


Genotype and residual enzyme activity in medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: Are predictions possible?

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Abstract

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is the most common defect of mitochondrial β -oxidation. Confirmation diagnostics after newborn screening (NBS) can be performed either by enzyme testing and/or by sequencing of the *ACADM* gene. Here, we report the results from enzyme testing in lymphocytes with gene variants from molecular analysis of the *ACADM* gene and with the initial acylcarnitine concentrations in the NBS sample. From April 2013 to August 2019, in 388 individuals with characteristic acylcarnitine profiles suggestive of MCADD the octanoyl-CoA-oxidation was measured in lymphocytes. In those individuals with residual activities <50%, molecular genetic analysis of the *ACADM* gene was performed. In 50% of the samples (195/388), MCADD with a residual activity ranging from 0% to 30% was confirmed. Forty-five percent of the samples (172/388) showed a residual activity >35% excluding MCADD. In the remaining 21 individuals,

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; MCADD, medium-chain acyl-CoA dehydrogenase deficiency; NBS, newborn screening; VLCAD, very long-chain acyl-CoA dehydrogenase.

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MCAD residual activity ranged from 30% to 35%. The latter group comprised both heterozygous carriers and individuals carrying two gene variants on different alleles. Twenty new variants could be identified and functionally classified based on their effect on enzyme function. C6 and C8 acylcarnitine species in NBS correlated with MCAD activity and disease severity. MCADD was only confirmed in half of the cases referred suggesting a higher false positive rate than expected. Measurement of the enzyme function in lymphocytes allowed fast confirmation diagnostics and clear determination of the pathogenicity of new gene variants. There is a clear correlation between genotype and enzyme function underlining the reproducibility of the functional measurement in vitro.

KEYWORDS

confirmation diagnostics, fatty acid oxidation disorders, MCAD deficiency, medium-chain acylcarnitines, newborn screening

1 | INTRODUCTION

Medium-chain acyl-CoA dehydrogenase (MCAD; EC1.3.8.7) catalyzes the first oxidation step of medium-chain fatty acids in the mitochondrial β -oxidation pathway. MCAD deficiency (MCADD, OMIM #201450) is the most common autosomal recessive disorder of fatty acid oxidation with an incidence between 1:4900 and 1:24900 newborns in Europe and North America.¹ Patients with MCADD are prone to metabolic decompensation usually triggered by catabolic stress such as prolonged fasting or febrile illnesses due to unmet energy supply by β -oxidation. Symptoms of metabolic decompensations comprise hypoketotic hypoglycemia, muscular hypotonia, impaired vigilance, Reye-like encephalopathy, coma, and death.²⁻⁵ The inclusion of this disease in neonatal screening programs in many countries worldwide has dramatically improved the clinical outcome and reduced the mortality since patients can usually be identified before the onset of symptoms.^{1,6,7} Elevation of the following acylcarnitine concentrations and ratios is considered suggestive of MCADD: C6, C8, C10, C10:1 as well as the ratios C8/C2, C8/C6, C8/C10, C8/C12, C8/C12, and C8/C16.^{8,9} Urinary excretion of hexanoylglycine, isohexanoylglycine, suberylglycine, and phenylpropionylglycine are indicative of MCADD as well, although confirmed patients lacking these metabolites in urine have also been reported.¹⁰⁻¹³ Confirmatory diagnosis of MCADD can be performed by molecular testing; however, the identification of one single or novel variant of unknown significance often leads to inconclusive results.⁴ Enzyme testing in lymphocytes, by measuring the oxidation rate of octanoyl-CoA or phenylpropionyl-CoA, is a faster and more efficient method for the confirmation of patients.¹⁴⁻¹⁶ There is evidence that residual enzyme function correlates with the

expected phenotype. Different biallelic and monoallelic variants in the *ACADM* gene are associated with variable reductions of cellular enzyme function and subsequently differences in the risk of metabolic decompensation.¹⁴⁻¹⁶ However, it remains often difficult to evaluate at which level of residual enzyme function there is a risk of a metabolic derangement as after the “diagnosis” has been made it is impossible not to treat. Despite the diagnosis, the clinical relevance of mild MCADD has remained uncertain.

In this study, we present data from confirmation diagnostics in MCAD patients performed from April 2013 to August 2019 in the Laboratory of Clinical Biochemistry and Metabolism at the Center for Pediatrics and Adolescent Medicine of Freiburg. In addition to the genotype and residual enzyme function we included initial disease-specific acylcarnitine concentrations from newborn screening (NBS).

2 | METHODS

Blood from 460 newborns with a positive NBS result suggestive of MCADD from Germany, Canada, and Italy was referred to our laboratory for confirmation diagnostics during the period from April 2013 to August 2019. In 171 out of 460 samples, we performed enzyme testing and molecular genetic analysis. Two hundred seventeen out of 460 samples received MCAD enzyme testing only, and in 72/460 samples we performed exclusively mutation analysis of the *ACADM* gene. Enzymatic data are available of 388 individuals. Results of enzyme testing were available between two and seven working days from arrival of the blood sample, with a median value of 2.5 days. The values described to exclude or confirm diagnosis were developed based on previous data.¹⁵ The

defined reference values for discrimination of patients, carriers, and healthy individuals are described in the results section. Individuals with enzyme activities <30% were only defined as “patients” if two sequence variants on *ACADM* gene were found; otherwise they were classified as healthy heterozygous carriers.

