MCAD deficiency
To be, or not to be at risk

Catharina Maria Louise Volker-Touw

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die in 1965 als een van de eersten een ziektebeeld beschreef,
dat later de naam 'MCAD deficiëntie' kreeg.

Paranimfen

Andrea Schreuder

Karen Anjema

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACAD	Acyl-CoA dehydrogenase
ACADM	Gene encoding medium-chain acyl-CoA dehydrogenase
ACADVL	Gene encoding very long-chain acyl-CoA dehydrogenase
ANT	Adenine nucleotide transporter
AT	Anaerobic threshold
ATP	Adenosine triphosphate
C_0-carnitine	Free carnitine
C_2-carnitine	Acetylcarnitine
C_4-CoA	Butyryl-CoA
C_6-CoA	Hexanoyl-CoA
C_8-CoA	Octanoyl-CoA
C_8-carnitine	Octanoylcarnitine
C_8/C_2	Ratio between octanoylcarnitine and acetylcarnitine
C_8/C_{10}	Ratio between octanoylcarnitine and decanoylcarnitine concentrations
C_{10}-carnitine	Decanoylcarnitine
$C_{10:1}$-carnitine	Decenoylcarnitine
$C_{14:1}$-carnitine	Tetradecenoylcarnitine
$C_{14:1}/C_{16}$	Ratio between tetradecenoylcarnitine and palmitoylcarnitine
C_{16}-carnitine	Palmitoylcarnitine
C_{16}-CoA	Palmitoyl-CoA
CAT	Catalase
CoASH	Free CoA
CPET	Cardiopulmonary exercise test
CPT	Carnitine palmitoyltransferase
DHA	Docosahexaenoic acid
ETC	Electron transport chain
ETF	Electron transfer flavoprotein
EMA	Ethylmalonic acid
ETF-QO	ETF:ubiquinone oxidoreductase
FAD	Flavin adenine dinucleotide
FFA	Free fatty acids
GPx	Glutathione peroxidase
GSH	Glutathione, reduced form
HADH	3-hydroxyacyl-CoA dehydrogenase
KB	Ketone bodies
KO	Knock-out

LCAD	Long-chain acyl-CoA dehydrogenase
L-carnitine	Free carnitine for supplementation
LCHAD	Long-chain 3-hydroxyacyl-CoA dehydrogenase
MADD	Multiple acyl-CoA dehydrogenase deficiency
MCAD	Medium-chain acyl-CoA dehydrogenase
mFAO	Mitochondrial fatty acid oxidation
MRS	Magnetic resonance spectroscopy
mTORC	Mammalian target of rapamycin
MTP	Mitochondrial trifunctional protein
NAD	Nicotinamide adenine dinucleotide
NBS	Newborn screening
OXPHOS	Oxidative phosphorylation
PAH	Physical Activity History
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PDHc	Pyruvate dehydrogenase complex
Pi	Inorganic phosphate
PPA	Phenylpropionic acid
PP-CoA	Phenylpropionyl-CoA
PP-glycine	Phenylpropionylglycine
RS	Reactive species
SCAD	Short-chain acyl-CoA dehydrogenase
SCHAD	Short-chain 3-hydroxyacyl-CoA dehydrogenase
SOD	Superoxide dismutase
TBA-RS	Thiobarbituric acid reactive species
TCA cycle	Tricarboxylic acid (citric acid) cycle
TKB	Total ketone body concentration
τPi	Pi recovery
TR	Repetition time
UQ	Ubiquinone
VLCAD	Very long-chain acyl-CoA dehydrogenase
VO_2 max	Maximal oxidative capacity
W_{max}	Maximal workload
WT	Wild type

Chapter 1

General introduction & outline of the thesis

MITOCHONDRIAL FATTY ACID OXIDATION

Mitochondria are organelles that serve to generate the energy that is used to sustain cellular processes. Energy is provided in the form of adenosine triphosphate (ATP). Oxidative phosphorylation (OXPHOS), mitochondrial fatty acid oxidation (mFAO) and the tricarboxylic acid (TCA) cycle function together in the generation of ATP.

Mitochondrial FAO is a complex cyclic pathway serving the stepwise shortening of saturated straight-chain fatty acids (Figure 1). The pathway includes uptake of fatty acids by cells; conversion of these fatty acids into acyl-CoA esters in the cytosol, and subsequently into acylcarnitines; transport of the acylcarnitines over the mitochondrial membrane; re-esterification into acyl-CoA esters; and the spiral of mitochondrial β-oxidation that shortens acyl-CoA esters stepwise and generates both reducing equivalents (NADH and $FADH_2$) and acetyl-CoA. Mitochondrial β-oxidation consists of four steps, catalyzed by 1) the FAD-dependent acyl-CoA dehydrogenases (ACADs), 2) 2-enoyl-CoA hydratases, 3) the NAD^+-dependent L-3-hydroxyacyl-CoA dehydrogenases, and 4) 3-ketoacyl-CoA thiolases. During each cycle the acyl-CoA ester that entered the cycle is shortened by two carbon atoms, which are released as acetyl-CoA. The number of cycles that an acyl-CoA ester goes through the mitochondrial β-oxidation depends on its chain-length [1].

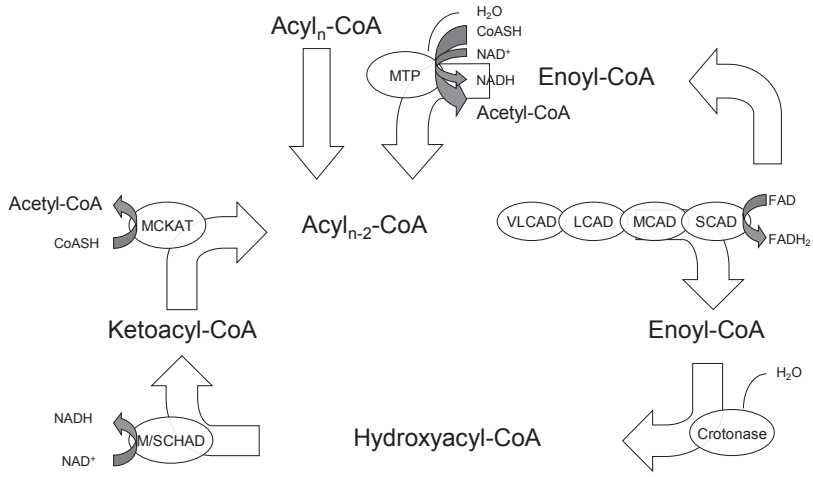

Figure 1. Schematic overview of mitochondrial fatty acid oxidation. CoASH: Free CoA; FAD: Flavin Adenine Dinucleotide; NAD: Nicotinamide adenine dinucleotide; MCAD: Medium-chain acyl-coA dehydrogenase; MCKAT: Medium-chain ketoacyl-CoA thiolase; MSCHAD: Medium-chain hydroxyacyl-CoA dehydrogenase; MTP: Mitochondrial trifunctional protein; LCAD: Long-chain acyl-CoA dehydrogenase; LCHAD: Long-chain hydroxyacyl-CoA dehydrogenase; SCAD: Short-chain acyl-CoA dehydrogenase; SCHAD: Short-chain acyl-CoA dehydrogenase; VLCAD: Very long-chain acyl-CoA dehydrogenase.

Acetyl-CoA can either be oxidized in the TCA cycle to generate $FADH_2$ and NADH, but can also be converted into the ketone bodies (KB) β-hydroxybutyrate and acetoacetate in liver [1] (Figure 2). KB have a glucose-sparing effect by reducing glucose utilization particularly in brain, possibly by inhibition of glycolysis due to citrate formation [2].

The NADH that is formed in the mFAO and TCA cycle can enter OXPHOS via complex I of the electron transport chain (ETC). The $FADH_2$ that is generated in mFAO and TCA cycle can enter OXPHOS via electron transfer flavoprotein (ETF) and ETF-CoQ oxidoreductase (ETF-QO) [1]. In OXPHOS, the five complexes of the ETC aid in the generation of ATP in complex V (F_1F_0-ATPase) by transferring protons across the inner mitochondrial membrane. The generated ATP can subsequently be transported to other organs and tissues to serve as a source of energy [3]. Mitochondrial FAO and OXPHOS are thus closely linked.

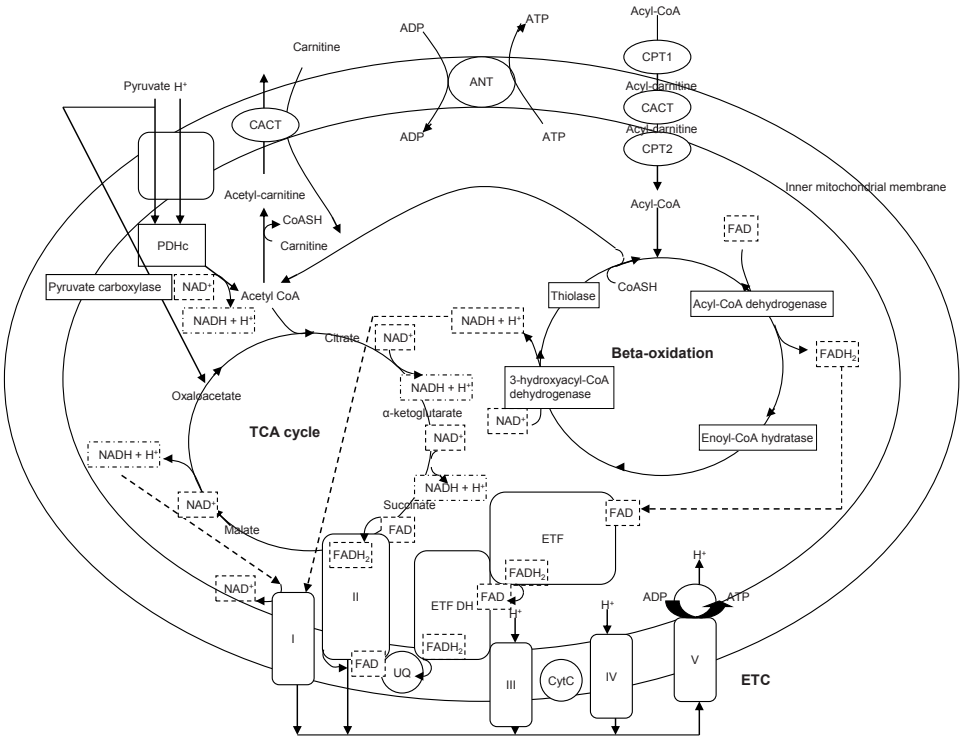

Figure 2. Simplified version of the metabolic pathways in the mitochondrion. ANT: Adenine nucleotide transporter; CPT: Carnitine palmitoyltranferase; Cytc: Cytochrome *c*; ETF(-DH): Elelctron transfer flavoprotein (dehydrogenase); FAD: Flavin Adenine Dinucleotide; NAD: Nicotinamide adenine dinucleotide; PDHc: Pyruvate dehydrogenase complex; UQ: ubiquinone.

