-CoA dehydrogenase (VLCAD) catalyses the first step in the β-oxidation spiral of long-chain fatty acids. Clinically, VLCAD deficiency (VLCADD) is a heterogeneous disease with involvement of highly oxidative organs.[1,2] Three phenotypes are apparent: a severe, early-onset presentation with cardiomyopathy and hepatopathy; a hepatic phenotype that usually becomes manifest in infancy with recurrent hypoketotic hypoglycemia similar to shorter-chain fatty acid oxidation (FAO) disorders; and a milder, later-onset, myopathic form with episodic muscle weakness, myalgia, and myoglobinuria.[1] Cardiomyopathy has been diagnosed as early as the first week of life[2] and as late as 5 years of age.[3] Onset with ventricular tachycardia has been reported on the first day of life.[4] The majority of patients with the myopathic phenotype has been diagnosed in adulthood, although, in some patients, episodes of myalgia are noted in early childhood or adolescence.[5,6]

The incidence of VLCADD is unknown, but more than 100 patients have been described, and a substantial group comprises initially normal adults in whom later-onset myopathic disease develops.[1] Despite molecular heterogeneity, VLCADD demonstrates genotype-phenotype correlation, with primarily missense mutations in milder forms and null mutations in lethal phenotypes.[1,2,7]

FAO	Fatty acid oxidation	VLCAD	Very long-chain acyl-CoA dehydrogenase
MCAD	Medium-chain acyl-CoA dehydrogenase	VLCADD	VLCAD deficiency
MCADD	MCAD deficiency		

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There is strong scientific support for MS/MS-based newborn screening for FAO disorders,[8] but, currently, only 13 US states mandate medium-chain acyl-CoA dehydrogenase (MCAD) screening by MS/MS, and only 5 require screening for VLCADD.[9] MCAD deficiency (MCADD) occurs in 1 in 10,000[10] to 1 in 15,000 newborn infants,[12] and MS/MS screening has been an excellent method for its detection.[11-13] Retrospective MS/MS-based analysis of a newborn screening card from a patient with the severe VLCADD phenotype demonstrated that acylcarnitine analysis can be diagnostic in the newborn period also for this disorder.[14]

We report that among newborn infants screened for FAO disorders in Germany and the United States, at least 7 unrelated asymptomatic individuals with elevated C14 long-chain acylcarnitines consistent with VLCADD were detected. In one family, a 3-year-old asymptomatic sibling was identified by family screening, making a total of 8 individuals. The two objectives of this study are to test whether the newborn infants with acylcarnitine profiles suggestive of VLCADD have molecularly and/or biochemically confirmed disease.

METHODS

Among all newborn infants screened for VLCADD and diagnosed with biochemical evidence of this disease within the last 3 to 5 years, 7 were followed in this study and characterized by enzyme and/or mutational analysis. In one family, a 3-year-old asymptomatic sibling was identified by family screening, making a total of 8 patients reported herein. The investigations were performed with the approval of the Vanderbilt University Institutional Review Board and with informed consent from the parents. The patients' clinical and family histories were obtained from the referring physicians.

Tandem Mass Spectrometry

Acylcarnitine analysis was performed in 6 different newborn screening laboratories (Table I) at 2 to 4 days of life.[10,11] A confirmatory second blood sample was usually drawn at 8 to 12 days of life.

Molecular Genetic Analysis

All 20 VLCAD exons of the patients' genomic DNA from whole blood were amplified by the polymerase chain reaction (PCR) and directly sequenced under standard conditions[15] with the use of 27-bp-long intronic primer pairs.

Enzyme Analysis

VLCAD activity was measured in lymphocyte homogenates by using the specific substrate palmitoyl-CoA. The reaction product was quantified by high-pressure liquid chromatography.[16]

RESULTS

We present 8 individuals with elevated C14 acylcarnitines suggestive of VLCADD detected by newborn and family screening. All individuals were asymptomatic at the time of diagnosis and during follow-up (Table I).