We performed enzyme testing in lymphocytes and mutation analysis, with informed consent of the parents. The study was performed according to the rules of the Declaration of Helsinki and approved under study number 219/19 by the ethical review board of Albert-Ludwig University of Freiburg. Whenever the initial acylcarnitine profiles were available, data were included in the study (Supplementary Table 1).

2.1 | Isolation of lymphocytes and octanoyl-CoA oxidation

The measurement of MCAD residual activity was achieved in lymphocytes by assaying the oxidation rate of octanoyl-CoA. Lymphocytes were isolated from 1 to 2 mL EDTA blood using Ficoll-Paque™ Plus (Amersham Bioscience, Freiburg, Germany) and Leucosep H (Greiner Bio One, Solingen, Germany) tubes as described previously¹⁷ and stored at -80°C . Octanoyl-CoA oxidation rate was measured as described previously¹⁵ with minor modifications. The turnover rate of octanoyl-CoA was calculated using the sum of the peak area of the products C8:1-CoA and C8:OH-CoA and compared with the peak area of the substrate C8:0-CoA set at an initial concentration of 20 nmol. All tests were run in triplicate in the presence of two negative controls chosen randomly from our pool. In order to confirm lymphocyte quality the measurement of very long-chain acyl-CoA dehydrogenase (VLCAD) function was measured by the oxidation rate of palmitoyl-CoA as described previously.¹⁸ The reaction products C8:1-CoA and C8:OH-CoA and C8:0-CoA were identified by high-performance liquid chromatography (HPLC) followed by UV-detection. A Phenomenex C18 Kinetex column (5 μm , 100A, 150 \times 4.6 mm) was used (Phenomenex, Aschaffenburg, Germany). For the separation of the metabolites, two mobile phases were applied, 25 mM KH_2PO_4 pH 6.9 and acetonitrile (ACN) with the following elution gradient maintained at 30°C : 0 to 4 minutes (10%-30% ACN), 4 to 8 minutes (30% ACN), 8 to 10 minutes (30%-80% ACN), 10 to 11 minutes (80% ACN), and 11 to 13 minutes (80%-10% ACN). The injection volume was 5 μL at a flow rate of 2 mL/min. The CoA-esters were detected at 254 nm. The interday variation was 4.3%. For practical reasons, it was not possible to measure the intraday value. As no external quality assessment programs exist so far for the MCAD

enzyme testing, known samples from healthy controls and patients are retested regularly to ensure the quality of our results.

2.2 | Mutation analysis

When requested, we performed also mutation analysis. Genomic DNA was extracted from EDTA blood and dried blood spots with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and with the Dried Blood spot DNA Isolation Kit (Norgen Biotek Corp, Ontario, Canada). All exons and part of the flanking intronic regions of the *ACADM* gene were amplified by PCR and analyzed by Sanger fluorescent sequencing. The amplified sequences were analyzed using the SeqPilot software analysis (JSI Medical Systems GmbH, Ettenheim, Germany). Single base pair variants of *ACADM* sequence (NM_000016.4) were assessed for pathogenicity by performing different *in silico* analyses using the Alamut Visual 2 software (<http://www.interactive-biosoftware.com/alamut-visual/>) that summarizes the results from four different tools covering different functional aspects (SIFT, <https://sift.bii.a-star.edu.sg/>; Mutation Taster, <http://www.mutationtaster.org/>; PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>; and Align GVDG, <http://agvgd.hci.utah.edu/>). All discovered variants were compared to data of The Human Gene Mutation Database professional version (<http://www.hgmd.cf.ac.uk/ac/index.php>) and the corresponding literature links. In the absence of variants or in case of heterozygosity in our samples, we performed additionally a multiplex ligation-dependent probe amplification (MLPA) analysis to exclude the presence of large deletions/duplications.

2.3 | Statistical analysis

Graphs, statistical evaluation of the enzyme activities, and Spearman *r* correlation of genotype-enzyme activities as well as of acylcarnitine species and ratios were performed using GraphPad Prism 8 (La Jolla, California). Enzymatic data are presented as means \pm SD.