Acyl-CoA dehydrogenases

The group of ACADs catalyzes the first step in mitochondrial β-oxidation. During this FAD-dependent reaction, an acyl-CoA ester is oxidized into an enoyl-CoA ester with concomitant reduction of FAD into $FADH_2$ (Figure 1). Four types of ACADs exist, at least as involved in mFAO, each handling acyl-CoA esters with a different chain-length: short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD) and very-long chain acyl-CoA dehydrogenase (VLCAD), respectively. In humans, SCAD, MCAD, and VLCAD are known for their role in the oxidation of saturated fatty acids, whereas LCAD is involved in oxidation of branched-chain fatty acids [4]. Unlike the other ACADs, LCAD is highly expressed in lung [4]. In mice, all four ACADs are known to be involved in the oxidation of saturated fatty acids. Defects in each of the ACADs have been described.

Medium-chain acyl-CoA dehydrogenase deficiency

The MCAD enzyme serves the first step in the mitochondrial β-oxidation of medium-chain length acyl-CoA esters (in humans specifically 6-12 carbon atoms, but this is species-specific). MCAD deficiency is the most common defect in mFAO. Patients typically present after a period of increased catabolic stress (e.g. prolonged fasting, or intercurrent illness) with symptoms that correspond to a hypoketotic hypoglycemia. Examples are lethargy, convulsions, but also coma and sudden infant death. Both the age of first clinical presentation, and the phenotypical spectrum vary considerably, ranging from death in the neonatal phase to remaining asymptomatic throughout life [1,5]. The reason for this clinical variation is currently unknown, as a genotype-phenotype correlation has thus far not been found and the pathophysiological mechanism behind the clinical symptoms has not been unraveled.

Besides acute clinical presentations with symptoms associated with a hypoketotic hypoglycemia, chronic complaints such as muscle pain, muscle fatigue, and a lowered exercise tolerance are also frequently reported, predominantly in the population of adult patients [6–8].

After diagnosis, the prognosis is excellent when prolonged fasting is avoided. Guidelines on the maximum duration of fasting with age have been developed [9]. During intercurrent illness an emergency regimen is advised, including upregulation of the feeding frequency and preventive admission to the hospital for glucose infusion.

Metabolites accumulating in MCAD deficiency

Medium-chain acylcarnitines typically accumulate in MCAD deficiency. Medium-chain acyl-CoA esters accumulate due to impaired MCAD enzyme functioning, and are transferred to free carnitine (C_0-carnitine) for subsequent excretion as acylcarnitines in blood and urine [10]. As a result of this process, elevated concentrations of medium-chain length acylcarnitines, in humans in particular octanoylcarnitine (C_8-carnitine), can be identified in plasma. The development of tandem mass spectrometry (MS/MS) has enabled quantitative identification of acylcarnitines, and this technique is currently used in diagnosing patients with mFAO defects [11]. Accumulation of acylcarnitines in mFAO defects can lead to secondary low C_0-carnitine concentrations in plasma [10].

Besides conversion to acylcarnitines, medium-chain acyl-CoA esters can undergo omega-oxidation, resulting in increased excretion as medium-chain dicarboxylic acids in the urine [12]. *N*-acylglycine conjugates of these dicarboxylic acids such as *N*-hexanoylglycine, *N*-suberylglycine and phenylpropionylglycine are typically found in the urine of MCAD deficient patients [12].

Newborn screening for MCAD deficiency

MCAD deficiency meets the criteria described by Wilson and Jungner, and was therefore included in the nationwide newborn screening program (NBS) in The Netherlands in 2007 [9,23]. Before NBS, only patients who presented clinically and their family members were diagnosed [5]. Since NBS, diagnosis is generally made in asymptomatic newborns based on abnormal metabolite patterns (i.e. elevated C_8-carnitine concentrations, and an elevated C_8/C_{10} ratio) in bloodspots [5,24].

In case of a positive NBS result for MCAD deficiency, MCAD enzyme analysis and molecular analysis of the *ACADM* gene are performed to confirm the diagnosis. Before NBS, approximately 80% of the patients was homozygous for the most common c.985A>G missense mutation in the *ACADM* gene (gene encoding MCAD), whereas the majority of the remaining patients carried one copy of this mutation [25]. Since NBS, the c.985A>G frequency has decreased, as new *ACADM* genotypes (i.e. variant *ACADM* genotypes) have been identified that have never been seen before in the population of clinically presenting patients [26–30]. Since these variant *ACADM* genotypes have not been seen before, their clinical relevance is questionable.

Mouse model for MCAD deficiency

Developments in genetic knowledge and genetic modification have in the past decades enabled the development of genetically modified mouse models for various metabolic diseases. For the group of mFAO defects several mouse models have been developed, i.e. for VLCAD deficiency, LCAD deficiency, MCAD deficiency, and SCAD deficiency [31–35].

In all of these mouse models the predominant phenotype is cold intolerance. Additionally, neonatal mortality, as can be seen in patients, is reported in the LCAD knock-out (KO) and MCAD-KO mouse models [34]. In the MCAD-KO mouse, hypoglycemia upon prolonged fasting alone has not been observed. Acylcarnitine profiles corresponding to the defect are seen in all mouse models; however, these patterns differ slightly from the human situation. This difference results from the role of LCAD in dehydrogenation of acyl-CoAs with a length of 6 to 20 carbon atoms in rodents, whereas in humans, LCAD predominantly participates in branched-chain fatty acid oxidation [36]. As a result, rodents may via LCAD be able to partially compensate for deficiencies in the dehydrogenation of long- and medium-chain acyl-CoAs [36], i.e. in the case of VLCAD-KO and MCAD-KO mice. Studies in mouse models for mFAO defects may aid in unraveling the pathophysiology behind the hypoketotic hypoglycemia in patients with these defects.

Systems biology

In the past decade, the field of systems biology has emerged rapidly. These developments have enabled the study of metabolic defects more in-depth, and on a more biochemical level, with the aid of computer models (*in silico* models). Systemic effects of enzyme deficiencies and accumulating intermediates can be analyzed *in silico*, and the effect of a metabolic defect on the accumulation of intermediates that cannot be measured in plasma or urine (i.e. acyl-CoA esters) can be predicted. Models of mFAO, glycogenolysis, and the TCA cycle have already been described [37–39]. *In silico* models can be used for the generation of hypotheses on possible pathophysiological mechanisms that can contribute to the development of a clinical phenotype in MCAD deficiency, as systems biology enables detailed analysis of metabolic networks at different levels [37–39].

Outline of the thesis

The pathophysiology of MCAD deficiency has not been unraveled fully, and new *ACADM* genotypes are identified upon NBS. As a genotype-phenotype correlation has not been described in MCAD deficiency, insight in the mechanisms responsible for the development of clinical symptoms is of major importance. Furthermore, as the population of adult patients with MCAD deficiency is increasing, insight into the long-term consequences of MCAD deficiency becomes more and more important.

Therefore, the following questions were addressed in this thesis:

- Is risk stratification in patients with MCAD deficiency possible after positive NBS? (chapter 2)
- What is the clinical relevance of the variant *ACADM* genotypes that are being identified since introduction of NBS for MCAD deficiency? (chapter 3)
- Is intramuscular energy metabolism perturbed during prolonged exercise in patients with MCAD deficiency? (chapter 4)
- Does MCAD deficiency lead to oxidative damage and an altered enzymatic antioxidative defense? (chapter 5)
- Can the mouse model for MCAD deficiency aid in unraveling the pathophysiology behind MCAD deficiency? (chapter 6)
- How are mitochondrial fatty acid oxidation and oxidative phosphorylation related in the generation of a clinical phenotype in case of defects in one of these systems? (chapter 7)

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Chapter 2

Risk stratification by residual enzyme activity after newborn screening for medium-chain acyl-CoA dehydrogenase deficiency: data from a cohort study

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ABSTRACT

Since the introduction of medium-chain acyl coenzyme A dehydrogenase (MCAD) deficiency in population newborn bloodspot screening (NBS) programs, subjects have been identified with variant *ACADM* (gene encoding MCAD enzyme) genotypes that have never been identified in clinically ascertained patients. It could be hypothesized that residual MCAD enzyme activity can contribute in risk stratification of subjects with variant *ACADM* genotypes.

We performed a retrospective cohort study of all patients identified upon population NBS for MCAD deficiency in the Netherlands between 2007-2010. Clinical, molecular, and enzymatic data were integrated.

Eighty-four patients from 76 families were identified. Twenty-two percent of the subjects had a variant *ACADM* genotype. In patients with classical *ACADM* genotypes, residual MCAD enzyme activity was significantly lower (median 0%, range 0-8%) when compared to subjects with variant *ACADM* genotypes (range 0-63%; 4 cases with 0%, remainder 20-63%). Patients with (fatal) neonatal presentations before diagnosis displayed residual MCAD enzyme activities <1%. After diagnosis and initiation of treatment, residual MCAD enzyme activities <10% were associated with an increased risk of hypoglycemia and L-carnitine supplementation. The prevalence of MCAD deficiency upon screening was 1/8,750 (95% CI 1/7,210–1/11,130).

Determination of residual MCAD enzyme activity improves our understanding of variant *ACADM* genotypes and may contribute to risk stratification. Subjects with variant *ACADM* genotypes and residual MCAD enzyme activities <10% should be considered to have the same risks as patients with classical *ACADM* genotypes. Parental instructions and an emergency regimen will remain principles of the treatment in any type of MCAD deficiency, as the effect of intercurrent illness on residual MCAD enzyme activity remains uncertain. There are, however, arguments in favour of abandoning the general advice to avoid prolonged fasting in subjects with variants *ACADM* genotypes and >10% residual MCAD enzyme activity.

INTRODUCTION

Medium-chain acyl-Coenzyme A dehydrogenase (MCAD [E.C.1.3.99.3]) deficiency (OMIM 201450) is the most common inherited disorder of mitochondrial fatty acid oxidation. The MCAD enzyme is responsible for the first step in the mitochondrial β-oxidation of CoA esters of medium-chain length fatty acids [1].

Before the introduction of population newborn bloodspot screening (NBS) for MCAD deficiency, patients presented clinically during periods of catabolic stress, precipitating acute symptoms [2,3]. Some patients developed seizures, coma or even presented with sudden death, associated with hypoketotic hypoglycemia. However, asymptomatic family members have also been recognized with the same disease-causing genotype [4]. Worldwide, approximately 80% of clinically presenting patients were homozygous for the c.985A>G missense mutation in the *ACADM* gene encoding the MCAD enzyme [5]. Early diagnosis significantly improves the outcome [3] and treatment is mainly dietary, consisting of avoidance of prolonged fasting and an emergency regimen during intercurrent illness [6]. Secondary free carnitine (C_0-carnitine) deficiency in blood may be corrected by L-carnitine supplementation in some patients, but evidence for this treatment is limited [7].

Since the introduction of the NBS programs, newborns are identified by the detection of increased concentrations of medium-chain length acylcarnitines and their ratios [8]. An elevated concentration of octanoylcarnitine (C_8-carnitine) is the most common biomarker for MCAD deficiency. Besides patients with classical *ACADM* genotypes, NBS identifies subjects with hyperoctanoylcarnitinemia and variant *ACADM* genotypes, i.e. genotypes that have not been recognized before in clinically ascertained patients [9]. As population NBS programs aim to prevent development of a phenotype, physicians feel forced to institute treatment, independent of the genotype. It can be questioned, however, whether subjects with variant *ACADM* genotypes have the same clinical risks compared to patients with classical *ACADM* genotypes. Or even, whether they should be regarded patients at all. Different studies used laboratory parameters to estimate the significance of variant *ACADM* genotypes [10–14]. Clinical follow-up parameters are difficult to interpret, because early diagnosis and treatment influence the natural clinical course of subjects with variant *ACADM* genotypes.

