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Free carnitine concentrations and biochemical parameters in medium-chain acyl-CoA dehydrogenase deficiency: Genotype–phenotype correlation

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Abstract

Biallelic variants in the *ACADM* gene cause medium-chain acyl-CoA dehydrogenase deficiency (MCADD). This study reports on differences in the occurrence of secondary free carnitine (C0) deficiency and different biochemical phenotypes related to genotype and age in 109 MCADD patients followed-up at a single tertiary care center during 22 years. C0 deficiency occurred earlier and more frequently in c.985A>G homozygotes (genotype A) compared to c.985A>G compound heterozygotes (genotype B) and individuals carrying variants other than c.985A>G and c.199C>T (genotype D) (median age 4.2 vs. 6.6 years; $p < 0.001$). No patient carrying c.199C>T (genotype C) developed C0 deficiency. A daily dosage of 20–40 mg/kg carnitine was sufficient to maintain normal C0 concentrations. Compared to genotype A as reference group, octanoylcarnitine (C8) was significantly lower in genotypes B and C, whereas C0 was significantly higher by 8.28 µmol/L in genotype C ($p < 0.05$). In conclusion, C0 deficiency is mainly found in patients with pathogenic genotypes associated with high concentrations of presumably toxic acylcarnitines, while individuals carrying the variant c.199C>T are spared and show consistently mild biochemical phenotypes into adulthood. Low-dose carnitine supplementation maintains normal C0 concentrations. However, future studies need to evaluate clinical benefits on acute and chronic manifestations of MCADD.

KEYWORDS

acylcarnitines, biochemical phenotype, medium-chain acyl-CoA dehydrogenase deficiency, newborn screening, secondary carnitine deficiency

1 | INTRODUCTION

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD; #OMIM 201450) is the most prevalent disorder of fatty acid oxidation and is caused by biallelic variations within the *ACADM* gene (GeneBank Accession M16827.1). Together with phenylketonuria, MCADD is the most frequent inborn error of metabolism (IEM) identified by newborn screening (NBS) worldwide. The prevalence of MCADD varies

significantly by geographical region and ethnic background.[1] In Germany, MCADD is detected with a prevalence of 1: 10.086 by NBS.[2] Before the implementation of NBS, a prevalent variation of the *ACADM* gene, c.985A>G, was identified with an allele frequency of 94% in clinically ascertained patients, with 80% of patients being homozygous.[3,4] This variant, however, has been shown to be considerably less frequent in individuals detected by NBS indicating the presence of hypomorphic variation. In NBS cohorts, many novel variants including a second prevalent variation, c.199C>T, have been identified.[5–9]

The enzyme medium-chain acyl-CoA dehydrogenase (MCAD) catalyzes the first step of the mitochondrial ß-oxidation of medium-chain fatty acids. Its impaired activity results in an accumulation of medium-chain acyl-CoA-esters and their corresponding acylcarnitines. The accumulation of octanoylcarnitine (C8) is the biochemical hallmark of the disease, and C8 is used as primary marker metabolite for the detection in NBS, often combined with analyte ratios to increase diagnostic specificity.[10,11]

Free carnitine (C0) is used to form acylcarnitines via conjugation from acyl-CoA-esters providing a sufficient intracellular CoA pool to maintain intermediary metabolism and to facilitate the renal elimination of accumulated metabolites. Accordingly, secondary carnitine deficiency (SCD) is frequently encountered in inborn errors of metabolism (IEM) which are characterized by an accumulation of acylcarnitines such as glutaric acidemia type 1, isovaleric acidemia, and methylmalonic aciduria.[12,13] In most of these disorders, carnitine is regularly administered as an integral part of the therapy,[13–16] however, without evidence from controlled clinical trials.[17] In MCADD, the supplementation of carnitine is still a subject of controversy. It has been postulated that carnitine supplementation may prevent metabolic decompensation,[13,18] but a beneficial effect has not been proven.[19]

Data on the supplementation practice of metabolic centers is limited and variable.[20–22] Infants with MCADD carrying the prevalent variation c.985A>G have been shown to frequently develop SCD.[23] However, no data on long-term substitution or SCD in other genotypes have been reported so far.

We report on biochemical phenotypes, SCD, and carnitine supplementation in 109 MCADD patients followed at a single tertiary care center during 22 years. We show that SCD occurs mainly in MCADD patients with pathogenic genotypes, but not in individuals carrying the missense variant c.199C>T. We provide pathophysiological and clinical considerations advocating for carnitine supplementation in MCADD patients with SCD. Our findings aim to aid treatment decisions and to support clinicians in counseling families and patients with regards to carnitine supplementation in the absence of reliable clinical evidence. Furthermore, we describe the development of biochemical parameters with age in different MCADD genotypes.

2 | PATIENTS AND METHODS

2.1 | Patient cohort

We retrospectively analyzed data from 109 MCADD patients followed at the metabolic center of the Munich university children's hospital between 1999 and 2021. The majority of patients ($n = 103$) was diagnosed by NBS, which was implemented in Bavaria on 1 Jan 1999. Few patients were born before the implementation of NBS and diagnosed by family screening ($n = 3$) or clinically after metabolic decompensation ($n = 3$). Confirmatory testing comprised the analysis of acylcarnitines in serum, sequencing of the *ACADM* gene, and enzymatic testing in leucocytes or fibroblasts, when molecular testing remained inconclusive (i.e., only one sequence variation detectable).

Treatment and clinical follow-up of the patients included the recommendation to avoid fasting and to follow an emergency treatment plan during episodes of intercurrent illness. The concentrations of C0 and acylcarnitines in serum were determined mostly annually. The supplementation of carnitine was recommended when C0 concentrations below the reference range (8.9–93.3 μmol/L) were found. Carnitine supplementation was started at a daily dose between 20 and 40 mg/kg body weight daily, divided into 2 to 3 doses, aiming to maintain C0 concentrations within the lower reference range.