3 | RESULTS

Despite a positive NBS suggestive for MCADD, only in 50% of the individuals (196 out of 388) MCADD was confirmed with a residual activity ranging from 0% to 30% (Figure 1A). In 21 out of 388 individuals, we measured an MCAD residual activity of 30% to 35%. Approximately one-fourth of the individuals displayed

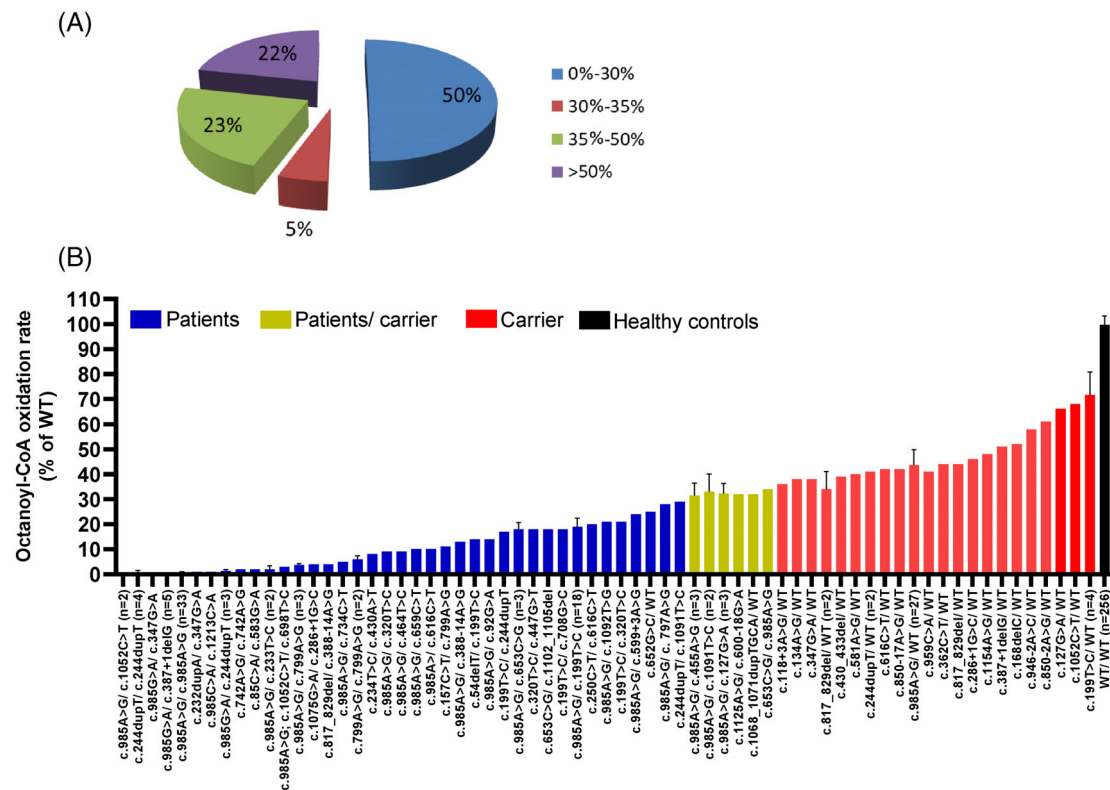


FIGURE 1 Distribution of medium-chain acyl-CoA dehydrogenase (MCAD) residual activity measured in 388 newborns with suspected MCAD deficiency (MCADD) on newborn screening (NBS) (A) and correlation of enzyme activity with the identified variants (B). The distribution of the range of residual activity was as follows: 0%-30% MCADD patients; 30%-35% MCADD patients and heterozygous individuals; 35%-50% heterozygous individuals; >50% healthy individuals. In 171 out of 388 samples, we performed enzyme testing and molecular genetic analysis. Correlation was considered significant if $P < .05$

a residual activity of 35% to 50% (88/388). In 84 out of 388, residual activity was >50% (Figure 1A). These data are suggestive of a high false positive rate in NBS also for MCADD.

3.1 | Correlation of gene variants with residual enzyme activity

The genotype of all sequenced patients identified by NBS is shown in Supplementary Table 1. Patients are listed in the order of the measured residual activity expressed as octanoyl-CoA oxidation rate. A total of 170 patients have been sequenced. In this cohort, 110 patients with a residual activity up to about 30% carried variants either in homozygous or compound heterozygous form confirming MCADD. We could observe a good correlation between genotype and enzyme function in that identical genotypes led to similar residual activities (Figure 1B; $P < .0001$). In particular, the most common mutation c.985G>A, found in 33 patients (patients #1-17, 28-42, and 48) in homozygosity was associated with a residual activity of $0.52\% \pm 0.44\%$, in line with previous reports of enzymatic measurements either with octanoyl-CoA or phenylpropionyl-CoA as substrate.^{15,16} A

further correlation could be observed also for patients #54 and #58 who carried the sequence variant c.985G>A in compound heterozygosity with the gene variant c.799A>G. In these cases, the residual activity was $3.67\% \pm 0.47\%$ (Figure 1B). Samples from patients carrying the homozygous mutation c.799A>G (patients #92 and #100) resulted in a residual activity of $6\% \pm 1\%$. When c.985G>A was associated in compound heterozygosity with the known mild gene variant c.199T>C,⁸ the residual activity increased up to $18.89\% \pm 2.66\%$ (Figure 1B). Several patients with residual activities in a range between 25% and 36% were either homozygous or compound heterozygous for variants in the *ACADM* gene. This group included five patients carrying five newly identified variants, namely c.652G>C, c.1068_1071dupTGCA, c.455A>G, c.653C>A, and c.1091T>C (Table 1). In case of the identification of variants in compound heterozygosity with c.985A>G, the measured residual activities allowed the classification of this newly identified mutation as being mild. All other sequenced individuals were heterozygotes and the variants were classified as severe (Table 1). Twenty-eight patients were heterozygous for the common mutation c.985G>A, and showed a residual activity of $44.25\% \pm 3.9\%$.