MS/MS-Based Acylcarnitine Analysis

All newborn infants of this study had mild elevations of C14 acylcarnitines (C14, C14:1, and/or C14:2) at the time of screening between 2 and 4 days of life. In all, the acylcarnitine profiles suggestive of VLCADD were confirmed in a second blood sample (Table I). In patient 7, only C14:2 acylcarnitine was indicative of VLCADD; C14:1 acylcarnitine was in the normal range. Patient 3 was diagnosed by family screening, and retrospective analysis of the newborn screening card from this individual also revealed an acylcarnitine profile suggestive of VLCADD (Table I).

In some patients, acylcarnitine analysis was used for treatment monitoring during follow-up. In patient 4, analysis at 1 and 2 months of age demonstrated persistent elevation of C14:1 and C14:2 acylcarnitines and a mildly reduced free carnitine (Table I). In patients 1, 2, and 3, long-chain acylcarnitines completely normalized within 2 weeks after institution of preventive measures, as described below. Follow-up biochemical data on patients 5 through 8 are not available.

The 7 newborn infants followed in this study were from 16 total identified as having VLCADD by expanded newborn screening in Germany and the United States. In Germany, an estimated 1,360,000 newborn infants have been screened for VLCADD, and a total of 11 patients with VLCADD, confirmed by two independent acylcarnitine profiles at different times, have been identified (personal communications with Dr J. Sander, Hannover; Dr R. Fingerhut, Munich; Dr A. Schulze, Heidelberg). Thus, the estimated VLCADD incidence is ~1:125,000 births. Recent data from the New England Screening program reveal that among 341,000 newborns screened, 4 have been detected with VLCADD confirmed in 2 independent screening samples and with documented mutations. An additional 4 presumptive cases with out-of-range primary marker (3 cases, C14:1) or an abnormal secondary marker (C14:2 and C14:1/C16 ratio) are currently being investigated. If these additional 4 cases are confirmed by molecular analysis, the incidence of VLCADD in New England would be at least 1:42,500 births.

Clinical and Biochemical Data

All patients had normal pregnancy and birth histories. Clinical signs such as hepatopathy, cardiomyopathy, arrhythmias, or muscular hypotonia were not present at the time of diagnosis or during follow-up. Cardiomyopathy was excluded by standard echocardiographic criteria.

Patient 3 is the only individual identified by familial screening and demonstrates a common natural history of mild VLCADD. By the time of diagnosis at 3 years of age, he was

Table I. Acylcarnitine profiles in asymptomatic patients with VLCADD

Patient	C14:1	Normal	C14:2	Normal	C14	Normal	Free carnitine† (10–60)	C14:1/C4† (0.03–0.54)	C14:1/Free carnitine† (<0.01)	Age at analysis	Uneventful follow-up
1	0.58	<0.21	0.23	<0.11	0.25	<0.24	Normal	1.71		72 h	9 mo
2*	2.13	<0.44	NA		2.38	<0.71	Normal		0.11	10 d	5 mo
3*	0.57	<0.44	NA		NA		Normal		0.03	3 y	3 1/2 y
	1.47‡	<0.36	NA		1.49‡	<0.65	Normal			3 d	
4	0.30	<0.21	0.21	<0.11	NA		9	3.09		1 mo	4 mo
	1.39	0.02–0.08								2 mo	
5	NA		NA		NA		NA			NA	2 y
6	1.4	<0.89	0.13	<0.17	1.25	<1.24	Normal			2 d	2 mo
7	0.71	<0.89	0.19	<0.17	NA		Normal			2 d	2 mo
	0.66	<0.89	0.25	<0.17	NA		Normal			7 d	
8	4.78	<0.89	0.30	<0.17	NA		Normal			2 d	2 mo

Quantitative screening results from patients 2, 4, and 5 were not available. The first available acylcarnitine profiles are summarized. Different newborn screening centers have been involved in the initial diagnosis and the follow-up, which explains the difference in reference values. Patient 1 was screened at the Newborn Screening Laboratory Becker, Olgemoeller and colleagues, Munich, Germany. In patient 2, the acylcarnitine profile at 10 days was performed at the Center for Metabolic Diagnostics, ZfS, Reutlingen, Germany. Patient 3 was tested at the age of 3 years at the Center for Metabolic Diagnostics, ZfS, Reutlingen, Germany. Retrospective analysis of the newborn screening card from this patient was performed at the Newborn Screening Laboratory at the University of Leipzig. In patient 4, acylcarnitine analysis at 2 months was performed at the Laboratory Genetic Metabolic Diseases, Amsterdam. Patient 5 was screened by NeoGen, Pittsburgh, Pa. Patients 6, 7, and 8 were tested in the New England Screening Program, University of Massachusetts, Boston, Mass. All measurements are in μmol/L.