2.2 | Biochemical parameters and genotyping

Analysis of C0 and acylcarnitine concentrations in serum samples was performed by flow injection analysis mass spectrometry (FIA-MS/MS). Plasma samples (10 μL) were spiked with internal standard solution (200 μL) containing carnitine-d_9 (C0-d_9, 0.82 nmol/L) and acylcarnitines-d_3 of different chain lengths: acetylcarnitine-d_3 (C2-d_3, 0.20 nmol/L), propionylcarnitine-d_3 (C3-d_3, 0.04 nmol/L), butyroylcarnitin-d_3 (C4-d_3, 0.04 nmol/L), isovalerylcarnitine-d_9 (C5-d_9, 0.04 nmol/L), hydroxyisovalerylcarnitine-d_3 (C5OH-d_3, 0.04 nmol/L), glutarylcarnitine-d_3 (C5DC-d_3, 0.09 nmol/L), octanoylcarnitine-d_3 (C8-d_3, 0.04 nmol/L), dodecanoylcarnitine-d_9 (C12-d_9, 0.04 nmol/L), tetradecanoylcarnitine-d_9 (C14-d_9, 0.04 nmol/L), palmitoylcarnitine-d_3 (C16-d_3, 0.09 nmol/L), 3-hydroxypalmitoylcarnitine-d_3 (C16-OH-d_3, 0.07 nmol/L) and stearoylcarnitine-d_3 (C18-d_3, 0.08 nmol/L). Protein precipitation was achieved by homogenization of the sample and subsequent centrifugation (5 min, 10 000 rpm). The supernatant (100 μL) was transferred to a microtiter plate and diluted with 100 μL of methanol: water (9:1, v/v) containing 0.025% formic acid. FIA-MS/MS analysis in positive ionization mode was performed on a tandem mass spectrometer TQ4500 (AB Sciex, Darmstadt, Germany) equipped with a Nexera LC-20ADXR HPLC system (Shimadzu, Kyoto, Japan). Injection of each sample (20 μL) was performed by a PAL-RSI (CTC Analytics AG, Zwingen, Switzerland) autosampler unit. A mixture of methanol: water (9:1, v/v) containing 0.025% formic acid was used as mobile phase at a flow rate of 0.25 mL/min. The instrument was controlled using Analyst 1.7 software (AB Sciex, Darmstadt, Germany). ChemoView 2.0.4 software (AB Sciex, Darmstadt, Germany) and Microsoft Excel (Microsoft Office, 2010) were used for data analysis. The concentrations of C0, C8, and the diagnostic ratios C8/acetylcarnitine (C2) and C8/decanoylcarnitine (C10), which are frequently applied in NBS, were used for the long-term evaluation of biochemical parameters.

TABLE 1 Variants in the *ACADM* gene (NM_000016.6).

	ACADM genotype					
	Allele 1			Allele 2		
Status of prevalent variant and no. of patients	cDNA	Location	Protein	cDNA	Location	Protein
Homozygous for c.985A>G (Group A) $n = 51$						
	c.985A>G	Exon 11	p.Lys329Glu	c.985A>G	Exon 11	p.Lys329Glu
Heterozygous for c.985A>G (Group B) $n = 35$						
1	c.985A>G	Exon 11	p.Lys329Glu	**c.31-9T>A**	**IVS 1**	**splice site variant**
2	c.985A>G	Exon 11	p.Lys329Glu	**c.86G>A**	**Exon 2**	**p.Arg29Gln**
2	c.985A>G	Exon 11	p.Lys329Glu	c.216 + 1G>T	IVS 3	splice site variant
1	c.985A>G	Exon 11	p.Lys329Glu	c.233T>C	Exon 4	p.Ile78Thr
1	c.985A>G	Exon 11	p.Lys329Glu	c.244dupT	Exon 4	p.Trp82Leufs*23
1	c.985A>G	Exon 11	p.Lys329Glu	c.347G>A	Exon 4	p.Cys116Tyr
1	c.985A>G	Exon 11	p.Lys329Glu	**c.348T>A**	**Exon 4**	**p.Cys116***
1	c.985A>G	Exon 11	p.Lys329Glu	c.472T>C	Exon 7	p.Tyr158His
3	c.985A>G	Exon 11	p.Lys329Glu	c.616C>T	Exon 8	p.Arg206Cys
1	c.985A>G	Exon 11	p.Lys329Glu	**c.529G>T**	**Exon 7**	**p.Glu177***
1	c.985A>G	Exon 11	p.Lys329Glu	c.600-18G>A	IVS 7	splice site variant
2	c.985A>G	Exon 11	p.Lys329Glu	c.734C>T	Exon 9	p.Ser245Leu
2	c.985A>G	Exon 11	p.Lys329Glu	c.797A>G	Exon 9	p.Asp266Gly
1	c.985A>G	Exon 11	p.Lys329Glu	c.799G>A	Exon 9	p.Gly267Arg
1	c.985A>G	Exon 11	p.Lys329Glu	**c.850–17A>G**	**IVS 9**	**splice site variant**
1	c.985A>G	Exon 11	p.Lys329Glu	c.928G>A	Exon 10	p.Gly310Arg
1	c.985A>G	Exon 11	p.Lys329Glu	c.946-2A>G	IVS 10	splice site variant
2	c.985A>G	Exon 11	p.Lys329Glu	c.999_1011dup	Exon 11	p.Gln338*
1	c.985A>G	Exon 11	p.Lys329Glu	c.1067T>C	Exon 11	p.Ile356Thr
1	c.985A>G	Exon 11	p.Lys329Glu	c.1091T>C	Exon 11	p.Ile364Thr
2	c.985A>G	Exon 11	p.Lys329Glu	c.1114dupG	Exon 11	p.Ala372Glyfs*11
2	c.985A>G	Exon 11	p.Lys329Glu	c.1237C>A	Exon 12	p.Arg413Ser
2	c.985A>G	Exon 11	p.Lys329Glu	c.1247T>C	Exon 12	p.Ile416Thr
2	c.985A>G	Exon 11	p.Lys329Glu	n.d.	n.d.	n.d.
Heterozygous for c.199T>C (Group C) $n = 14$:						
10	c.199T>C	Exon 3	p.Tyr67His	c.985A>G	Exon 11	p.Lys329Glu
2	c.199T>C	Exon 3	p.Tyr67His	c.320T>C	Exon 5	p.Leu107Ser
1	c.199T>C	Exon 3	p.Tyr67His	c.799G>A	Exon 9	p.Gly267Arg
1	c.199T>C	Exon 3	p.Tyr67His	c.1237C>A	Exon 12	p.Arg413Ser
c.985A>G and c.199T>C absent (Group D) $n = 9$:						
1	c.157C>T	Exon 3	p.Arg53Cys	**c.608T>G**	**Exon 8**	**p.Leu203***
4	c.244dupT	Exon 4	p.Trp82Leufs*23	c.244dupT	Exon 4	p.Trp82Leufs*23
1	c.244dupT	Exon 4	p.Trp82Leufs*23	c.542A>G	Exon 7	p.Asp181Gly
1	c.742A>G	Exon 9	p.Arg248Gly	**c.637_638delinsAA**	**Exon 8**	**p.Ala213Asn**
1	**c.792del**	**Exon 9**	**p.Ile264Metfs*19**	**c.792del**	**Exon 9**	**p.Ile264Metfs*19**
1	c.799G>A	Exon 9	p.Gly267Arg	c.799G>A	Exon 9	p.Gly267Arg