TABLE 1 New variants identified in this study. New variants are marked in bold

| Individual Nr ^a | Allel 1 | Predicted aminoacid change | Allel 2 | | Predicted aminoacid change 2 | Residual activity | | Severity ^b |
|----------------------------|---------------------------|----------------------------|-----------|----------------------|------------------------------|-------------------|-----------------------|---------------------------|
| | | | Exon | Allel 2 | | Exon | Severity ^b | |
| 1 | c.232dupA | p.(Ile78Asnfs*2) | 5 | c.347G>A | p.(Cys116Thr) | 5 | 1 | Severe |
| 2 | c.985G>A | p.(Lys329Glu) | 11 | c.1213C>A | p.(Gln405Lys) | 12 | 1 | Severe |
| 3 | c.85C>A | p.(Arg29Arg) | 2 | c.583G>A | p.(Gly195Arg) | 7 | 2 | Severe |
| 4 | c.1075G>A | p.(Ala359Thr) | 11 | c.286+1G>C | | 4 | 4 | Severe |
| 5 | c.234T>G | p.(Ile78Met) | 5 | c.430A>T | p.(Lys144*) | 6 | 8 | Both severe |
| 6 | c.659C>T | p.(Thr220Ile) | 8 | c.985G>A | p.(Lys329Glu) | 11 | 10 | Severe |
| 7 | c.54delT | p.(His19Ilefs*17) | 2 | c.199T>C | p.(Tys67His) | 3 | 14 | Severe |
| 8 | c.199T>C | p.(Tys67His) | 3 | c.708G>C | p.(Lys239Asn) | 8 | 18 | Severe |
| 9 | c.985G>A | p.(Lys329Glu) | 11 | c.1092T>G | p.(Ile364Met) | 11 | 21 | Mild |
| 10 | c.652G>C | p.(Ala218Pro) | 8 | WT ^a | | | 25 | Severe^a |
| 11 | c.455A>G | p.(Lys152Gly) | 6 | c.985G>A | p.(Lys329Glu) | 11 | 28 | Mild |
| 12 | c.1068_1071dupTGCA | p.(Lys358Cysfs*15) | 11 | WT | | | 32 | Severe |
| 13 | c.653C>A | p.(Ala218Asp) | 8 | c.985G>A | p.(Lys329Glu) | 11 | 34 | Mild |
| 14 | c.118+3A>G | | | WT | | | 36 | Severe |
| 15 | c.581A>G | p.(Asn194Ser) | 7 | WT | | | 40 | Severe |
| 16 | c.850-17A>G | | | WT | | | 42 | Severe |
| 17 | c.1154A>G | p.(Tyr385Cys) | 11 | WT | | | 48 | Severe |
| 18 | c.168delC | p.(Arg57Lysfs*21) | 3 | WT | | | 52 | Severe |

^aA possible variant on WT allele of individual Nr. 10 may escaped detection by Sanger sequencing and MLPA; in this case the variation p.(Ala218Pro) may be also considered as mild.

^bThe severity of the sequence variation is referred to the newly identified sequence variants.

The allele frequency for the common variants c.985G>A and c.199T>C⁸ was 52.6% and 7.7% of the total alleles, respectively. As shown in Table 1, we found a total of 20 novel *ACADM* variants, including 13 missense variants, 4 small deletions/duplications, and 3 splice site variants (Table 1). In 12 patients, the novel variants were found in compound heterozygosity with a second either severe mutation, mostly c.985G>A, or a mild mutation, mostly c.199T>C. Patients #57 and #66 were carrying two novel variants each, c.1075G>A/c.286+1G>C and c.234T>G/c.430A>T, respectively. These four newly identified variants could be classified as severe since they resulted in a residual activity of 4% and 8%, respectively (Table 1). All further newly identified variants found in heterozygous form could also be classified as severe as affected patients showed a residual activity in the range of 36% to 52%.

Overall, measurement of the enzyme function in lymphocytes allowed determination of the pathogenicity of new gene variants. In addition, there is a clear correlation between genotype and enzyme function demonstrating also the reproducibility of the functional measurement in vitro.

3.2 | Acylcarnitine species on NBS and enzyme activity

In order to investigate the correlation between acylcarnitine levels in NBS samples with MCAD activity, NBS results were analyzed. Individuals were clustered in different groups according to their residual activity. As the samples were collected from 19 different screening laboratories the method of analysis and the cutoffs for the reported acylcarnitines differed remarkably. As shown in Figure 2, a good correlation between residual activity and initial acylcarnitine values could be observed only for the species C6 and C8, in line with previous reports.^{8,19} Patients with severe MCADD (range of residual activity 0%-10%) showed the highest C6 and C8 concentrations with reported values up to 54.3 μmol/L. A higher residual activity correlated with lower concentrations of C6 and C8 measured in NBS (Figure 2A,B). No correlation with enzyme residual activity was observed for the concentrations of C10 and C10:1 acylcarnitines. Indeed, similar concentrations of these acylcarnitine species were measured in false positive samples with a residual activity >30% and MCADD samples (Figure 2C,D).