It could be hypothesised that residual MCAD enzyme activity is a prognostic parameter in risk stratification of patients with variant *ACADM* genotypes. Therefore, we performed a cohort study integrating NBS test results, molecular studies, and clinical data with enzymatic data of all patients from the Dutch birth cohorts 2007-2010 identified in the population NBS program for MCAD deficiency. The results from subjects with variant *ACADM* genotypes were stratified, using data from patients with classical *ACADM* genotypes as a reference.

PATIENTS AND METHODS

The Medical Ethical Committee of the University Medical Centre Groningen approved the study (METc

2011/133). Parents provided written informed consent.

Population NBS protocol

In The Netherlands, the epidemiology and natural history of MCAD deficiency have been well characterized before introduction of the population NBS program in 2007 [2,4,15]. In our country, 99.75% of all newborns are screened between 72 and 168 hours after birth (www.rivm.nl). In dried blood spots the following parameters are determined: concentrations of C_0-carnitine, C_8-carnitine, and decanoylcarnitine (C_{10}-carnitine), and the C_8/C_{10} ratio. For MCAD deficiency, the decision is based on the C_8-carnitine concentration only as clinical and laboratory follow-up is initiated in newborns with C_8-carnitine ≥ 0.50 µmol/l within 24 hours.

Protocol for laboratory follow-up

After an initial positive NBS test for MCAD deficiency, the newborns are referred to a metabolic centre. Laboratory follow-up includes a complete acylcarnitine profile in plasma and/or a dried blood spot by tandem mass-spectrometry, and urinary organic acid analysis using gas chromatography–mass spectrometry, as described by Derks et al [9]. Determination of MCAD enzyme activity is performed in leukocytes or lymphocytes, with an HPLC-based assay using 3-phenylpropionyl-CoA (PP-CoA) as a substrate [9,16]. Residual MCAD enzyme activities are expressed as percentage from healthy controls. Analysis of the *ACADM* gene (OMIM 607008) is performed by sequencing all exons and adjacent intron regions. The nucleotide numbering starts from the first adenine of the ATG translation initiation codon of the *ACADM* cDNA sequence and amino acid numbering starts from the methionine encoded by this translation initiation codon. The abovementioned analyses are preceded by parental informed consent.

A 'variant *ACADM* genotype' was defined as an *ACADM* genotype that has not been recognized before in clinically ascertained patients in either The Netherlands [2] or in literature.

Cohort

In this study, we included children from the Dutch birth cohorts 2007-2010 with clinical follow-up in a metabolic centre after population NBS for MCAD deficiency. For most children the diagnosis has been confirmed by MCAD enzyme and/or *ACADM* gene analysis. If these analyses were not performed, the confirmatory acylcarnitine profile was characterized by C_8-carnitine concentrations >50 z-scores (i.e. ≥ 1.65 µmol/l) and a C_8/C_{10} ratio ≥10 z-scores (i.e. ≥ 8.3) above the threshold for NBS, based on reference data from the National Institute for Public Health and the Environment.

Clinical and laboratory data from all patients were retrospectively retrieved from the Dutch Diagnosis Registration Metabolic Diseases database (www.ddrmd.nl), and medical and laboratory files by one investigator (CT). Data from the patients in which the diagnosis had been confirmed enzymatically were included in the analysis of the prognostic value of MCAD enzyme analysis.

Data analysis

Using data from the Dutch Central Bureau for Statistics (CBS) (www.cbs.nl), the prevalence P was calculated by dividing the total number of patients by the total number of newborns N. For calculation of the 95% confidence interval (95% CI) the following formula was used:

$$95\%\ CI = P \pm 1.96 \times ((P \times (1-P))/N)^{1/2}$$

Differences between normally distributed continuous data were analyzed using parametric tests. Data that were not normally distributed were analyzed using nonparametric tests. For dichotomous data, a chi-squared test was used. For analysis of correlations, Spearman's rank test was used. The significance level was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., version 5.00, 2007).

RESULTS

Cohort

In the period 2007-2010, the diagnosis MCAD deficiency was confirmed in 84 patients, after 108 initial positive screening results for C_8-carnitine ≥ 0.50 µmol/l. Data from the NBS test, molecular studies and clinical follow-up were compared to the residual MCAD enzyme activity, in order to determine the prognostic value. Results from patients with a classical *ACADM* genotype were used as a reference.

Relationship between ACADM genotypes and residual MCAD enzyme activities

ACADM genotypes were available from 68 of the 84 patients (Table 1). Homozygosity for the common c.985A>G mutation was observed in 62% (42/68), the c.985A>G allele frequency was 77% (104/136). Variant *ACADM* genotypes were observed in 22% (15/68).

Data on residual MCAD enzyme activity could be obtained from 64 of the 84 patients. Median residual MCAD enzyme activity in patients with classical *ACADM* genotypes was 0% (range 0-8% and 0-5% in leukocytes and lymphocytes, respectively). Subjects with variant *ACADM* genotypes displayed significantly higher MCAD residual enzyme activities (median 25%, range 0-63%, Mann-Whitney U test, $p<0.01$) (Figure 1).

For further comparison of groups, patients were stratified based on residual MCAD enzyme activity. A threshold of 10% was considered practical and safe, because the highest residual MCAD enzyme activity determined in a patient with a classical *ACADM* genotype was 8%.

Relationship between population NBS test results and MCAD enzyme activities

Figure 2A demonstrates that C_8-carnitine concentrations were significantly higher in patients with residual MCAD enzyme activities <10% than in subjects with residual MCAD enzyme activities ≥10% (median 3.96, range 0.77–14.80 µmol/l vs. median 1.11, range 0.56– 2.82 µmol/l, respectively, Mann-

Table 1. *ACADM* genotypes in 68 patients, identified upon population NBS for MCAD deficiency in The Netherlands.

GENOTYPE		ALLELE 1			ALLELE 2			MCAD ACTIVITY (%)		REFERENCES
	Number	Nucleotide change	Exon	Coding effect	Nucleotide change	Exon	Coding effect	A	B	
CLASSICAL	42	c.985A>G	11	p.K329E	c.985A>G	11	p.K329E	0	0	25–27
	5	c.985A>G	11	p.K329E	c.233T>C	4	p.I78T	2	2	23
	2	c.985A>G	11	p.K329E	c.789A>G	9	p.L263F	0	n.d.	2
	1	c.985A>G	11	p.K329E	c.799G>A	9	p.G267R	8	n.d.	28,29
	2	c.233T>C	4	p.I78T	c.233T>C	4	p.I78T	<1	3	23
	1	c.233T>C	4	p.I78T	c.789A>G	9	p.L263F	0	n.d.	2,23
VARIANT	2	c.985A>G	11	p.K329E	c.158G>A	3	p.R53H	n.d.	23	This study
	3	c.985A>G	11	p.K329E	c.199T>C	3	p.Y67H	58	31	30
	1	c.985A>G	11	p.K329E	c.216+1G>T	4	Splice site variant	n.d.	0	This study
	2	c.985A>G	11	p.K329E	c.238A>G	4	p.R80G	n.d.	36	This study
	1	c.985A>G	11	p.K329E	c.470C>T	7	p.A157V	n.d.	0	This study
	1	c.985A>G	11	p.K329E	c.493G>A	7	p.A165T	n.d.	20	This study
	1	c.985A>G	11	p.K329E	c.600-18G>A	8	p.XXX?	n.d.	63	10
	1	c.985A>G	11	p.K329E	c.928G>A	10	p.G285R	0	n.d.	23
	1	c.233T>C	4	p.I78T	c.1066A>T	11	p.I356F	n.d.	0	This study
	1	c.250C>T	4	p.L84F	c.199T>C	3	p.Y67H	n.d.	58	30,31
	1	c.799G>A	9	p.G267R	c.865G>A	10	p.V289I	n.d.	25	This study

A, measured in leukocytes; B, measured in lymphocytes; n.d., not determined. Median residual MCAD enzyme activities are depicted.

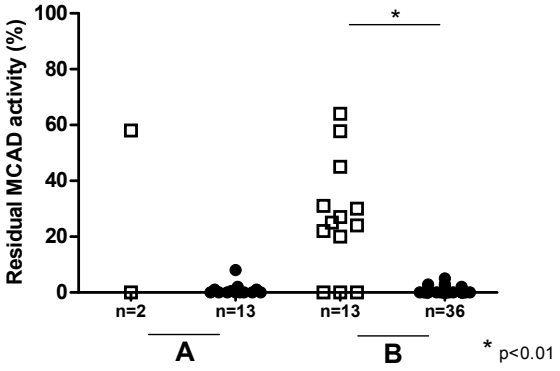

Figure 1. Residual MCAD enzyme activities measured in leukocytes, and lymphocytes. Residual MCAD enzyme activities measured in leukocytes (A), and lymphocytes (B) from true positives with variant *ACADM* genotypes (squares) and classical *ACADM* genotypes (dots).

Whitney U test, $p<0.01$). Additionally, C_8/C_{10} ratios were significantly higher in the group of patients with residual MCAD enzyme activities <10% (median 13.00, range 3.21–18.50), when compared to the group of subjects with residual MCAD enzyme activities ≥10% (median 3.41, range 1.81–8.03, Mann-Whitney U test, $p<0.01$). A strong negative linear correlation was found between the NBS C_8/C_{10} ratio and the residual MCAD enzyme activity measured in lymphocytes (Spearman r = -0.67, $p<0.001$) (Figure 2B).

Relationship between clinical phenotypes and MCAD enzyme activities

Seven newborns had a clinical presentation in the neonatal period before diagnosis (Table 2). Two of these newborns died before arrival in the hospital. Five had been admitted to the hospital before

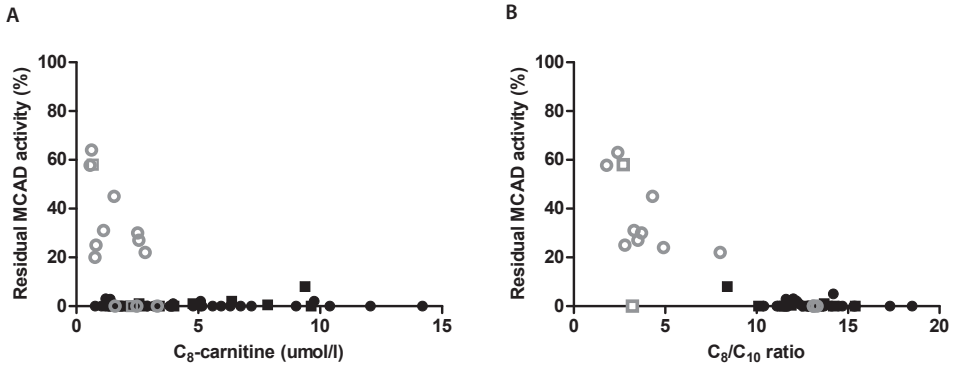

Figure 2. Relationship between residual MCAD enzyme activity and C_8-carnitine (A) and C_8/C_{10} (B) upon NBS. Classical *ACADM* genotypes depicted in black, variant *ACADM* genotypes in grey. Squares: measured in leukocytes; Circles: measured in lymphocytes.