Note: n.d. no second, pathogenic variation was identified; MCADD confirmed by enzymatic testing in leucocytes and fibroblasts, respectively. Novel variants are marked in bold.

Molecular testing of the *ACADM* gene was performed by Sanger sequencing of all exons and parts of the adjacent intronic regions as previously described (GeneBank Accession M16827.1; RefSeq ID NM_000016.6).[8] The activity of MCAD in lymphocytes or fibroblasts was measured in the Laboratory for Genetic Metabolic Diseases (UMC Amsterdam) and the children's university hospital Freiburg.

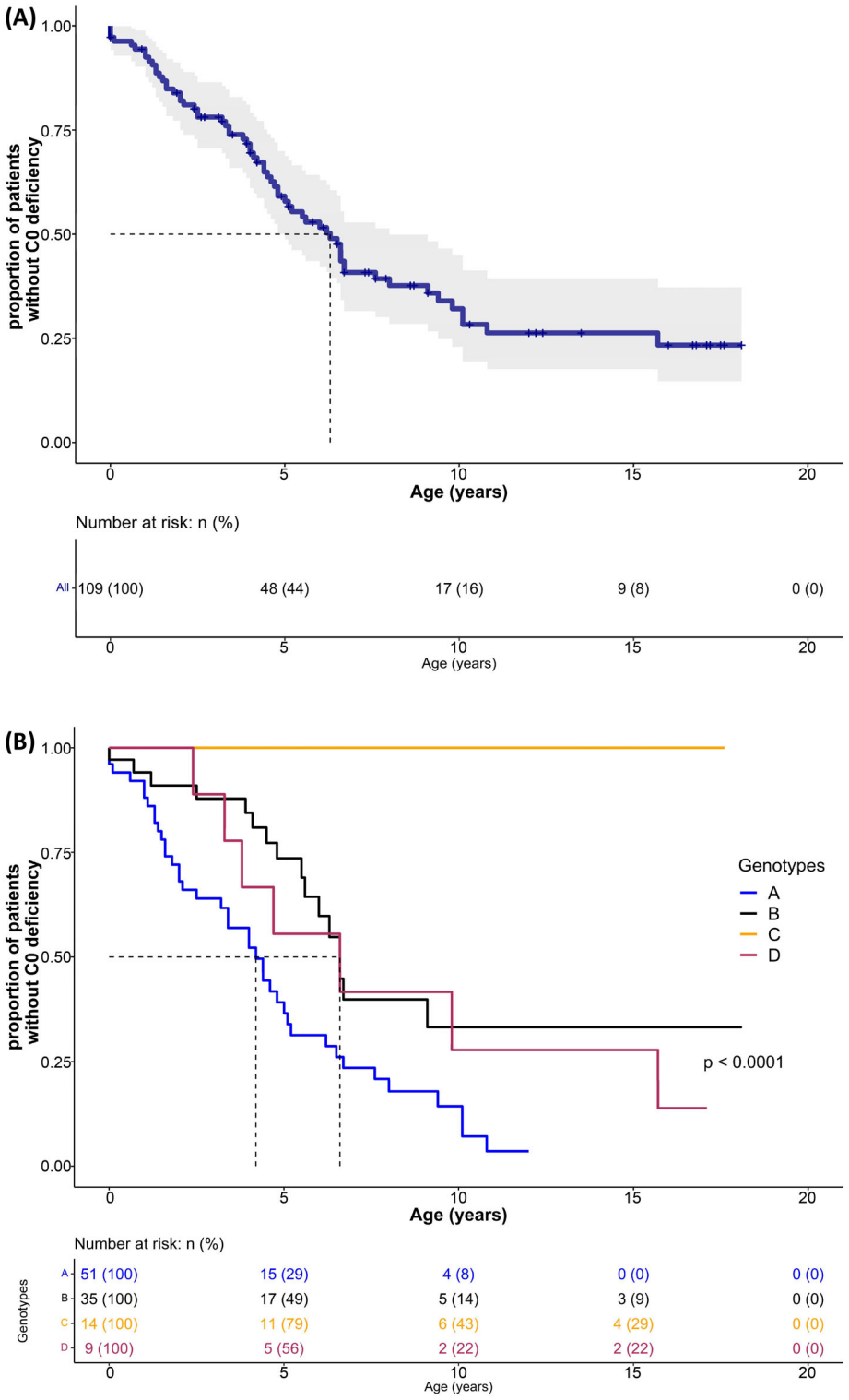

FIGURE 1 Kaplan–Meier curves depicting the distribution of age at first diagnosis of secondary carnitine deficiency in medium-chain acyl-CoA dehydrogenase deficiency patients. (A) All patients of the cohort. The confidence interval is shaded in gray. (B) Patients stratified by *ACADM* genotype: homozygous for c.985A>G (genotype A), compound heterozygous for c.985A>G but not harboring variant c.199C>T (genotype B), compound heterozygous for c.199C>T (genotype C) and pathogenic variants other than c.985A>G or c.199C>T (genotype D). Differences between the genotypes were analyzed using log-rank test. Median ages (years) at diagnosis of secondary carnitine deficiency (SCD) are indicated as dashed lines. Tables in the bottom parts of the figure give the numbers (percentages) of patients at risk to develop SCD. Patients were censored when SCD was diagnosed or at last follow-up. [Colour figure can be viewed at wileyonlinelibrary.com]

2.3 | Statistical analyses

For the statistical analyses MCADD patients were categorized according to their genotype into four groups (see below).

Age was categorized into four groups according to the numbers of acylcarnitine measurements: younger than 1 year, 1 year to below 3 years, 3 years to below 6 years, 6 years and older. The proportion of measurements within each age group are balanced. The distribution of biochemical parameters grouped by age and genotype are presented as boxplots.

Time to first diagnosis of SCD was investigated by Kaplan–Meier curves, which were compared by log-rank tests. The impact of genotype on the risk of developing SCD was analyzed using Cox proportional hazard modeling with relative risks (RR) representing the change in the risk of SCD in a genotype compared to the reference genotype A.