All ratios usually used in NBS for the diagnosis of MCADD appeared to be correlated with the measured residual enzyme activity (Figure 3). However, the exclusion of the group 0% to 10% showed clearly that for the ratios C8/C10 and C8/C16 this correlation was lost (Supplementary Figure 3B,D).

3.3 | Reduction of the octanoyl-CoA oxidation rate is not accompanied by an upregulation of the palmitoyl-CoA oxidation rate

In order to evaluate the quality of the isolated lymphocytes and investigate whether VLCAD may in part

compensate for a reduced octanoyl-CoA oxidation rate in MCADD we correlated the octanoyl-oxidation rate (corresponding to MCAD enzyme activity) with the palmitoyl-oxidation rate (corresponding to the VLCAD enzyme activity) (Figure 4; $P = .08051$). Surprisingly, we observed a palmitoyl-CoA oxidation rate $>100\%$ in few samples, especially in MCAD patients with a residual activity in the range 0% to 30%. However, since almost all the samples displayed a palmitoyl-CoA oxidation rate $<100\%$, we assume that no compensatory upregulation of VLCAD enzyme activity occurred in case of MCADD. In analogy to cases reported previously,¹⁸ three patients #25 (0% MCAD; Supplementary Table 1), #26 (0% MCAD; Supplementary Table 1), and #106 (5% MCAD) showed a remarkably reduced VLCAD residual enzyme activity