NBS test results became available, three of them with documented hypoglycemia. All newborns with a neonatal presentation had a C_8/C_{10} ratio ≥10 upon NBS and residual MCAD enzyme activities <1%. Six of these patients had a classical *ACADM* genotype. The seventh case had the variant c.985A>G/c.216+1G>T genotype, corresponding with 0% residual MCAD enzyme activity.

After the diagnosis was established, no fatal manifestations of MCAD deficiency were reported. Patients with residual MCAD enzyme activities <10% were 1.2 times more frequently admitted to the hospital preventively, compared to subjects with residual MCAD activities ≥10% (Table 2). All patients with documented hypoglycemia had residual MCAD enzyme activities <10%. During follow-up, no L-carnitine was prescribed for low plasma C_0-carnitine concentrations in subjects with residual MCAD enzyme activities ≥10%. In contrast, 51% (27/51) of the patients with residual MCAD enzyme activities <10% received L-carnitine supplementation.

Table 2. Prevalence of clinical symptoms and interventions in patients, organised by residual MCAD enzyme activity.

	Residual MCAD enzyme activity	
	< 10%	≥ 10%
Neonatal presentation (%)	13% (7/53)	0%
Hypoglycemia (%)	8% (4/51)	0%
L-carnitine supplementation (%)	51% (27/51)	0%
Patients with hospital admissions (%)	82% (42/51)	55% (6/11)*
Hospital admissions (n)	116	14*
Hospital admissions per life year	0.64/life year	0.55/life year

Data were processed as described in 'Patients and Methods'. Hypoglycemia was defined as blood glucose concentration <2.6 mmol/L or reported hypoglycemia in medical charts. * p<0.05.

Epidemiology

Between 2007-2010, 84 patients were identified from 76 families. The prevalence of MCAD deficiency was 1/8,750 (95%CI 1/7,210–1/11,130), which is considered to be a high estimate (Table 3). When subjects with residual MCAD activities ≥10% were excluded, the prevalence was 1/10,070 (95%CI 1/8,190-1/13,070).

Table 3. Epidemiology of MCAD deficiency in The Netherlands before and after the introduction of population NBS.

MCAD deficiency in The Netherlands	Frequencies	(95% CI)
ACADM mutation carrier frequency[a]	1 / 55	(1 / 46 – 1 / 68)
Expected prevalence[a]	1 / 12,100	(1 / 8,450 – 1 / 18,500)
Observed prevalence 1985-1999[b]	1 / 27,400	(1 / 23,000 – 1 / 33,900)
Screened newborns 2007-2010[c]	735,282	
Patients 2007-2010[d]	84	
Prevalence upon neonatal screening[d]	1 / 8,750	(1 / 7,210 – 1 / 11,130)

Legend: [a] According to De Vries et al [15]; [b] According to Derks et al [4]; [c] According to CBS; [d] This study.

DISCUSSION

This is the first report in which residual MCAD enzyme activities in a large cohort of patients identified upon population NBS for MCAD deficiency have been used for risk stratification. Within the group of newborns with hyperoctanoylcarnitinemia, a broad spectrum of *ACADM* genotypes has been identified. As the clinical significance of many variant *ACADM* genotypes is incompletely known, we integrated clinical, molecular and enzymatic data from a well-defined population. Residual MCAD enzyme activity correlated well with *ACADM* genotype and phenotype, and could therefore aid in the risk stratification of patients after positive NBS test.

In this study, residual MCAD enzyme activity was measured with PP-CoA, which is a very specific substrate to determine residual MCAD enzyme activity *in vitro* [16,17]. Traditionally, natural substrates such as hexanoyl-CoA and octanoyl-CoA were used to determine MCAD enzyme activity. However, even in patients who were homozygous for the classical c.985A>G *ACADM* mutation, high residual enzyme activities were found when using these substrates, possibly due to the overlap in substrate specificity with other acyl-CoA dehydrogenases [9]. Therefore, in our country these substrates have been replaced in the confirmatory enzymatic studies after positive NBS test results. However, the possible role of PP-CoA in the pathophysiology of MCAD deficiency is currently unknown and it is important to realize that the pathophysiology is far more complex than just deficient MCAD enzyme activity.

Before NBS test results became available, a neonatal presentation occurred in seven patients with residual MCAD enzyme activities <1%. Two of these patients presented with a fatal event. Despite the important benefits of population NBS programs for MCAD deficiency [3], the question remains justified, whether we detect *all* patients before clinical presentation and whether they are all *patients*. When compared to other NBS programs, blood sampling for the NBS test is relatively late in our country, i.e. between 72 and 168 hours of life. However, similar percentages of (fatal) neonatal presentations have been reported in studies from other countries, where the NBS test is performed earlier in the neonatal period [3]. Since several inherited metabolic diseases may present with a fatal neonatal presentation, escaping early detection by population NBS programs, we recommend dried blood spot analysis of acylcarnitines and amino acids in all newborns who die before the NBS test has been performed.

In a subset of the children with variant *ACADM* genotypes, residual MCAD enzyme activity is relatively high (Table 1). This raises the question whether these subjects are at risk of developing clinical symptoms as patients with classical genotypes, and should be considered "patients". If the main arguments for a population NBS program are strictly considered [18], subjects with variant *ACADM* genotypes may not be regarded "true-positives", because their genotypes have not been observed before in clinically ascertained patients. However, some of these mutations may have never been detected due to low carrier frequencies, but may give the same clinical risks as classical *ACADM* mutations [19]. Already before the introduction of NBS for MCAD deficiency, the term "patient" was a matter of debate. Reduced penetrance of the disorder is a well-recognised phenomenon, reflected by asymptomatic family members with the same classical *ACADM* genotype as clinically ascertained probands [4]. Moreover, it is recognized that single nucleotide polymorphisms (in combination with a classical *ACADM* mutation) may contribute to the development of hyperoctanoylcarnitinemia by modulating mitochondrial fatty acid oxidation [19].

In this study, 15 subjects had a 'variant *ACADM* genotype'. Three newborns (with c.985A>G/c.216+1G>T; c.985A>G/c.470C>T and c.233T>C/c.1066A>T genotypes) displayed C_8/C_{10} ratios >10 and residual MCAD enzyme activity <1% (Figure 2b), as observed in patients with classical *ACADM* genotypes. In our opinion, they should be considered and treated like patients with classical *ACADM* genotypes. The patient with the c.985A>G/c.928G>A genotype displayed a C_8/C_{10} ratio <10. Since MCAD enzyme activity was absent, he was considered "patient" like patients with classical *ACADM*

genotypes. In the remaining 11 subjects with variant *ACADM* genotypes, residual MCAD enzyme activities ranged between 20%–63%. Should they receive (the same) treatment as patients with classical genotypes? In previous studies that aimed to determine risk stratification for subjects with variant *ACADM* genotypes, different sets of data have been used. Examples include correlation of metabolite concentrations to *ACADM* genotypes [10,11], or heterologous overexpression studies to determine the effect of various *ACADM* mutations on residual MCAD enzyme activity and the thermal stability of the MCAD enzyme [12–14]. In most cases, however, the effect of one single *ACADM* mutation was studied, although MCAD deficiency is an enzyme deficiency caused by genetic alterations on both *ACADM* alleles.

In this study, classical *ACADM* genotypes were associated with residual MCAD enzyme activities <10%. Substantial octanoate oxidation [20] and normal ketone body metabolism [21] were demonstrated in previous stable isotope studies under normal fasted conditions in patients with classical *ACADM* genotypes. It is well recognized that clinically ascertained patients tolerated overnight fasting without problems before establishment of the diagnosis, as recently reviewed [22]. Similar observations were made in our country, since overnight fasting per se has never precipitated symptoms in patients [2,6]. As a subset of subjects with variant *ACADM* genotypes was associated with a milder biochemical and clinical phenotype, and relatively high residual MCAD enzyme activity, it could be argued that the general advice on avoidance of overnight fasting in subjects with residual MCAD enzyme activities ≥10% can be abandoned. However, the major argument in favour of avoiding prolonged fasting is early anticipation in circumstances of intercurrent illness with fever in young patients. Some (variant) *ACADM* mutations have shown to lead to temperature sensitive MCAD folding variants *in vitro* [14,23]. The clinical *in vivo* effects remain to be determined. Therefore, an emergency regimen and clinical follow-up remain basic principles of the treatment, regardless of *ACADM* genotype.

The prevalence of MCAD deficiency upon NBS in The Netherlands is threefold higher than found after clinical presentation (Table 3) [4], and in line with previous reports [24]. Prior to the introduction of the NBS program, the expected prevalence of MCAD deficiency was calculated to be 1/12,100 (95%CI: 1/8,450–1/18,500) in our country, based on the *ACADM* c.985A>G carrier frequency in the general population and the assumption of a 94% allele frequency for this common mutation in clinically ascertained cases[15]. The latter was confirmed in patients from the Dutch birth cohorts 1985-1999 [4]. The current study displays a more heterogeneous *ACADM* mutational spectrum with an observed allele frequency of the c.985A>G *ACADM* mutation of only 77%. Although the observed prevalence is comparable to the expected prevalence [15], it is important to realize that the availability of molecular tests for the complete *ACADM* gene has significantly increased since the 1990s. This might have biased identification of patients in our previous studies, hence, causing an underestimation of the expected prevalence. In addition, demographic alterations within the same geographic area might have contributed (www.cbs.nl).

CONCLUSIONS

Population-wide NBS programs identify newborns with hyperoctanoylcarnitinemia and variant *ACADM* genotypes. These newborns are currently regarded patients, in whom follow-up and dietary treatment are initiated similarly to the group with classical *ACADM* genotypes. This study demonstrates the prognostic value of residual MCAD enzyme activity in newborns with hyperoctanoylcarnitinemia and a variant *ACADM* genotype. Residual MCAD enzyme activities <10% were associated with clinical symptoms, regardless of *ACADM* genotype. Clinical symptoms or classical *ACADM* genotypes have thus far not been reported in subjects with residual MCAD enzyme activities ≥10%. For all positively screened newborns with any form of MCAD deficiency, basic principles of the treatment are parental instructions, cautious clinical follow-up, and application of an emergency regimen. In subjects with variant *ACADM* genotypes and ≥10% residual MCAD enzyme activities, the necessity to avoid overnight fasting is debatable.

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Chapter 3

In vitro and in vivo consequences of variant medium-chain acyl-CoA dehydrogenase genotypes

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ABSTRACT

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common inherited disorder of the mitochondrial fatty acid oxidation, caused by mutations in the *ACADM* gene. Since the introduction of neonatal screening for MCAD deficiency, a subgroup of newborns have been identified with variant *ACADM* genotypes that had never been identified before in clinically ascertained patients. *In vitro* residual MCAD enzyme activity has been found to facilitate risk stratification. In this study we integrated results of *in vitro* (residual MCAD enzyme activities) and *in vivo* (clinical fasting tolerance tests, and phenylpropionic acid loading tests) tests in this subgroup of newborns, defining the consequences of variant *ACADM* genotypes.