TABLE 2 Biochemical parameters of medium-chain acyl-CoA-dehydrogenase deficiency patients in different age groups.

			Biochemical parameters				
Genotype	Age group (years)	Number of observations	Ref.	C0 (μmol/L) (8.9–93.3) Mean ± SE	C8 (μmol/L) (<0.26)	C8/C2 (<0.9)	C8/C10 (<1.4)
A ($n = 40$)	< 1	47		22.39 ± 9.80	5.98 ± 3.87	1.08 ± 0.57	10.88 ± 2.52
	1 – < 3	59		15.21 ± 8.17	3.78 ± 2.20	1.21 ± 0.61	11.57 ± 2.62
	3 – < 6	32		14.06 ± 7.48	2.83 ± 1.90	1.11 ± 0.50	10.71 ± 2.81
	≥ 6	20		15.02 ± 9.33	3.63 ± 2.80	1.30 ± 0.59	11.47 ± 4.74
B ($n = 33$)	< 1	38		24.58 ± 7.81	3.69 ± 3.21	0.63 ± 0.56	7.60 ± 4.55
	1 – < 3	37		21.65 ± 9.00	2.86 ± 2.37	0.87 ± 0.76	8.01 ± 5.06
	3 – < 6	47		17.47 ± 10.41	1.70 ± 1.66	0.64 ± 0.57	6.98 ± 4.70
	≥ 6	40		31.65 ± 14.94	0.90 ± 0.77	0.23 ± 0.27	2.54 ± 2.02
C ($n = 14$)	< 1	24		25.88 ± 10.26	1.20 ± 0.65	0.18 ± 0.13	1.96 ± 0.56
	1 – < 3	25		27.13 ± 8.34	0.76 ± 0.58	0.12 ± 0.06	1.88 ± 0.43
	3 – < 6	27		30.19 ± 9.88	0.75 ± 0.47	0.11 ± 0.08	1.59 ± 0.56
	≥ 6	56		26.38 ± 7.87	0.97 ± 1.01	0.19 ± 0.18	2.41 ± 1.03
D ($n = 9$)	< 1	7		22.72 ± 11.58	3.66 ± 3.30	1.30 ± 0.51	8.80 ± 3.66
	1 – < 3	5		10.71 ± 2.16	2.31 ± 1.37	1.06 ± 0.46	9.68 ± 5.44
	3 – < 6	9		14.62 ± 5.71	5.10 ± 3.79	1.39 ± 0.79	10.73 ± 5.57
	≥ 6	19		14.51 ± 4.46	2.80 ± 2.31	0.93 ± 0.50	8.32 ± 4.30

Abbreviations: C0, free carnitine; C8, octanoylcarnitine; C2, acetylcarnitine; C10, decanoylcarnitine; Ref., reference value; SE, standard error.
Note: Patients are grouped according to the *ACADM* genotype as follows: homozygous for c.985A>G (genotype A), compound heterozygous for c.985A>G but not harboring variant c.199C>T (genotype B), compound heterozygous for c.199C>T (genotype C) and pathogenic variants other than c.985A>G or c.199C>T (genotype D). Data are included until the occurrence of free carnitine deficiency.

The data structure in this study is based on repeated measurements of a single individual. Thus, multilevel modeling for repeated measures with interactions was employed to investigate the development of different biochemical parameters over age within genotypes. Data was selected only until the age of first diagnosis of SCD for those who had a deficiency and until the last observation time for patients without a deficiency.