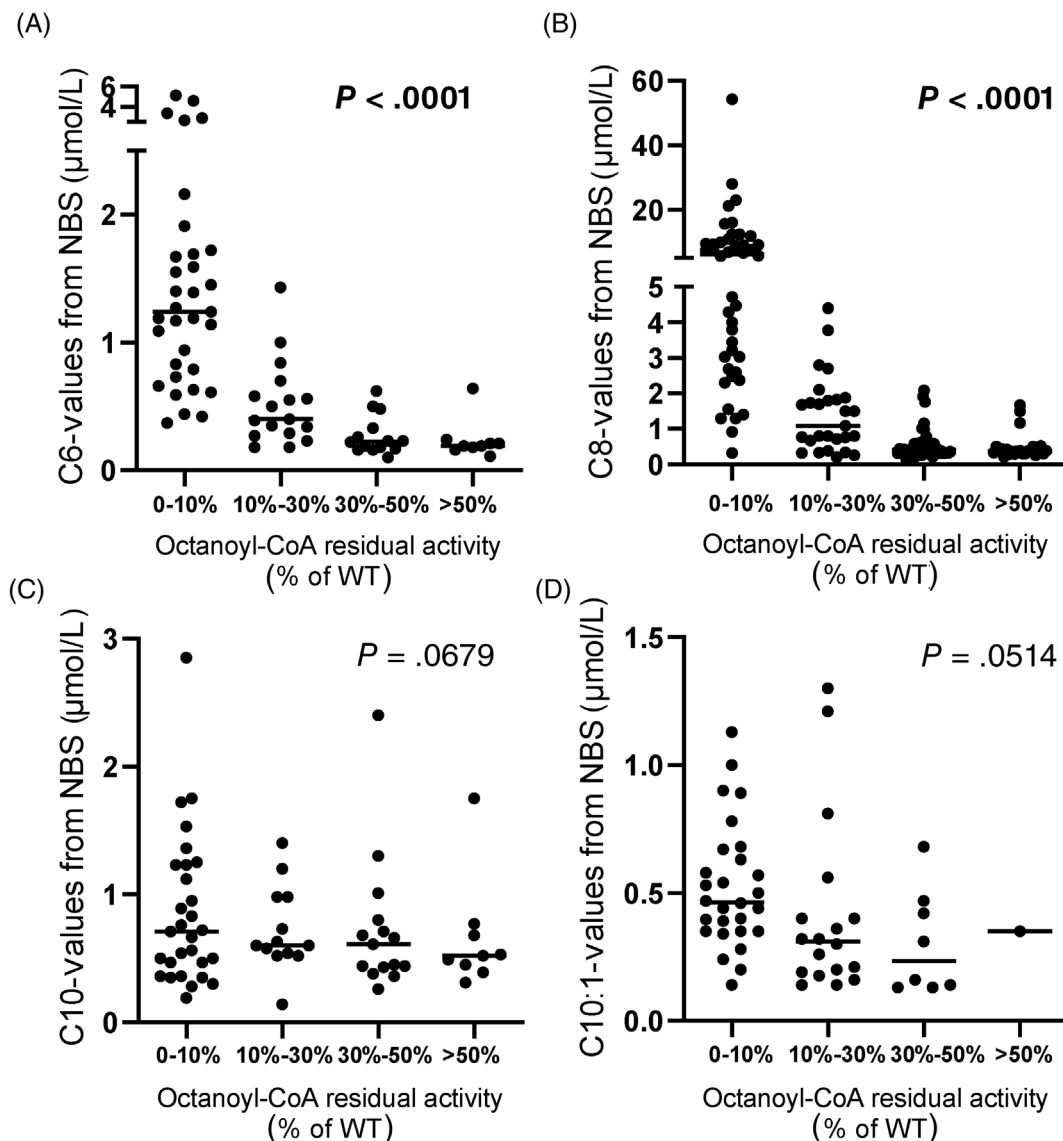


FIGURE 2 Correlation of medium-chain acyl-CoA dehydrogenase (MCAD) residual enzyme activity and medium-chain acylcarnitines in newborn screening (NBS) among patients with residual activity $<10\%$, 10% - 30% , 30% - 50% , and $>50\%$. Acylcarnitine values (when available) are given as $\mu\text{mol/L}$ and are listed in Supplementary Table 1. Correlation was considered significant if $P < .05$

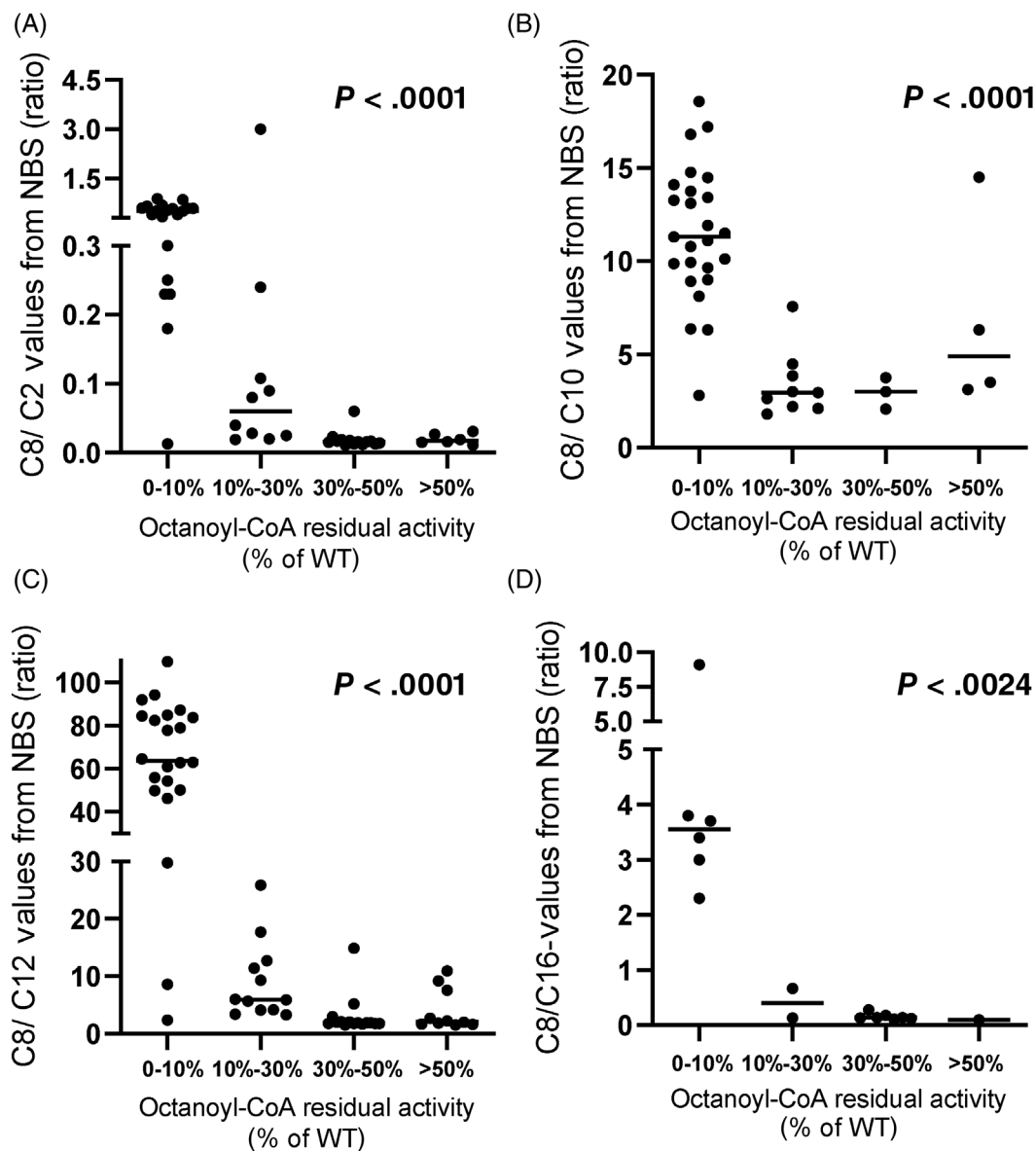


FIGURE 3 Correlation of medium-chain acyl-CoA dehydrogenase (MCAD) residual enzyme activity and medium-chain acylcarnitine ratios in newborn screening (NBS) among patients with residual activity <10%, 10%-30%, 30%-50%, and >50%. Acylcarnitine values (when available) are given as $\mu\text{mol/L}$ and are listed in Table 1. Correlation was considered significant if $P < .05$

with values of 30%, 41%, and 37%, respectively. Sequencing of *ACADVL* confirmed heterozygosity for an *ACADVL* variant in these patients. The clinical significance of these findings such as synergistic heterozygosity is yet unclear.