Enzyme analyses were performed in leukocytes with: hexanoyl-CoA (C_6-CoA) +/- butyryl-CoA (C_4-CoA), and phenylpropionyl-CoA (PP-CoA). *In vitro* studies were performed in 9 subjects with variant *ACADM* genotypes, *in vivo* functional tests in 6 of these subjects.

Enzyme analyses with C_6-CoA, C_6-CoA + C_4-CoA, and PP-CoA identified significantly higher residual MCAD enzyme activities in subjects with variant *ACADM* genotypes when compared to patients with classical *ACADM* genotypes. After prolonged fasting (range 15-19 hours) no hypoglycaemia was observed. Increasing concentrations of free fatty acids indicated lipolysis, and ketone body concentrations were sufficient for blood glucose concentrations in 5 out of 6 subjects. Phenylpropionic acid loading clearly demonstrated *in vivo* residual MCAD enzyme activity in all studied subjects.

Subjects with variant *ACADM* genotypes and residual MCAD enzyme activities >10% display residual MCAD enzyme activities *in vitro* and *in vivo*. Our findings support the hypothesis that the guidelines on maximal duration of fasting might be abandoned in subjects with residual MCAD enzyme activities >10% under normal conditions. An emergency regimen and parental instructions remain necessary in all subjects with MCAD deficiency, regardless of residual MCAD enzyme activity.

INTRODUCTION

Inherited disorders of mitochondrial fatty acid oxidation (mFAO) are a group of acute presenting, life-threatening disorders among which medium-chain acyl coenzyme A dehydrogenase (MCAD [E.C.1.3.99.3; OMIM 201450]) deficiency is the most common [1]. Worldwide, population neonatal bloodspot screening (NBS) programs have become available for the disorder.

Before the introduction of MCAD deficiency in NBS programs, patients presented clinically with symptoms associated with a life-threatening hypoketotic hypoglycemia, such as seizures, coma or even sudden death. The c.985A>G missense mutation in the *ACADM* gene (gene encoding MCAD, OMIM 607008) was the most common mutation. Since the introduction in NBS programs, the spectrum of *ACADM* genotypes has changed dramatically. Novel *ACADM* genotypes were identified in prospectively screened asymptomatic newborns (i.e. variant *ACADM* genotypes), of which the clinical consequences are currently unknown. In previous studies our group and others emphasized the importance of determination of residual MCAD enzyme activity, both for diagnostic and prognostic purposes [2,3]. However, studies integrating laboratory (*in vitro)* and clinical (*in vivo)* data are scarce [4].

Currently, a late evening meal is advised during the first two years of life for patients with MCAD deficiency, regardless of the *ACADM* genotype [2]. Based on *in vitro* data, the necessity to treat all subjects with MCAD deficiency similarly is debatable, in particular with regards to the advice on maximum duration of fasting [2]. However, *in vivo* fasting tolerance under controlled conditions has not been studied before in this group of subjects. It can be hypothesized that residual MCAD enzyme activities and clinical fasting tolerance tests reflect the *in vitro* and *in vivo* role of these variant *ACADM* genotypes. In order to enable personalized care after diagnosis, clinical fasting tolerance tests were performed in our centre in subjects with variant *ACADM* genotypes after informed consent of the parents. In this study, we integrated data on *in vitro* MCAD enzyme assays, and *in vivo* functional tests from 9 subjects with variant *ACADM* genotypes.

METHODS

The Medical Ethical Committee of the University Medical Centre Groningen approved the study (METc 2011/133). Parents agreed on participation by written informed consent.

Cohort

In the Netherlands MCAD deficiency has been included in the national population NBS program since 2007. Free carnitine (C_0-carnitine), octanoylcarnitine (C_8-carnitine), decanoylcarnitine (C_{10}-carnitine), and the C_8/C_{10} ratio are determined in dried blood spots obtained from newborns 72 to 168 hours after birth (www.rivm.nl). Clinical and laboratory follow-up is initiated within 24 hours in newborns in case of elevated C_8-carnitine concentrations. During the pilot NBS program in 2003-2006 the cut-off concentration for C_8-carnitine was 0.30 µmol/l [5]. Since 2007, the cut-off concentration for C_8-carnitine

has been ≥ 0.50 µmol/l [2]. Diagnosis of MCAD deficiency is made after abnormal NBS, based on persisting abnormal metabolite profiles, the presence of 2 mutations in the *ACADM* gene, and/or residual MCAD enzyme activity <50% when measured with hexanoyl-CoA (C_6-CoA) in leukocytes. Laboratory analysis is always performed in family members of a proband, including all siblings.

Subjects carrying 'variant *ACADM* genotypes', who were diagnosed at the Beatrix Children's Hospital, UMC Groningen, Groningen, The Netherlands between 2003-2011 were included in our cohort. A 'variant *ACADM* genotype' was defined as an *ACADM* genotype that had not been described before in clinically ascertained patients in either The Netherlands [6] or in literature.

Maximum percentage of weight loss was calculated as the ratio between the birth weight and the lowest weight observed in the first neonatal period.

Enzyme analysis

Residual MCAD enzyme activity was determined in leukocytes. Before 2007, a GC-MS based analysis using C_6-CoA +/- butyryl-CoA (C_4-CoA) as substrates was the standard in our centre [7,8]. The assay with C_6-CoA has been described extensively, and was modified for more accurate analysis of MCAD enzyme activity and elimination of the contribution of short-chain acyl-CoA dehydrogenase (SCAD; OMIM 606885). In this assay 785.7 µM C_4-CoA was added as a substrate, besides C_6-CoA. At least one commercially available leukocyte pellet (Sanquin, The Netherlands) was used as control in each enzyme assay. Molecular analysis of the *ACADM* gene was performed in all subjects with residual MCAD activity <50% when determined with C_6-CoA. Since 2007, an HPLC-based assay using 3-phenylpropionyl-CoA (PP-CoA) as a substrate is used [5,9]. Residual enzyme activities are expressed as a percentage from healthy controls.

Clinical fasting tolerance tests

All clinical fasting tolerance tests were performed in the clinical function test ward of the Beatrix Children's Hospital, UMC Groningen, according to established protocols [10,11]. Hypoglycemia was defined as blood glucose concentrations <2.6 mmol/l [12]. The fasting tolerance test was combined with a 3-phenylpropionic acid (PPA) loading test, where 25 mg/kg PPA was administered orally [13]. Two portions of urine were collected during the fasting tolerance test. Portion 1 was obtained after overnight fasting in the first 4 hours following PPA loading; the second portion was obtained in the following 4 hours of the fasting tolerance test. All PPA loading tests were performed after the age of 6 months [14]. Urinary phenylpropionylglycine was determined by gas-chromatography-mass spectrometry (GC-MS), according to Chalmers *et al* [15].

Data analysis

Clinical and laboratory data, and data from functional tests were retrospectively retrieved from the medical charts and laboratory files of the subjects by one investigator (CT).

Differences between normally distributed continuous data were analysed using parametric tests. Data that were not normally distributed were analysed using nonparametric tests. For analysis of cor-

relations, Spearman's rank test was used. The significance level was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., version 5.00, 2007).

RESULTS

Cohort

From the birth cohort 2003-2011, 50 newborns were diagnosed with MCAD deficiency in our centre after referral for a positive NBS (in either the pilot screening program, or the regular nationwide NBS). In 9 of these 50 subjects (18%) a variant *ACADM* genotype was identified (Table 1). In two of these subjects (cases 8 & 9), only one *ACADM* mutation was detected after sequencing of all exons and adjacent introns. However, acylcarnitine profiles in these subjects were indicative of MCAD deficiency, and residual MCAD enzyme activities were <50% when measured with C6-CoA.

Two subjects (cases 3 & 5) were diagnosed with C_8-carnitine concentrations below 0.50 µmol/l. Case 3 was the younger sister of case 2, carrying the c.985A>G/c.199T>C *ACADM* genotype. Case 5 was identified during the pilot screening program. Maximum percentage of weight loss in the neonatal phase in our cohort of subjects with variant *ACADM* genotypes correlated with C_8-carnitine concentrations upon NBS (Spearman r -0.89, $p<0.05$; median 5.9%; range 4.7-9.8%). In cases 3 and 5 the maximum percentage of weight loss was respectively 4.7% and 5.7%; in cases 8 and 9 with only one identified *ACADM* mutation it was respectively 7.0% and 5.9%. None of the subjects with a variant *ACADM* genotype presented clinically with hypoketotic hypoglycemia, either in the neonatal period or after diagnosis.

In vitro studies – Residual MCAD enzyme activities

Results from *in vitro* analyses in the 9 subjects with variant *ACADM* genotypes are depicted in Table 1 and Figure 1. In 7 of these subjects MCAD enzyme analyses were performed in leukocytes with three different substrates: C_6-CoA, C_6-CoA+C_4-CoA, and PP-CoA. Irrespective of the substrate, significantly higher residual MCAD enzyme activities were measured in subjects with variant *ACADM* genotypes, when compared to patients with classical *ACADM* genotypes (Figure 1). However, when measured with PP-CoA, case 7 demonstrated no residual MCAD enzyme activity. With the natural substrates C_6-CoA and C_6-CoA+C_4-CoA, the group of subjects with variant *ACADM* genotypes differed clearly from both the group with classical *ACADM* genotypes, and from carriers of the c.985A>G mutation (i.e. siblings or parents of patients). The observed proportional change in enzyme activity after addition of C_4-CoA to the C_6-CoA assay correlated with the MCAD enzyme activity measured with PP-CoA (Spearman r -0.72, $p<0.05$) (Figure 1d). Additionally, strong correlations were identified between residual MCAD enzyme activities measured with PP-CoA and C_6-CoA+C_4-CoA, and the C_8/C_{10} ratio identified upon NBS (Spearman r -0.65; $p<0.001$ and Spearman r -0.54; $p<0.01$, respectively) (Figure 2).

Table 1. Results fasting test and PPA loading test in subjects with variant *ACADM* genotypes.