Statistical significance was evaluated at a significance level of 0.05 in all statistical tests (i.e., $p < 0.05$).

All statistical analyses were performed by statisticians (U.B., M.H.) using R version 3.6.2 (http://www.r-project.org).

3 | RESULTS

3.1 | Patient features and descriptive analysis

The study cohort of 109 MCADD patients comprised 51 (46.8%) males and 58 (53.2%) females. The median observation time for all patients was 10.24 years (95% CI: 7.93,12.21) ranging from 0.01 years up to 26.14 years with more than 25% of the patients being observed beyond the age of 15 years (Supplementary Figure 1).

Molecular testing was available in all patients and showed two pathogenic variations in all but two patients, in whom only the frequent variant c.985A>G was identified in a heterozygous state. In both patients, diagnosis of MCADD was confirmed by enzymatic testing and revealed no detectable activity in fibroblasts in one patient, and a markedly reduced residual activity of 0.34 mU/mg protein (11%) in leukocytes in the other.

The *ACADM* variants identified in the patients are listed in Table 1. According to their genotype, MCADD patients were categorized as follows: patients homozygous for the prevalent variation c.985A>G (genotype A) ($n = 51$; 46.8%); patients compound heterozygous for c.985A>G but not harboring variant c.199C>T (genotype B), ($n = 35$; 32.1%); patients compound heterozygous for c.199C>T (genotype C) ($n = 14$; 12.8%); patients carrying pathogenic variants other than c.985A>G or c.199C>T (genotype D) ($n = 9$; 8.3%). Eight novel variations were identified: 2 nonsense, 1 missense, 2 small deletion/insertions, and 3 splice site variants. Except for two variants, all novel variants are predicted to be likely pathogenic (for pathogenicity scores see Supplementary Table 1).

3.2 | Secondary carnitine deficiency in MCADD patients

SCD was found in 63 of 109 MCADD patients with a median age at first diagnosis of 6.3 years (95% CI 5, 8) (Figure 1A). The lowest C0 concentration encountered was 2.7 μmol/L in a patient with genotype A. The risk of SCD differed significantly between genotypes ($p < 0.001$) being highest in patients with genotype A (Figure 1B). At the age of 4.2 years (95% CI 3.2, 5.2), 50% of patients with this