*Siblings; †normal; ‡retrospective analysis of the newborn screening card; C12 was 0.72 μmol/L (normal, <0.49), C16:1 was 0.69 μmol/L (normal, <0.64). *NA*, Not available.

a healthy, normally developed boy; and, even during periods of infectious illness, did not have any VLCADD-associated symptoms.

In all patients, preventive measures were instituted after diagnosis. In newborn infants, the mainstay of treatment was the avoidance of fasting with regular meals every 3 to 4 hours, a diet with reduced long-chain fatty acids, and supplementation with medium-chain triglycerides (MCT). Patient 1 was breast-fed without supplementation of MCT during the first 3 months of life and stayed well. At 3 months, milk rich in MCT was started in equal amounts with breast milk. The same dietary regime was implemented in patient 2 at 3 weeks of age. In patient 4, the total intake of long-chain fat was restricted to 3 g/kg per body wt after diagnosis, and MCT fats were supplemented. Patient 3 was only advised to avoid fasting periods longer than 10 hours at 3 years of age. During follow-up, mild, common infectious illnesses did not result in metabolic decompensation in any patient.

Liver function tests and creatine kinase (CK) concentrations were normal at the time of diagnosis and during follow-up in all patients tested.

VLCAD Enzyme Assay

VLCAD assays in lymphocytes from patients 1 through 4 demonstrated reduced activity to 6% to 11% of normal (Table II). American patients did not have VLCAD enzyme assays performed.

Molecular Genetic Analysis

In 7 of the total of 8 patients, molecular genetic analysis of the VLCAD gene was done and confirmed the diagnosis (Table II). All mutations were missense mutations. Two of 6 unrelated patients had homozygous mutations; in one family, consanguinity was reported (patient 1). Four patients were compound heterozygotes. The previously reported V243A mutation was found in 5 of 6 unrelated patients on 7 of 12 alleles. To evaluate the carrier frequency of this most common mutation, 100 normal individuals were screened, and the mutation was not present in any. The other mutations in our patients have either not been previously reported (T161C, G249R) or are known only in isolated cases (A450P, R419W, P25L) (2, 7, unpublished results).

Mutation Localization Within the MCAD Structural Model

To better understand the mutation effects on protein structure and function, we determined the VLCAD mutation sites within the protein. The molecular structure of human MCAD has been defined by x-ray crystallography to high resolution.[17] Because alignment of the amino acids 98-433 of human VLCAD with human MCAD[2,18] reveals 34% similarity, the human MCAD structure is an excellent predictive model for VLCAD. In human MCAD, the catalytic base responsible for the α-proton abstraction from the thioester substrate is E376[17]; this residue is conserved in human VLCAD at position 422[19] (Fig 1).

Table II. Molecular and biochemical investigations in asymptomatic patients with VLCADD

	Allele 1		Allele 2		VLCAD activity		
Patient	Mutation	Amino acid change	Mutation	Amino acid change	nmol/min/mg protein	Normal	% of Normal
1	848T > C	V243A	848T > C	V243A	0.5	4.7 ± 1.7	11
2*	602A > G	Y161C	1468G > C	A450P	0.4	4.4 ± 1.6	9
3*	602A > G	Y161C	1468G > C	A450P	0.3	4.4 ± 1.6	7
4	ND		ND		0.3	4.7 ± 1.7	6
5	848T > C	V243A	848T > C	V243A			
6	848T > C	V243A	865G > A	G249R			
7	848T > C	V243A	1376G > A	R419Q			
8	194C > T	P25L	848T > C	V243A			

*Siblings.
ND, Not determined, mutational analysis was not performed in this patient.