Case	Genotype		NBS		MCAD activity (%)				Fasting test							PPA loading test
	Allele 1	Allele 2	C_8	C_8/C_{10}	Organic acids	C_6-CoA	$+C_4$-CoA	PP-CoA	Age (mo)	Duration (h)	Glc (t=15 h)	KB (t=15 h)	Glc*KB (t=15 h)	FFA/KB (t=15 h)	C_8 (t=15h)	C_8/C_{10}	PP-glycine
1	c.985A>G	c.199T>C	2.85	2.3	Normal	39	36	-	-	-	-	-	-	-	-	-	-
2	c.985A>G	c.199T>C	0.67	2.7	DC, HG	44	29	58	-	-	-	-	-	-	-	-	-
3	c.985A>G	c.199T>C	0.35	2.3	Trace N-HG, SG, DC	48	43	48	-	-	-	-	-	-	-	-	-
4	c.985A>G	c.199T>C	0.74	2.8	Trace HG	36	34	44	10	18	4.5	1.1	5.0	1.0	3.7	3.4	Trace
5	c.985A>G	c.473A>G	0.39	2.3	Normal	38	25	39	6	19	2.8	0.3	0.8	2.2	2.0	3.8	Absent
6	c.985A>G	c.734C>T	1.41	3.0	Trace DC, 5-OH-C, HG, SG	41	17	11	13	17	4.4	0.8	3.3	1.9	3.6	4.2	Trace
7	c.985A>G	c.928G>A	2.28	3.2	5-OH-H, trace HG	48	23	0	16	15	3.2	3.5	10.6	-	1.9	2.9	Absent
8	c.985A>G	Not found	0.70	1.3	Normal	28	-	-	25	18.5	4.0	0.9	3.7	1.5	2.9	2.1	Trace
9	c.985A>G	Not found	0.50	0.8	Normal	15	11	86	27	16.5	2.9	1.4	5.8	0.8	0.5	2.2	Trace
*	Classical *ACADM* genotype		2.98	12.7	5-OH-H, HG, PPG, SG, DC	28	14	0	-	17-24	3.1	0.6	2.2	5.7	-	-	High
#	Control population		-	-	-	-	-	-	0-24	15	4.1 (3.1-4.8)	1.3 (0.4-3.2)	5.5 (1.2-15.4)	1.1 (0.7-2.4)	-	-	Absent
#	Control population		-	-	-	-	-	-	25-84	15	4.6 (3.8-5.3)	0.5 (0.1-1.7)	2.1 (0.4-8.9)	1.8 (0.8-6.4)	-	-	Absent

Characteristics of subjects with variant *ACADM* genotypes during fasting tolerance tests and PPA loading tests. MCAD activity is determined in leukocytes. Residual MCAD enzyme activities are depicted as percentage from controls. At least one control was included in each enzyme assay. Ref, reference value; -, not determined; C8, C_8-carnitine; DC, dicarboxylic acids; Glc, glucose; HG, *N*-hexanoylglycine; SG, *N*-suberylglycine; PPG, phenylpropionylglycine; 5-OH-C, 5-OH-capronic acid; 5-OH-H, 5-OH-hexanoic acid; PPA, phenylpropionic acid; KB, ketone bodies; FFA, free fatty acids. * Characteristics of patients with classical *ACADM* genotypes during fasting according to Bonnefont et al.[10], median values are depicted. For NBS and enzymatic data median values from the UMCG cohort are depicted. # Reference values according to van Veen et al.[11] Median reference values are depicted. p10-p90 are indicated between brackets.

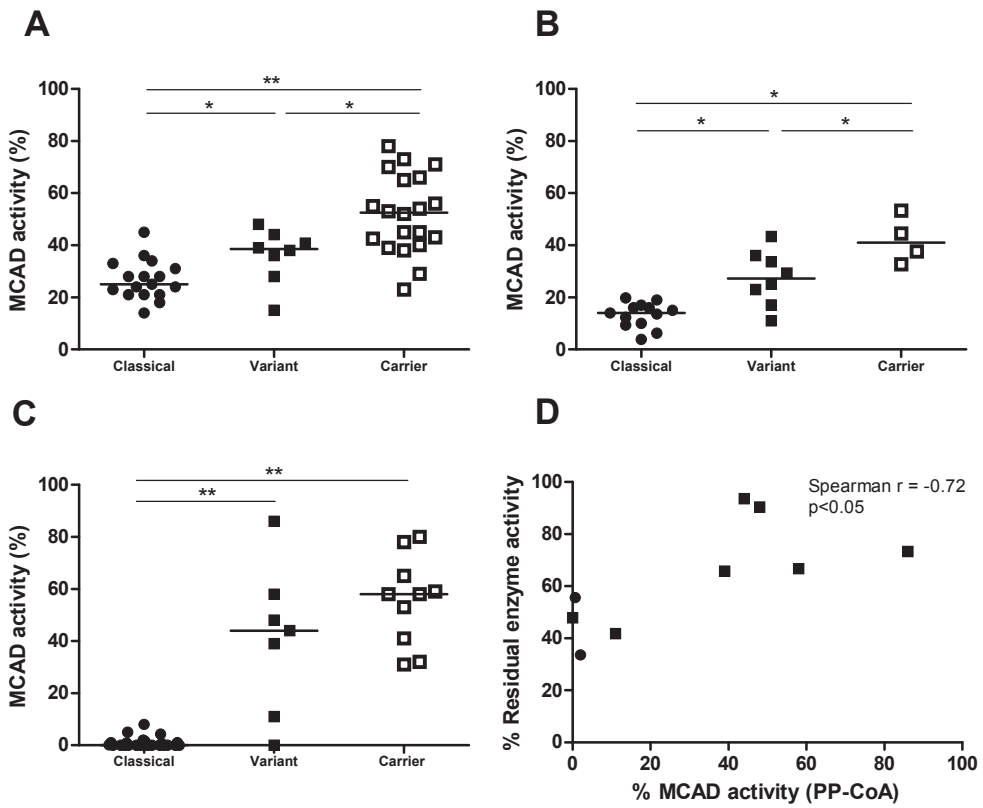

Figure 1. Residual MCAD enzyme activities measured with different substrates in leukocytes. Residual MCAD enzyme activities measured with C_6-CoA (A); Residual MCAD enzyme activities measured with C_6-CoA+C_4-CoA (B); Residual MCAD enzyme activities measured with PP-CoA (C); MCAD enzyme activity with PP-CoA correlates with the effect of C_4-CoA on C_6-CoA enzyme activity (D). In 1D, enzyme activity measured with C_6-CoA is set at 100% on the y-axis. Residual MCAD enzyme activities are depicted as percentage from controls. At least one control was included in each enzyme assay. Medians are indicated. * $p<0.05$; ** $p<0.001$.

In vivo studies – Fasting tolerance test

Clinical fasting tolerance tests were performed in 6 subjects with a variant *ACADM* genotype, at a median age of 14.5 months (range 6-27 months). The duration of fasting was supra-physiological in all subjects, as regular overnight fasting duration was extended. Case 7 fasted for 15 hours and the remaining 5 subjects for 16.5 – 18.5 hours (Table 1). Concentrations of glucose, free fatty acids (FFA), ketone bodies (KB), and their ratios after 15 hours of fasting are depicted in Table 1. All subjects could complete the fasting tolerance tests without clinical symptoms and/or hypoglycemia.

Case 5 demonstrated normal fasting parameters after overnight fasting but hypoketosis after 18.5 hours. Low FFA concentrations that were not clearly increasing in time reflected a minimal role of mFAO under these circumstances in this subject, with subsequent low concentrations of KB (squares

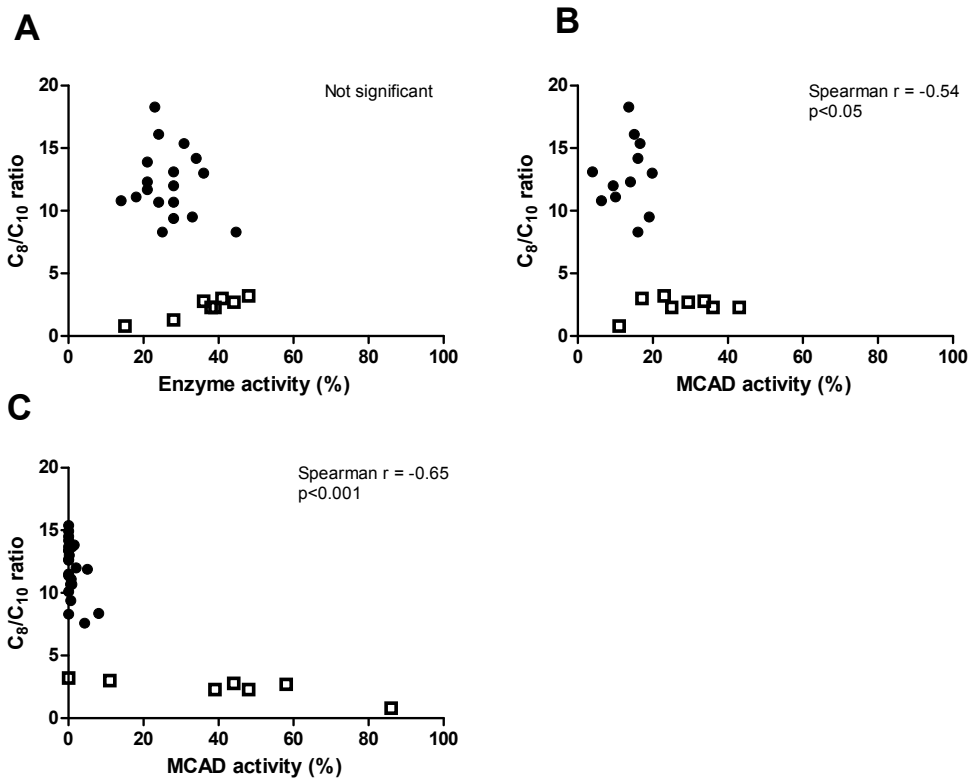

Figure 2. Correlation between residual MCAD enzyme activities measured in leukocytes and C_8/C_{10} upon NBS. Residual MCAD enzyme activities measured with C_6-CoA (A); Residual MCAD enzyme activities measured with C_6-CoA+C_4-CoA (B); Residual MCAD enzyme activities measured with PP-CoA (C). Residual MCAD enzyme activities are depicted as percentage of controls. At least one control was included in each enzyme assay. Variant *ACADM* genotypes are depicted as squares, classical *ACADM* genotypes as dots.

in Figure 3) [10,11]. Case 7 and case 9 also showed blood glucose concentrations that were below p10 for age and duration of fasting after 15 hours of fasting. FFA/KB ratios were at p10 for age and duration of fasting, with increasing concentrations of FFA and KB around p90. All other included subjects had KB concentrations that corresponded to blood glucose concentrations upon fasting (dots in Figure 3C). A relationship between biochemical response to fasting and residual MCAD enzyme activity with either substrate could not be identified.

During fasting, C_8-carnitine concentrations increased in time. Additionally, observed concentrations during fasting were considerably higher when compared to the concentrations that were seen during regular visits to the outpatient clinic (median during follow-up 0.6 µmol/l; median after 15h fasting 3.2 µmol/l, p=0.07). C_8-carnitine concentrations during fasting did not correlate with concentrations found in the initial NBS test.

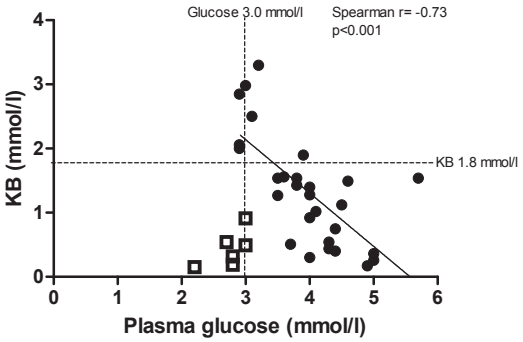

Figure 3. Correlation between plasma glucose and KB during fasting tolerance test. Relationship between blood glucose and KB concentrations is indicated. Case 5 is depicted in squares, as KB were low for glucose concentrations. The other cases (dots) are pooled.

The C_8/C_2 and C_8/C_{10} ratios during fasting were slightly higher than the ratios that were identified upon NBS and during regular visits to the outpatient clinic (Table 1). During the fasting tolerance test the ratios remained stable in time in all subjects [16].