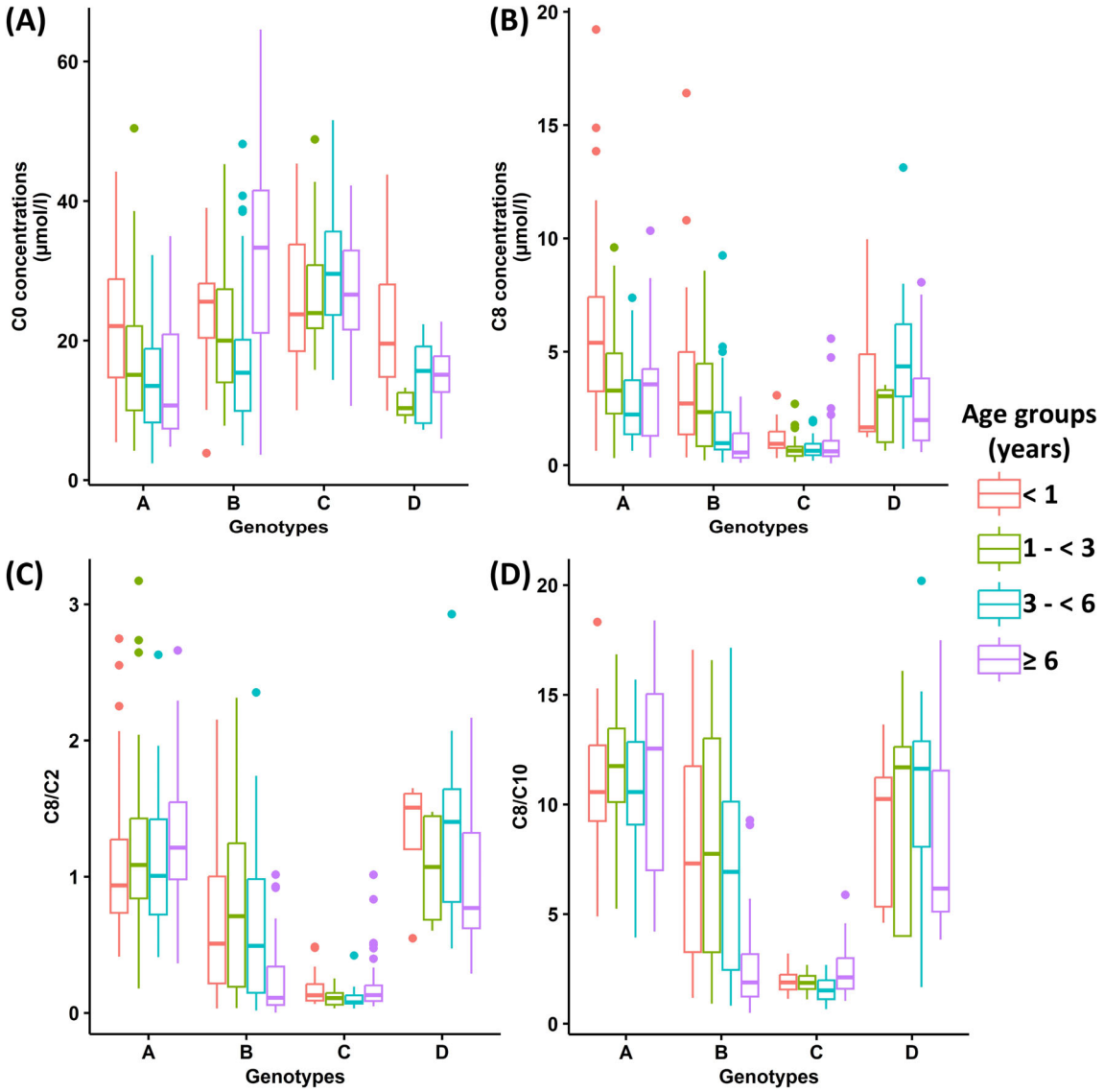

FIGURE 2 Biochemical parameters of medium-chain acyl-CoA dehydrogenase deficiency patients grouped according to age and genotype. Data of patients are included until the occurrence of secondary carnitine deficiency. Patients were stratified by *ACADM* genotype: homozygous for c.985A>G (genotype A), compound heterozygous for c.985A>G but not harboring variant c.199C>T (genotype B), compound heterozygous for c.199C>T (genotype C) and pathogenic variants other than c.985A>G or c.199C>T (genotype D). Boxplots display the 1st and the 3rd quartile as upper and lower boundaries of the boxes with the horizontal line within the boxes representing the median. The vertical lines above and below the boxes indicate the range, outlying parameters are shown as dots. (A) free carnitine (C0), (B) octanoylcarnitine (C8), (C) diagnostic ratio C8/acetylcarnitine (C2), (D) diagnostic ratio C8/decanoylcarnitine (C10). [Colour figure can be viewed at wileyonlinelibrary.com]

genotype had already been diagnosed with SCD, whereas no deficiency was found among patients with genotype C. The median age at first diagnosis of SCD was 6.6 years for genotypes B (95% CI 5.6, –) and D (95% CI 3.8, –).

Stratified by genotype, 63.5% of children with genotype A were diagnosed with SCD before the age of 5 years. This proportion increased to 85.7% by the age of 10 years. Within genotype B, 26.4% showed SCD until the age of 5 years, and 66.8% until the age of 10 years. Accordingly, 44.4% and 72.3% of children with genotype D had a deficiency until the age of 5 and 10 years, respectively.

Cox proportional hazard modeling showed that the relative risk of developing SCD was 60% lower among patients with genotype B compared to patients with genotype A (RR 0.40, $p = 0.002$). Genotypes C had no risk of developing SCD, and genotypes D about half of the risk compared to genotypes A (RR 0.53, $p = 0.123$). However, the effect was not significant due to the small number of observations.

3.3 | Supplementation of carnitine

Patients with SCD were recommended to take carnitine supplementation. A dose adjustment with increasing weight was not required in the majority of patients, and some patients maintained normal C0

TABLE 3 Effect of genotype and age genotype interaction on biochemical parameters of medium-chain acyl-CoA dehydrogenase deficiency patients.

	C0	C8	C8/C2	C8/C10
	Effect (SE) (μmol/L)			
Genotype A	Ref. group	Ref. group	Ref. group	Ref. group
Genotype B	3.32 (2.10)	−2.03 (0.44)*	−0.435 (0.113)*	−3.91 (0.822)*
Genotype C	8.28 (2.43)*	−4.15 (0.49)*	−0.953 (0.126)*	8.28 (2.43)*
Genotype D	−2.09 (3.58)	−0.46 (0.75)	0.297 (0.194)	−0.2 (1.36)
	Effect (SE) (μmol/L × year)			
Age - genotype A	−1.24 (0.29)*	−0.28 (0.07)*	0.030 (0.016)	0.111 (0.083)
	Ref. group	Ref. group	Ref. group	Ref. group
Age - genotype B	1.29 (0.35)*	0.081 (0.087)	−0.047 (0.02)*	−0.22 (0.102)*
Age - genotype C	1.28 (0.33)*	0.285 (0.082)*	−0.025 (0.018)	−0.06 (0.093)
Age - genotype D	0.90 (0.46)	0.158 (0.109)	−0.043 (0.025)	−0.033 (0.137)

Abbreviations: C0, free carnitine; C8, octanoylcarnitine; C2, acetylcarnitine; C10, decanoylcarnitine; Ref. group, reference group; SE, standard error.

Note: Patients were stratified by *ACADM* genotype: homozygous for c.985A>G (genotype A), compound heterozygous for c.985A>G but not harboring variant c.199C>T (genotype B), compound heterozygous for c.199C>T (genotype C) and pathogenic variants other than c.985A>G or c.199C>T (genotype D). Data are included until the occurrence of carnitine deficiency for those who had a deficiency and until the last observation time for patients without a deficiency.

*$p < 0.05$. A multilevel modeling for repeated measures was applied.

concentrations with dosages as low as 10 mg/kg body weight. Twenty-one patients tried to withdraw therapy after some years of supplementation. Thirteen (61.9%) of them had to restart supplementation due to a recurrence of the SCD. Two patients out of 63 decided against carnitine supplementation.

3.4 | Effect of age on biochemical parameters

The mean concentrations and standard errors of the biochemical parameters C0, C8, and the diagnostic ratios C8/C2 and C8/C10 are listed in Table 2. The distribution of analyte concentrations and ratios are depicted as boxplots in Figure 2. Biochemical parameters were included until the occurrence of SCD to reflect the natural course of the parameters and to avoid effects of carnitine supplementation on biochemical parameters.

Overall, in genotypes A, B, and D, patients above 1 year of age showed lower concentrations of C0 and C8 as compared to the first year of life. Patients with genotype B without SCD above the age of 6 years revealed high concentrations of C0 and low concentrations of C8 indicating mild biochemical phenotypes. Patients with genotype C displayed consistently normal concentrations of C0 throughout the different age groups. Concentrations of C8 and levels of diagnostic ratios were apparently lower in this genotype.