Locating all known VLCAD missense mutations (References 2 and 7, our unpublished results) within the alignment of MCAD and VLCAD sequences demonstrates that most of the disease-causing mutations are in conserved regions, although none is located within the catalytic domain. The alignment within the structural model (Fig 2) reveals that the predicted locations are not only in β-sheets and random coils, as previously reported,[2] but also in α-helixes.

The T161C, V243A, G249R, and R419Q missense mutations present in our patients are also located in conserved regions of the VLCAD gene (Fig 1) that probably have structural importance, suggesting that all these mutations are significant ones. The positions of the P25L and the A450P mutations are outside the homologous region shared by MCAD and VLCAD (Fig 1).

The T161C, V243A, and G249R mutations hold positions in the structural model (Fig 2) on outer loops that appear not to interfere with the active site pocket and the flavin cofactor binding region (Fig 2). The R419Q mutation is located in the dimerization domain.

DISCUSSION

In this study, we present 8 asymptomatic patients with confirmed VLCADD detected by newborn and family screening with MS/MS. Our study indicates that MS/MS-based newborn screening detects asymptomatic VLCADD at 2 to 4 days of life. On the basis of molecular and enzymatic findings, these asymptomatic individuals detected by screening probably are at risk of future dangerous, metabolic crises and/or later-onset disease.

To date, in both Germany and Massachusetts, no individual with clinically diagnosed VLCADD has been reported who had a normal acylcarnitine profile on newborn screening. This strongly suggests that MS/MS-based screening is highly sensitive, although further follow-up is needed to document sensitivity. Among β-oxidation defects, sensitivity and specificity of prospective MS/MS screening appear excellent for MCADD.[11-13] Retrospective analysis of the newborn screening cards from affected patients with mitochondrial trifunctional protein (TFP)[20] and isolated LCHAD deficiency[20] suggests that diagnosis of these disorders is also feasible at 2 to 4 days of age.

The most common mutation (7 of the 12 alleles tested in unrelated individuals), V243A, is a mild mutation in previously reported patients that has always resulted in a clinical phenotype.[2,7,18]. Symptoms in clinically diagnosed patients with this mutation (n = 10) included neonatal[18] and infantile-onset[2] hypoketotic hypoglycemia and later-onset myopathy.[18] Transfection of the V243A mutant VLCAD protein into COS-7 cells documented reduced protein mass in comparison to cells transfected with normal VLCAD,[18] proving that this mutation is a significant one, and enzyme activity of the expressed V243A mutant VLCAD was only 20% to 25% of normal.[18] Among the 5 other missense mutations delineated in our group of asymptomatic patients, 3 have previously been reported to cause clinical phenotypes and 2 are novel. Expression of these 5 mutations either in patients or transfected cells has not been studied. Based on the molecular and enzymatic findings with residual enzyme activities of 6% to 11% of normal in our asymptomatic newborn infants, we believe that these individuals are at substantial risk of later-onset disease or future metabolic crisis triggered by fasting or illness. This conclusion is also strongly supported by previous reports of disease manifestations in VLCADD. A previously completely well child with unrecognized VLCADD died at the age of 5 years after perioperative fasting and sedation.[21] Other asymptomatic FAO disorders frequently become manifest as sudden and unexpected death.[22-24] Many individuals with initially asymptomatic VLCADD have later-onset disease, with enzymatic and molecular findings comparable to our patients. Later-onset disease is usually characterized by myopathic symptoms, and onset is in adolescence or adulthood.[5,25]

Fig 1. Alignment of human MCAD and VLCAD amino acid sequences and location of known VLCAD missense mutations. *Yellow*, Location of missense mutations reported in the literature.[2,8] *Red letter on yellow*, Not previously reported missense mutations from our own results. *Green*, Location of missense mutations in patients of the study. *Blue*, Active site at VLCAD position E422. *Vertical line* marks end of the region of homology between MCAD and VLCAD amino acid sequences.