4 | DISCUSSION

MCADD is the most common defect of fatty acid oxidation. The introduction of MCADD as a target disease in NBS programs in many countries worldwide has remarkably reduced the risk of mortality and psychodevelopmental

delay.²⁰ Due to the high prevalence of the common classical mutation c.985A>G on exon 11, sequencing of the *ACADM* gene is often the first approach for confirmation diagnostics in case of positive NBS. However, diagnostic algorithms at least in Germany also include the performance of a functional test to quantify residual enzyme activity (<https://www.awmf.org/leitlinien/detail/ll/027-021.html>). In this study, we could show a clear correlation between enzyme activity and genotype in MCADD that allowed the unambiguous discrimination of patients and carriers as well as the classification of newly identified variants with respect to their impact on enzyme function.

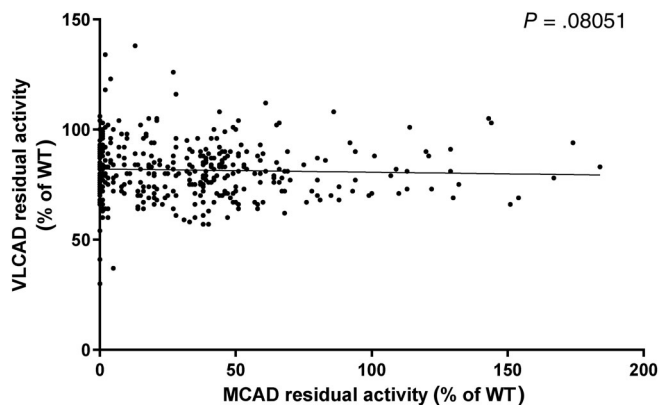


FIGURE 4 Correlation of medium-chain acyl-CoA dehydrogenase (MCAD) with very long-chain acyl-CoA dehydrogenase (VLCAD) residual activity. Values are expressed as percent of the enzyme activity of healthy individuals. The red line represents the VLCAD enzyme activity of 100%. Correlation was considered significant if $P < .05$

Elevation of specific medium-chain acylcarnitines and related ratios is strongly indicative of MCADD.²¹ Despite the assumed high specificity of these metabolites only 50% of cases with a positive NBS suggestive of MCADD referred to our laboratory could be confirmed as true MCADD. The remaining samples showed either only a mild functional impairment typical for heterozygous carriers or no alteration of enzyme function. In the past, the correlation between biochemical phenotype and genotype has been extensively discussed especially with the known environmental factors influencing disease phenotype. Some studies described the lack of correlation between genotype and initial octanoylcarnitine levels.^{8,15} However, the presence of the common homozygous mutation c.985A>G or other known highly deleterious variants is often associated with very high values of initial octanoylcarnitine.^{19,22-24} In line with these studies, our results very clear showed that especially the initial levels of C6 and C8 acylcarnitines at NBS correlated with residual enzyme function. In fact, the highest C6 and C8 values have been reported in the group of patients with the lowest enzyme residual activity in the range of 0% to 10%. These data are strongly consistent with previous reports, although we plotted values of initial NBS from different screening laboratories that use different cutoffs for the identification of MCADD patients. With increasing residual activity we observed an overlapping of similar acylcarnitine values among mild patients (range, 10%-30%) but also in heterozygous carriers and healthy individuals. In this regard, Lehotay et al²⁵ mentioned already in 2004 the difficulty of interpreting borderline elevation of acylcarnitines and the consequences for the decision of biochemical and clinical follow-up in these cases, especially with heterozygotes for the common c.985A>G

mutation also presenting with elevated C8 levels.²⁵ Also several other variables such as infectious diseases and metabolic distress due to prematurity and low birth weight may indeed trigger β -oxidation and induce accumulation of acylcarnitines.^{25,26} In contrast, C10 and C10:1 species also currently used in NBS, appeared not to correlate with MCAD residual activity. It is conceivable that during catabolism when the degradation of fatty acids is upregulated, the accumulation of certain medium-chain acylcarnitines such as C10 and C10:1 indeed may reflect a physiological response of the organism as result of an overload of fatty acids in the mitochondrial β -oxidation. In a very similar way, the long-chain acylcarnitines C14:1 and C14:2 are typically increased also in healthy infants and individuals during fasting or metabolic situations.²⁷

The use of ratios can remarkably increase the sensitivity of NBS tests and thereby the discrimination of patients with different severities of MCADD.^{12,19,22,25} In this regard, C8/C2 and C8/C10 are considered most reliable with values about 10-fold higher in MCAD patients than in controls.²⁸ This is only in part in line with our data. Although all reported ratios clearly correlate with enzyme activities in the range of classical MCADD, in patients and individual with a residual activity >10% values of ratios are very variable. Especially the ratios C8/C10 and C8/C16 showed overlapping values among patients, carriers, and healthy individuals that complicates the interpretation of the NBS results. However, the low number of reported values obtained by different NBS laboratories as well as the different methods of acylcarnitine methods in the different NBS laboratories and different used cutoff makes an accurate evaluation of the data difficult.