According to the multilevel model for repeated measurements, C0 concentrations developed differently within the genotypes and showed a significant interaction effect of genotype and age (Table 3). In genotype A, with the increase of 1 year in age the concentration of C0 decreased on average by 1.24 μmol/L. This differed significantly for genotypes B and C, where the age dependency was leveled out by the interaction effect to around zero. Patients with genotype C showed C0 concentrations significantly higher by 8.28 μmol/L compared to genotype A as reference group. Concentrations of C8 were significantly lower in genotype B and C compared to genotype A. In patients with genotype A, C8 concentrations decreased with age by 0.28 μmol/L per year. The age genotype interaction effect was not significantly different in patients with genotype B, but significantly differed in genotype C patients, where the age dependency was leveled out by the interaction effect to around zero. The ratios of C8/C2 and C8/C10 were significantly lower in genotype B and C patients compared to genotype A patients. In genotype B patients the ratio decreased significantly with age by 0.047 and 0.22 per year, respectively.

For the nine patients of genotype D we could not find any significant difference to the reference genotype A in any of the four biochemical parameters.

4 | DISCUSSION

In this study, we describe the occurrence of SCD in MCADD depending on age and genotype. Furthermore, we report on differences in the biochemical phenotype of MCADD related to age and genotype.

In our cohort, the risk of SCD to occur was strongly associated with the genotype (Figure 1B). SCD was found early and most frequently in patients homozygous for the prevalent variation c.985A>G (genotype A). Patients compound heterozygous for c.985A>G (genotype B) and patients carrying variations other than c.985A>G and c.199C>T (genotype D) developed SCD less frequently. No patient carrying the variant c.199C>T (genotype C) was found to have SCD.

Genotypes A and D mainly comprise pathogenic *ACADM* genotypes. Homozygosity for the prevalent variation c.985A>G accounted

for 80% of clinically ascertained MCADD patients in the pre-screening era and has been described to be a risk factor for both fatal outcome despite diagnosis by NBS and fatal neonatal decompensation before NBS results were reported.[4,24–27] Genotype D comprises variants which have been reported in symptomatic patients or result in premature termination codons.[5,28,29] In contrast, the heterogeneous genotype B includes different variations in a compound heterozygous state with the pathogenic variant c.985A>G. These variants comprise deleterious variants as well as missense variants previously described only in NBS cohorts, and novel variants. The risk of SCD in this genotype was 2.5-times lower as compared to genotype A.

Severe genotypes, c.985A>G homozygotes and compound heterozygotes in particular, have been described to be associated with pronounced biochemical phenotypes and to accumulate higher concentrations of C8 as compared to other genotypes.[8,20,30] Acylcarnitines are formed to bind acyl-CoA derivatives and allow for the excretion of accumulating metabolites. The increased formation and urinary excretion of acylcarnitines, however, results in a concomitant loss of C0. Thus, the observation of SCD to occur frequently in genotypes with high concentrations of C8 is a coherent finding. Accordingly, genotype C is associated with a consistently mild biochemical phenotype and significantly lower concentrations of C8. No patient with this genotype was found to have SCD. Our data add to the evidence of c.199C>T being a functionally mild variation. Harboring this variation on one allele provides sufficient enzyme function to mitigate acylcarnitine accumulation and urinary loss. This holds true even if the severe variant c.985A>G is found on the second allele. In line with these findings, the urinary excretion of the characteristic metabolite hexanoylglycine is lower in patients with mild MCADD.[31,32] Of note, approximately one third of patients with genotype B did not show SCD at the age of 10 years and beyond. These patients carry rare variants on their second allele, that are presumably equally mild as the variant c.199C>T. This assumption is supported by the finding of apparently milder biochemical parameters comparable to genotype C in this age group (Figure 2).

Biochemical parameters at the age of NBS and confirmation testing have been described on many occasions,[8,27,30,31,33] but little is known on their natural history over time.[20] We analyzed the long-term development of biochemical parameters in our MCADD patients stratified by genotype and found a decrease of C0 and C8 concentrations in genotypes A, B, and D after the first year of life (Figure 2, Table 2). In genotype A, a decrease of C0 and C8 concentrations by 1.24 and 0.29 µmol/L per year of age was found applying a multilevel model for repeated measurements. In genotype C, C8 concentrations were considerably lower with C0 concentrations being consistently normal and significantly higher by 8.28 µmol/L as compared to reference genotype A. The markedly mild biochemical phenotype of genotype C is clearly depicted in the boxplots of C8/C2 and C8/C10, analyte ratios used to identify MCADD patients in NBS and to discriminate patients from healthy individuals in NBS.[10,11,27,33] Our data partially confirm the observations of Anderson and colleagues, who described a moderate, but significant decrease of C0 with age in MCADD patients but found no changes in C8. In this study, however, all patients received carnitine supplementation and different genotypes were not considered.[20] The marked decrease of C8 concentrations after the first year of life in our cohort is explained in parts by the fact that the data set below 1 year of age includes acylcarnitines sampled during the catabolic newborn period. The biochemical parameters of fatty acid oxidation defects have been shown to be most pronounced during the first days of life, when the newborns' energy demand relies on the beta-oxidation pathway, and decrease afterwards with increasing adaptation and adequate feeding.[7,33,34] In addition, significant age dependent variations with higher concentrations of C0 and other acylcarnitines involved in fatty acid oxidation (C2, C16, and C18:1) have been reported to occur also in healthy individuals during the first year of life.[35]

SCD is a frequent finding in both symptomatic[36,37] and asymptomatic[19,23] patients. Nevertheless, data on carnitine supplementation practice of metabolic clinics are limited and variable.[21,22] Some clinics do not recommend carnitine supplementation in MCADD,[21,22] some only for patients with SCD,[23] and some prescribe carnitine routinely for all MCADD patients.[20] In addition, there is controversy as to whether MCADD patients benefit from carnitine supplementation. Clinical evidence for a beneficial effect such as enhanced muscle strength or endurance is lacking.[19] However, even though muscular symptoms are not predominant in MCADD, they have not been systematically assessed in patient cohorts.