Many patients with VLCADD become and remain asymptomatic with treatment and preventive measures; therefore, it is possible that treated individuals described here may never exhibit any clinical phenotype. In severe cardiac phenotypes, the cardiomyopathy is reversible and does not reappear with dietary management and avoidance of fasting.[14,26-28] Episodes of hypoketotic hypoglycemia in patients with the hepatic phenotype and recurrent myoglobinuria in adult-onset forms are prevented if precipitating factors are avoided.[2]

In VLCADD, there is a proven genotype-phenotype correlation.[1,2,7] Premature termination mutations, changes causing shifts in the reading frame, or multiple amino acid deletions on both alleles that result in no residual VLCAD activity are present in 80% of patients with the severe, neonatal phenotype[2] and are absent in the later-onset, myopathic form.[7] In contrast, missense mutations or single amino acid deletions on both alleles result in residual VLCAD activity and are mainly present in milder phenotypes. In 72% of patients with missense mutations, however, onset of symptoms was reported within the first 4 years of life.[7] Thus, we predict that molecular and enzymatic characterization will be helpful in determining the probable severity of the clinical phenotype soon after screening results have been released to adjust treatment. That is, the presence of null mutations may necessitate immediate prophylactic and therapeutic measures, but missense mutations that are more predictive for a milder clinical phenotype may require less severe dietary restrictions.

In contrast to MCADD,[12] the mutational spectrum in clinically diagnosed patients with VLCADD is similar to individuals detected by screening and reported here. In MCADD, the frequency of the prevalent 985A > G mutation that 80% of clinically affected patients carry in homozygous form was much lower in newborn infants detected by screening.[12,29] A new MCAD mutation (199T > C) (a