Overall, functional enzyme testing is necessary to discriminate severe from mild MCADD in cases of new genetic *ACADM* variants, and to discriminate healthy carriers and healthy individuals when initial acylcarnitine profiles are borderline and of unclear interpretation. With the optimized method based on Sturm et al,¹⁵ diagnostic quality was improved remarkably. However, the prediction of metabolic derangement may still remain difficult as this depends genetically on how the variance affects enzyme residual activity but also on several other variables such as lifestyle and environmental factors. It remains, therefore, a matter of debate whether MCADD patients with relatively high residual activity are at risk of metabolic decompensation and it still remains difficult to define the limit below which the development of symptoms is expected. The consistent enzymatic results obtained with the high number of samples with homozygosity for c.985A>G ($0.52\% \pm 0.44\%$), compound heterozygosity for c.199T>C/c.985A>G ($18.89\% \pm 2.66\%$) or heterozygosity for c.985A>G ($44.25\% \pm 3.9\%$) allow a clear correlation between residual activity and genotype. With this method, compound heterozygous patients carrying the common mutation c.985A>G with a new

variant or patients with two new variants can be easily diagnosed and classified based on the effect of the variation on the protein function. In this regard, we identified the variants c.653C>A/p.A218D involving the same codon and nucleotide of a known described pathogenic mutation (c.653C>G).²⁹ Both variants were classified as pathogenic by the prediction software Alamut Visual 2; however, their impact on protein function was different. While the variant c.653C>G in compound heterozygosity with c.985A>G resulted in a residual activity of about 18% in our patient cohort, the newly identified variation showed a residual activity of 34%. Since this activity has been described also for the combination c.127G>A/c.985A>G, it is conceivable that despite the compound heterozygous presence of this variant the patients may very likely remain asymptomatic. Indeed, heterozygosity for an MCAD deficiency variant is not associated with a risk of metabolic decompensation. We believe that MCADD due to compound heterozygosity with at least one “high residual function” variant resulting in a residual activity >30% will also remain asymptomatic.

Initially meant as internal quality control for the viability of the lymphocytes, we also measured palmitoyl-CoA oxidation in the same patient samples. As acyl-CoA dehydrogenases share in part overlapping substrate specificity,³⁰ we also investigated the effects of reduced MCAD enzyme activity on the palmitoyl-CoA oxidation rate. Of interest was the finding that palmitoyl-CoA oxidation rate was mildly reduced in many samples. Biochemical and computational data have demonstrated that higher concentration of palmitoyl-CoA as substrate for the β -oxidation induced a decline in flux and led to the accumulation of acylcarnitines.³¹ Taking advantage of the parallel measurement of VLCAD enzyme activity, we could also identify three MCAD patients with a VLCAD enzyme activity in the range of healthy carriers. These results were confirmed by molecular sequencing of *ACADVL* and revealed the presence of two common missense variants and one splice site variants. All three variants were previously described as disease-causing.¹⁸ The clinical consequences of variants in two different genes affecting the same metabolic pathway are still unknown.

5 | CONCLUSION

The suspicion of MCADD in NBS is based on accumulating medium-chain acylcarnitines and respective ratios. Hexanoylcarnitine and octanoylcarnitine seem to be good predictors in the diagnosis of MCADD and high values associate with disease severity. However, in half of the referred suspected cases from NBS to our laboratory the disease could not be confirmed when functional assays were applied. These results are surprising since MCAD screening was reported to be relatively specific with few false positives.^{29,32}

In high patient numbers, we can demonstrate that the measurement of the enzyme function in lymphocytes allows clear determination of the pathogenicity of new gene variants. In addition, there is a clear correlation between genotype and enzyme function demonstrating also the reproducibility of the functional measurement in vitro. MCAD enzyme testing allows fast confirmation diagnostics for necessary prophylactic or therapeutic interventions.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Sara Tucci designed the research and drafted the manuscript, guarantor; Christine Wagner summarized the data and was responsible for the first draft of the manuscript; Sarah C. Grünert, Natalie Weinhold, Jeannette Klein, Francesco Porta, Marco Spada, Andrea Bordugo, Francesca Furlan, Francesca Menni, were primarily responsible for patient data collection; Uta Matysiak, Giulia Rodella and Anna Sajeva supported analysis and interpretation of the molecular genetics data; Ute Spiekerkötter co-drafted the article and revised it critically. Sara Tucci had primary responsibility for final content. All authors read and critically revised the article and approved the final version of the manuscript.

ETHICAL STATEMENT

The study was performed according to the rules of the Declaration of Helsinki and approved under study number 219/19 by the ethical review board of Albert-Ludwig University of Freiburg.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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