The major clinical manifestation of MCADD, which led to the implementation of NBS for this condition, is life-threatening metabolic decompensation and death during catabolic stress such as febrile illness with low food intake during infancy or prolonged fasting during anesthesia and surgery later in life. The role of accumulating toxic metabolites in the pathophysiology of MCADD is not yet fully elucidated, but their impact is conceivable and has been attributed to the clinical observation that acute MCADD symptoms such as lethargy, encephalopathy, and hepatopathy are not always associated with hypoglycemia or hyperammonemia and often persist for a longer period even after the correction of catabolism.[38] This hypothesis is supported by growing experimental evidence pointing to a disruption of mitochondrial functions in brain, liver, and skeletal muscle caused by accumulating toxic medium-chain fatty acids and acylcarnitines.[39] Additionally, carnitine depletion is supposed to have a negative impact on other metabolic pathways by the sequestration of CoA. Carnitine depletion might be exacerbated by additive effects such as the exposure to carnitine wasting medications like pivalate prodrugs or valproic acid and lead to severe adverse events.[40] Fatal liver failure was reported in a child with undiagnosed MCADD on valproate therapy.[41]

Our rationale behind treating SCD was to provide a sufficient supply for the excretion of presumably harmful metabolites and to preserve metabolic pathways affected by carnitine depletion. In view of the fact, that carnitine is a natural compound, safe, and well tolerated with very few side effects,[12] the assumed benefit outweighed the potential harm of long-term medication according to our estimation. The monitoring of C0 concentrations and carnitine supplementation in case of SCD were part of the routine clinical follow-up and the prophylactic therapeutic approach we set up for our MCADD patients

with the implementation of NBS. The lack of evidence, reasons in favor and against carnitine supplementations were discussed with the parents. Notably, 61 of 63 parents decided on carnitine supplementation for their asymptomatic offspring. However, coming from our data, follow-up on C0 is redundant in patients carrying the variant c.199C>T. It can most probably also be stopped in patients with other genotypes displaying constantly mild biochemical phenotypes and normal C0 concentrations (see genotype B). This strategy is, of course, not without alternatives and might apply for clinics that decide to monitor C0 and to treat SCD in their patients. Given the lack of reliable evidence, metabolic physicians together with the patients and their families may also decide not to monitor C0 at all, or to check for and treat SCD only in patients with muscular symptoms.

In our cohort, low doses of carnitine starting at 20 to 40 mg/kg body weight daily were sufficient to maintain C0 concentrations within the lower reference range. An adjustment of the dose with gaining weight was rarely necessary allowing to lengthen intervals between laboratory controls. This dosage is considerably lower than recommended in other IEM such as glutaric acidemia type 1[14] or MCADD reported by other authors.[20,23] One might hypothesize that using a low dose of carnitine might reduce the risk of the formation of trimethylamine-N-oxide (TMAO) in relevant amounts. TMAO is a metabolite that is formed by intestinal bacteria from dietary and supplemented carnitine and has been described to be associated with cardiovascular disease and thrombosis in a dose-dependent manner.[42,43] Whether long-term carnitine supplementation in IEM confers an increased risk for atherosclerosis is unknown to date. Concentrations of TMAO could not be determined in our MCADD patients.

In conclusion, this study shows that SCD mostly occurs in childhood and is mainly found in patients with pathogenic genotypes displaying high concentrations of presumably toxic acylcarnitines. Individuals carrying the mild variation c.199C>T are spared. A low-dose supplementation of carnitine is sufficient to maintain C0 concentrations within the normal range. However, reliable data on the clinical benefit of carnitine supplementation remain lacking. Future studies, at the best prospective and multi-center studies, will need to evaluate the potential clinical benefit of carnitine supplementation on acute and chronic manifestations of MCADD. Since SCD in fatty oxidation defects is often related to the occurrence of muscular or cardiac symptoms, these symptoms need to be carefully assessed in routine follow-up of MCADD patients. Eventually, one needs to keep in mind, that the absence of evidence does not necessarily imply an absence of effectiveness. Only few clinical practice in the treatment of rare metabolic diseases is based on high-level reliable evidence from controlled clinical trials but rather reflects clinical experience in conjunction with pathophysiological considerations. Thus, for the time being, we propose that metabolic physicians comprehensively inform MCADD patients and their families on the existing uncertainties and controversy about SCD and the necessity of its treatment and decide on a treatment strategy according to their preferences.

AUTHOR CONTRIBUTIONS

Katharina J. Weiss: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Ursula Berger:** Formal analysis, Writing – review & editing. **Maliha Haider:** Formal analysis, Writing – review & editing. **Matias Wagner:** Data curation, Writing – review & editing. **E. M. Charlotte Märtner:** Data curation, Writing – review & editing. **Stephanie Regenauer-Vandewiele:** Data curation, Writing – review & editing. **Amelie Lotz-Havla:** Data curation, Writing – review & editing. **Elfriede Schuhmann:** Investigation. **Wulf Röschinger:** Data curation, Investigation, Writing – review & editing. **Esther M. Maier:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

We will supply our data and materials upon request.

ETHICS STATEMENT

The study was conducted in accordance with the World Medical Association Declaration of Helsinki. Informed consent was waived by the local ethics committee (Ludwig-Maximilians-Universität München, Munich, Germany) for the review of medical records and biochemical data in a retrospective and anonymized way (approval no 18-734).

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