In case 8, longer-chain acylcarnitines accumulated in time, besides medium-chain acylcarnitines (acylcarnitines after 18.5 hours of fasting: C_{12}-carnitine 0.60 µmol/l, C_{14}-carnitine 0.23 µmol/l, $C_{14:1}$-carnitine 0.75 µmol/l).

Prolonged fasting was associated with increasingly abnormal excretion patterns of organic acids, with *N*-hexanoylglycine, and dicarboxylic acids in the urine of all subjects with variant *ACADM* genotypes during fasting, as opposed to the observations during regular visits to the outpatient clinic, when few or no abnormal metabolites were detected.

PPA loading test

The fasting tests were combined with a PPA loading test. After oral administration of PPA, no phenylpropionylglycine (PP-glycine) or trace amounts were excreted in the urine, together with large amounts of hippuric acid.

DISCUSSION

Since the introduction of NBS for MCAD deficiency, a new subgroup of newborns has been identified with variant *ACADM* genotypes that have not been seen before in clinically ascertained patients with classical *ACADM* genotypes. It remains unclear whether subjects with these variant *ACADM* genotypes are at risk for the development of a clinical phenotype. Prevention of prolonged fasting was found to be debatable when MCAD enzyme activities >10% were measured with PP-CoA [2]. In the current study, additional support was provided to abandon the advice on prevention of prolonged fasting under

normal conditions in subjects with residual MCAD enzyme activities >10%. All included subjects could tolerate an overnight controlled fasting tolerance test for at least 15 hours under healthy conditions. An additional PPA loading test determined *in vivo* residual MCAD enzyme activity. These functional tests were performed after the age of 6 months in all cases, when weaning naturally occurs and PPA loading tests can be performed reliably [14].

Several factors cause diversity between the subjects with variant *ACADM* genotypes that have been identified since introduction of NBS. Firstly, different *ACADM* genotypes are represented in this group. Secondly, the NBS protocol has been adapted in 2007, and has thus not been similar for all included subjects. Thirdly, treatment in the newborn period has not been the same in all subjects in this group. Identification of affected newborn siblings of probands was associated with dietary interventions from birth onwards in this group, instead of starting after positive NBS results.

Interestingly, two cases (case 3 and case 5) were identified upon NBS with C_8-carnitine concentrations below the current cut-off concentration of 0.50 µmol/l, despite *ACADM* mutations on both alleles. Case 3 was identified upon family screening and case 5 during the pilot NBS program. With the elevation of the C_8-carnitine cut-off concentration in 2007, the national criteria for subjects who should be regarded "patients" have also been adapted. However, due to family screening we still identify subjects with *ACADM* mutations on both alleles, but C_8-carnitine concentrations below the cut-off value. How can we interpret these observations? C_8-carnitine concentrations in newborns can theoretically be influenced by nutritional state [5], prematurity and/or very low birth weight [17], heterozygosity for the c.985A>G mutation [18], and the time at which NBS is performed [19]. In our cohort, nutritional state could have affected NBS C_8-carnitine concentrations, as these concentrations clearly correlated with percentage of maximum weight loss in the neonatal phase. Except for case 3, all subjects with a variant *ACADM* genotype in our cohort were firstborns who were breastfed. With breastfeeding caloric intake cannot be monitored, as opposed to during formula feeding. Especially in firstborns onset of lactation can be delayed, leading to suboptimal food intake in the first days of life [20]. Catabolism and subsequent increase in C_8-carnitine concentrations can result from this. In case 3, C_8-carnitine concentrations may have remained <0.50 µmol/l due to the positive family history and subsequent frequent feeding regimen that was started at birth.

Two subjects in our study (case 8 and case 9) displayed only one *ACADM* mutation after sequencing of the *ACADM* gene. Why have these subjects been identified upon NBS, as heterozygotes are usually not identified with the current C_8-carnitine cut-off concentration?[18] Case 8 and case 9 were not considered normal false-positives, and where included in the cohort based on the combination of (1) persisting abnormal plasma and urinary metabolites, and (2) residual MCAD enzyme activity determined with C_6-CoA. Results from fasting tolerance tests were remarkable in both subjects. In case 8, acylcarnitines with increasing chain-lengths up to C_{16}-carnitine accumulated upon fasting. The C_8-carnitine concentration increased considerably to a maximum of 4.7 µmol/l, whereas the C_8/C_{10} remained <2.5. Theoretically, these findings may indicate another phenotype-modifying mutation on a gene that plays a role in mFAO, a phenomenon known as synergistic heterozygosity [21,22]. Vockley *et al.* described the concept of synergistic heterozygosity, i.e. multiple partial defects in more

than one metabolic pathway, leading to clinical symptoms that correlate with the affected pathways [22]. In combination with the classical c.985A>G *ACADM* mutation, theoretical candidate genes to cause accumulation of acylcarnitines with medium and long chain-length are *ETFA* (HGNC:3481), *ETFB* (HGNC:3482), *ETFDH* (HGNC:3483), *ACADS* (HGNC:90) or *ACADVL* (HGNC:92) especially during episodes with increased catabolic stress such as during prolonged fasting. In case 8, $C_{14:1}$-carnitine concentrations increased to a maximum of 0.75 µmol/l upon prolonged fasting, with a corresponding $C_{14:1}/C_{16}$ ratio of 4.4. The interpretation of these findings is complicated as, currently, no pediatric reference values for acylcarnitines during prolonged fasting are available. In case 9, the *in vivo* and *in vitro* observations might be explained by other molecular mechanisms (e.g. deep-intronic mutations, mutations in the promoter region, and deletion/duplication mutations) that can be missed upon gene sequencing, next to synergistic heterozygosity [23,24].

We observed a normal fasting tolerance in subjects with variant *ACADM* genotypes and residual MCAD enzyme activities >10% under normal conditions after the age of 6 months. Based on these results, the need to prescribe a standard late evening meal in these subjects is debatable. PPA loading tests cannot be reliably performed before the age of 6 months [14]. As PPA loading tests were in this study combined with the fasting tolerance tests, no data are currently available on fasting tolerance in subjects with variant *ACADM* genotypes and residual MCAD enzyme activities >10% before the age of 6 months. It is well known that even patients with classical *ACADM* genotypes can tolerate overnight fasting already at a young age [6,25]. Besides, stable isotope studies demonstrated normal FFA and KB metabolism in patients with classical *ACADM* genotypes [26,27]. Administration of a late evening meal has several disadvantages: First of all, it can be considered a burden for both parents and the child, especially in the first year of life when the maximum duration of fasting is 6-8 hours [28]. Additionally, administration of a late evening meal increases the risks on overfeeding and dental caries [6,29]. In our opinion, abovementioned arguments, combined with the current data, justify abolishing a standard late evening meal in subjects with variant *ACADM* genotypes and residual MCAD enzyme activities >10% after the age of 6 months. *In vivo* functional tests can facilitate development of individualized patient-based guidelines for follow-up reliably from the age of 6 months. However, until the pathophysiology of MCAD deficiency and its variants is unravelled, an emergency regimen and parental instructions remain indispensable in the follow-up of all subjects with a positive NBS for MCAD deficiency.

CONCLUSIONS

Variant *ACADM* genotypes with residual MCAD enzyme activities >10% *in vitro* are associated with normal residual MCAD enzyme activities *in vivo* (defined by PPA loading) and normal fasting tolerance. The general advice to prevent prolonged fasting can be abolished in subjects with residual MCAD enzyme activities >10% after the age of 6 months. However, an emergency regimen and parental

instructions remain necessary in all subjects with MCAD deficiency, regardless of residual MCAD enzyme activity, at least until the pathophysiology of MCAD deficiency has been elucidated.

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Chapter 4

Abnormal energy balance during endurance exercise in quadriceps muscle of patients with MCAD deficiency

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Work in progress

ABSTRACT

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common defect in mitochondrial fatty acid oxidation. A subset of the adult patient population has subjective complaints of fatigue, muscle pain and/or reduced exercise tolerance. Here, we investigated if any abnormalities in muscle ATP metabolism in MCAD deficiency may underlie these complaints.

Eight patients with MCAD deficiency (all homozygous for the c.985A>G mutation) and 7 age- and gender-matched healthy controls performed an endurance task consisting of 80 minutes of exercise at 45% W_{max} on an upright bicycle ergometer, immediately followed by dynamic ^{31}P-MRS analysis during a 10-minute exercise bout inside a 3.0T MR scanner on a supine MR-compatible bicycle ergometer at an equivalent workload. Blood samples were obtained at various time points in the protocol for metabolite analysis.

Three patients could not complete the 80-minute exercise task due to symptoms of extreme fatigue. Blood metabolite profiles over the entire protocol revealed significantly lower ketone body, total carnitine and C_0-carnitine concentrations in the patient group when compared to healthy controls. In the subsequent 10-minute exercise bout inside the MR scanner, accumulation of Pi and decrease in PCr concentrations in exercising quadriceps muscle were found to be significantly greater in the patient group compared to controls. Pi recovery dynamics were similar in patients and controls. Quadriceps pH during exercise was more heterogeneous in patients than in healthy subjects, ranging from moderate muscle acidification (-0.2 pH units) in two patients to muscle alkalinization (+0.2 pH units) in a single patient.

We have obtained evidence for impaired muscle function in human MCAD deficiency. ^{31}P-MRS recordings in quadriceps muscle revealed an abnormal energy balance during exercise in the patients with MCAD deficiency. Mitochondrial ATP synthetic function was normal, as evidenced by unaltered recovery dynamics in patients compared to healthy controls. A moderate shift in fiber-type composition from slow-twitch oxidative fibers towards fast-twitch glycolytic fibers may explain these findings.

INTRODUCTION

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common inborn error of mitochondrial fatty acid oxidation (mFAO) [1]. Patients usually present with a hypoketotic hypoglycemia upon periods of catabolic stress. The clinical spectrum can vary from sudden infant death in the neonatal phase, to remaining asymptomatic throughout life [2]. The pathophysiology of MCAD deficiency remains enigmatic, and genotype-phenotype correlations have not been identified. After diagnosis, prognosis is excellent when prolonged fasting is prevented, and an emergency regimen is administered in case of catabolic stress, such as during intercurrent illness [3]. In some patients, L-carnitine is prescribed for secondary low free carnitine (C_0-carnitine) concentrations in plasma. However, evidence for this treatment is limited [4]. Complaints of reduced exercise tolerance (e.g. due to muscle pain or fatigue during or after exercise) are commonly reported in the patient population [2]. Yet, any functional impairment of muscle in MCAD deficiency has thus far remained to be objectified. Two investigations in respectively 5 and 4 patients with MCAD deficiency reported normal exercise capacity at various levels of intensity (i.e. a 2-hour exercise test at 60% of predicted maximal heart rate based on age, and a short incremental ramp test of a maximum of 15 minutes, respectively) after an overnight fast [5,6]. However, another investigation in 4 patients with MCAD deficiency reported impaired total fatty acid oxidation and palmitate oxidation *in vivo* upon a 1-hour cycling task at a workload of 55% of maximal oxidative capacity (VO_2 max) [7]. The aim of this study was to further investigate exercise tolerance and intramuscular energy balance during exercise in MCAD deficiency using ^{31}P-MRS. Eight patients with MCAD deficiency and 7 age- and gender-matched controls were subject to an endurance exercise challenge consisting of a total of 90 minutes bicycling exercise at a workload corresponding to 45% of the maximal workload (W_{max}). The last ten minutes of the bicycling exercise test were performed in supine position inside a magnetic resonance scanner during which ^{31}P-MRS spectra were recorded serially from the quadriceps muscle.