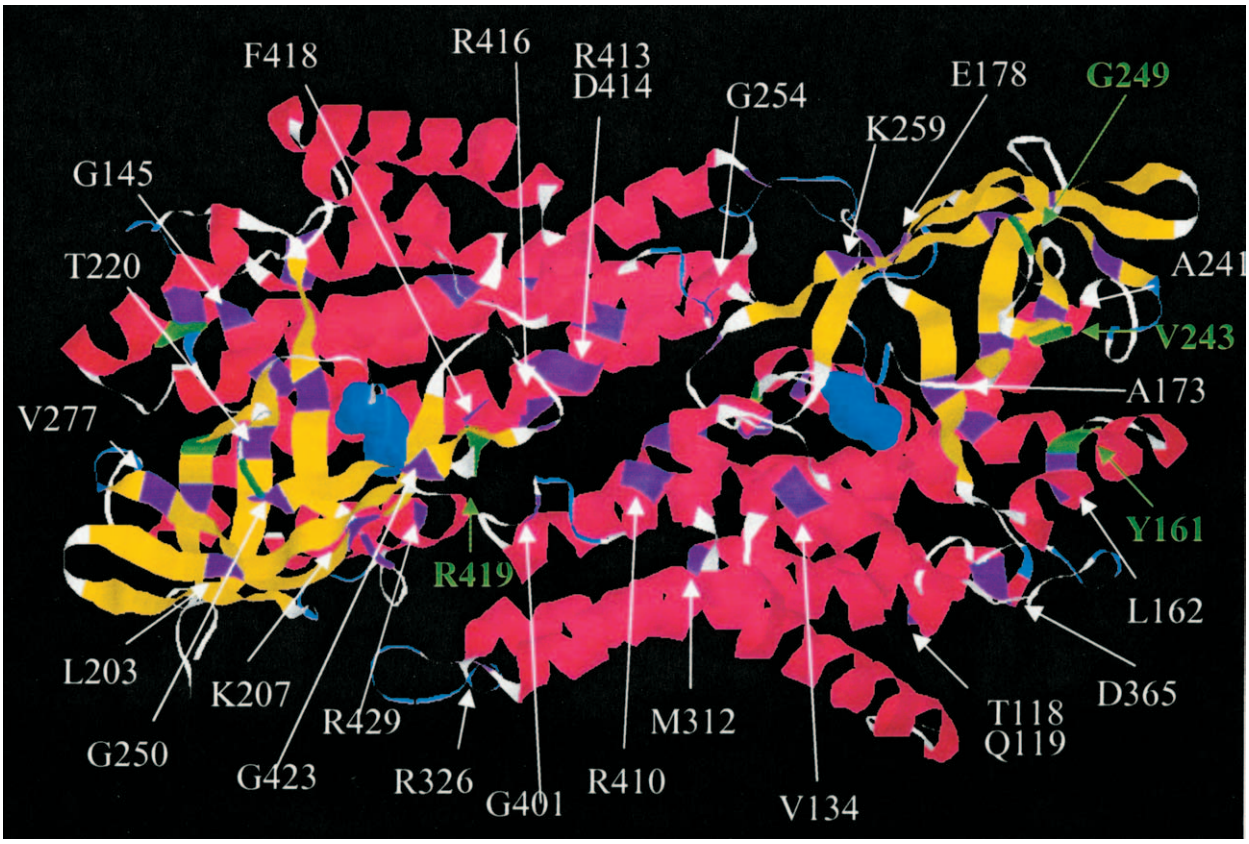

Fig 2. Crystal structure of human MCAD and homologous positions of reported VLCAD missense mutations. Two MCAD monomers are shown. An MCAD monomer consists of extended domains of α-helixes and β-sheets, connected by random coils and separated by the active site pocket and the flavin cofactor binding region. According to the extensive alignment homology between human MCAD and VLCAD amino acid sequences, the sites of all known VLCAD missense mutations within the MCAD protein are determined. *Blue* (spacefill mode), Catalytic region at amino acid position E422 (VLCAD numbering); *green*, mutations delineated in our patients; *purple*, missense mutations reported in the literature; *red*, α-helixes; *yellow*, β-sheets; *white and blue*, random coils.

mild folding mutation) was identified in this group and has not been observed in patients with clinically manifest disease.[12]

To better understand the mutations' effect on protein structure and function, we determined the VLCAD mutation sites within the structural model of human MCAD because amino acid sequences of both proteins share a high degree of homology. The T161C, V243A, and G249R mutations hold positions on outer loops (Fig 2), explaining the observed mild effect on protein stability. In contrast, mutations within the dimerization domain, such as the R419Q mutation that we delineated in patient 7, are likely to result in more instability. VLCAD instability and more rapid intramitochondrial protein degradation are the suspected mechanisms producing deficiency in patients with missense mutations and single amino acid deletions.[2,30] The patient with this R419Q mutation carries the V243A mutation on the other allele that probably determines the milder clinical phenotype.

Although we cannot exactly predict the mutations' effects on protein structure and stability, it is important to emphasize that the mutations we delineated in asymptomatic newborn infants are not different from previously reported VLCAD mutations in ill patients, both in relation to locations within the structural model and biochemical features.

Currently, routine MS/MS screening for VLCADD is required in only five American states.[9] Economic and ethical considerations are the main obstacles to the implementation of public newborn screening programs.[8] Initial results demonstrate that MS/MS is an excellent method for the detection of MCADD[11-13] and that the incidence of MCADD is with 1 in 10,000[10] to 1 in 15,000 newborn infants,[12] comparable to phenylketonuria, a disorder for which many countries and all American states require screening. Because MCADD has a high mortality rate at diagnosis in clinically diagnosed patients (30%-60%) and because >90% of prospectively detected patients have done well,[11,12] newborn screening for MCADD has obviously prevented deaths.

In the initial New England Newborn Screening Program, MS/MS screening of 164,000 newborns detected only one case of presumptive VLCADD.[11] More recent data from the New England Screening program and initial data from 3 German newborn screening laboratories revealed a VLCADD incidence of 1 in 42,500 to 1 in 125,000 births.

Thus, our results prove that the incidence of VLCADD is much higher than previously known.

Benign phenotypes that remain asymptomatic throughout life despite biochemical deficiency may exist in VLCADD, but because life-threatening metabolic derangement can occur as the primary manifestation even in patients with milder phenotypes and missense mutations, we advocate universal newborn screening programs for VLCADD to detect affected patients and prevent development of severe crisis. By prospective identification of patients with VLCADD, we will gain more knowledge about the morbidity of VLCADD and the definite risk of future disease, the spectrum of disease phenotypes, and the treatment options and indications.

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