METHODS

Subjects

The Medical Ethical Committee of the University Medical Centre Groningen approved of the study (METc 2012/262). After written and verbal explanation of the study aims, study procedures and risks, subjects gave written consent for participation. Eight adult patients with MCAD deficiency (all homozygous for the c.985A>G mutation and diagnosed after clinical presentation), and seven healthy gender- and age-matched controls were included in this study. All included patients with MCAD deficiency showed metabolite profiles with elevated concentrations of medium-chain acylcarnitines and C_8/C_{10} ratios >10, corresponding to their *ACADM* genotype. Except for MCAD deficiency, all participating patients were healthy, as were all participating controls. Exclusion criteria besides the presence of a disease other than MCAD deficiency were *1)* MCAD deficiency due to a genotype other than the

c.985A>G/c.985A>G *ACADM* genotype, *2)* general contra-indications for MR-analysis (i.e. the presence of metal objects in the body), and *3)* the use of hormonal therapy affecting glucose metabolism.

Training history was analyzed based on the Physical Activity History (PAH) questionnaire [8]. Subjects were asked to abstain from food intake 3 hours before both study sessions, and stop vitamin supplementation at least 2 weeks prior to the tests. Patients who received L-carnitine or riboflavin supplementation were also asked to stop supplementation at least 2 weeks prior to the tests. In the week prior to ^{31}P-MRS analysis, subjects kept a food diary, in order to determine dietary habits and the percentage of fat and carbohydrate intake in the individual diet per subject. Diaries of the food intake on the day before ^{31}P-MRS analysis were calculated using the Dutch Food Composition Table version 2011 (NEVO-table 2011, RIVM/Voedingscentrum, Den Haag 2011).

Study protocol – Cardiopulmonary exercise test (CPET)

Each subject participated in two exercise sessions: a CPET to determine W_{max} and VO_2 max (study session 1), and an endurance test at moderate intensity (45% of W_{max}) combined with ^{31}P-MRS analysis (study session 2). At least 2 days without measurements were scheduled between both sessions. The first session consisted of an exhaustive incremental exercise test on a braked bicycle ergometer (Jaeger, Wurzburg, Germany) to determine W_{max} and VO_2 max at the pediatric functional test ward of the Beatrix Children's Hospital, University Medical Centre Groningen, Groningen, The Netherlands. Participants performed exercise on a braked upright bicycle ergometer with increasing load, until exertion. Expected maximum load and the rate at which load was increased were determined based on anthropometric measures (and calculated with standard calculations provided by the supplier of the braked upright bicycle ergometer). We aimed at reaching the expected maximum load within 10 minutes. Subjects cycled at a speed of 60 rpm. During the test, subjects were fitted with a 12-lead ECG, a facemask for analysis of respiratory gas exchange, a cuff for automatic blood pressure analysis on the right upper arm, and an oximeter on the index finger. Forced expiratory volume in 1 second and forced vital capacity were determined during a pulmonary function test prior to the incremental exercise test. Anaerobic threshold was determined from the inclination in the VO_2/VCO_2 plot, and was displayed as a percentage of determined VO_2 max.

Study protocol – Endurance test at moderate intensity

I: 80-minute upright bicycling task.

Subjects exercised outside the MR scanner for 80 minutes on a braked bicycle ergometer (Corival, Lode, The Netherlands). Cycling load was set at 45% of predetermined W_{max}, which is considered to be an exercise burden with high fatty acid utilization [9]. Subjects cycled with a speed of 60 rpm, corresponding to the cycling speed at which VO_2 max was determined. Heart rate was monitored and documented every 15 minutes, and subjects had free accessibility to water. At the start of the endurance test (t=0), after 40 minutes of exercise (t=40), and at the end of study session 2 small blood

samples were obtained from an intravenously placed cannula for determination of glucose, free fatty acids (FFA), ketone bodies (KB), and acylcarnitines. Samples were stored at -80° until further analysis.

II: 10-minute supine bicycling inside MR scanner.
Immediately after the 80 minutes of moderate-intensity exercise outside the MR scanner, subjects changed into bicycle racing shoes equipped with plastic binding plates, and were moved to the adjacent MR scanner. In the MR scanner they completed the final 10 minutes of the endurance exercise task in supine position on an MR-compatible bicycle ergometer [10]. The braking weight, W (in kg), for the MR-compatible bicycle ergometer was individually determined at 45% of W_{max}, according to (eqn (1)):

$$W = W_{max}/275 \times 0.45 \times 5.5 \qquad \text{eqn (1)}$$

where W_{max} = maximum workload determined by the CPET, 275 corresponds to the mean W_{max} of an untrained healthy person during a CPET, 0.45 corresponds to the target 45% W_{max} workload, and 5.5 corresponds to the typical weight (in kg) that reproducibly causes fatigue to exhaustion in healthy untrained subjects on this particular supine MR-compatible bicycle ergometer (unpublished data). Subjects cycled at a speed of 60 rpm upon auditory instruction by a metronome and supervision by a coach inside the scanner room.

Technical aspects and data collection - ^{31}P-MRS

All ^{31}P-MRS measurements were conducted in a 3.0T whole body MR scanner (Intera; Philips Medical Systems, Best, The Netherlands). An MR-compatible bicycle ergometer described in detail elsewhere [10,11] was mounted on the front-side of a dedicated scanner table. Subjects were positioned on the table head-first in supine position, supported by a wedge-shaped pillow under the head and upper back, and attached to the ergometer pedals by cleat bindings. During exercise, subjects stabilized their body by holding on to handlebars adjusted to their individual specifications. A 6 cm diameter single-turn ^{31}P-surface coil was fastened over the quadriceps muscle (m. vastus lateralis) of the right leg using Velcro straps. Subjects first performed a short practice bout of bicycling in unloaded brake state to familiarize them with bicycling inside the magnet bore, after which the right upper leg was stabilized in extended position by heel fixation and supportive cushions. A series of ^{1}H-MR images was then acquired to evaluate correct positioning of the surface coil over the vastus lateralis muscle and subsequent image-based shimming of the magnetic field over the muscle volume sampled by the surface coil. Next, a resting ^{31}P-MRS spectrum was acquired from the m. vastus lateralis using an adiabatic half-passage excitation pulse (repetition time (TR) 3 s, 24 summed free induction decays (FIDs), spectral bandwidth 3,000 Hz 1024 points). During exercise (10 minutes) and subsequent recovery (15 minutes), ^{31}P-MRS spectra were acquired serially with a time resolution of 6 s (TR 3 s, 2 summed FIDs, spectral bandwidth 3,000 Hz, 1024 points). Spectral acquisition during exercise was gated to the cyclic motion of the leg as described previously [10].

^{31}P-MRS data processing and quantification

Free Induction Decays (FIDs) were processed and analyzed in the time domain using the AMARES algorithm in the public jMRUI software environment (version 3.0) as described elsewhere [10]. Kramer-Rao bounds of the AMARES lorentzian model fitting were used as statistical information on accuracy of peak area estimation. Absolute PCr and Pi concentrations were calculated after correction for signal saturation and assuming total adenylate nucleotide and creatine pool sizes of 8.2 and 42.7 mM, respectively, as previously described [12]. Intramuscular pH was determined from the resonance frequency of Pi using standard methods [10]. Steady-state PCr and Pi concentrations during exercise were determined from summed FIDs collected minimally 60 s after onset of bicycling. Datasets were analysed in a blinded fashion.

Metabolite analysis

Glucose was measured in plasma with the Biosen_C line (EKF Diagnostic, Surrey, United Kingdom) according to standard laboratory procedures. FFA and KB concentrations, i.e. total ketone body (TKB), 3-hydroxybutyrate, and acetoacetate concentrations, were determined with the Vitalab E Selectra (reagents from DiaSys, Holzheim, Germany) according to protocols provided by the manufacturer. Acylcarnitines were analyzed by tandem mass spectrometry according to Derks *et al.* [13].

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego California USA; www.graphpad.com) and SPSS version 20 (International Business Machines Corp., Armonk New York, USA). Normality of the data was assessed using the Shapiro-Wilk normality test. Differences between the complete group of patients and controls were analyzed two-tailed with either the unpaired Student's t-test (in case of normally distributed data) or Mann-Whitney test (in case of non-normally distributed data). Differences between parameters that were measured repeatedly at different time points in either the patient group or the control group were tested with the Wilcoxon's matched pairs test. Correlations were analyzed with either Pearson's analysis (in case of normally distributed data) or Spearman's analysis (in case of non-normally distributed data). Differences were considered statistically significant at $p < 0.05$. Timecourses of variables were analyzed and characterized kinetically using non-linear curve-fitting (Origin 6.0, Caltech Pasadena, US). Whenever possible, statistical information on data accuracy was incorporated in the curve-fitting by means of statistical weighting.

RESULTS

Subjects

Baseline characteristics of the patient group and the control group are presented in Table 1. Upon inclusion, four of the included patients (all female, #1-4, Table 1) reported subjective complaints of lowered exercise tolerance and muscle pain when performing sports. The main complaint was pain or a burning

sensation in the upper legs and reduced endurance capacity as a result. There were no reports of rhabdomyolysis. The other four patients (2 male, 2 female, #5-8, Table 1) reported no complaints and could tolerate both short- and long-term exercise. All subjects were considered to be low to moderately trained based on the Physical Activity History (PAH) questionnaire [8]. Caloric intake was normal in all included subjects. The percentage of fat and carbohydrate in the total diet was similar in patients and controls.

Table 1. Baseline characteristics of participants in rest.

Subject no.	Age (yr)	Gender	MCAD deficiency	Muscular complaints	BMI	HR/min	Activity (MET/wk)
1	20	F	Y	Y	26.0	92	72
2	21	F	Y	Y	24.3	121	504
3	18	F	Y	Y	23.4	73	0
4	43	F	Y	Y	32.4	87	0
5	24	M	Y	N	25.4	55	288
6	20	M	Y	N	27.6	93	360
7	23	F	Y	N	20.6	80	396
8	21	F	Y	N	24.0	73	504
Patients	21 (18-43)	2 M, 6 F	-	-	24.8 (20.6-32.4)	83.5 (55-121)	324 (0-504)
Controls	22 (18-43)	1 M, 6 F	N	N	23.0 (19.7-29.8)	95 (68-108)	216 (72-324)

Characteristics of the patients are presented in separate rows. For the control group only medians and ranges are presented (bottom row). F, female; M, male; Y, yes; N, no; HR, heart rate. 0 MET/week for activity indicates no reported regular participation in physical activity

Exercise testing

I: CPET

All subjects completed the short and high-intensity CPET without any clinical symptoms or subjective complaints of lethargy or muscle pain. W_{max} was as expected in all untrained and moderately trained subjects, based on standard calculations provided by the manufacturer of the bicycle ergometer taking gender, age, height, and weight into account. W_{max}, VO_2 max, and AT were similar in the patient and control group (Table 2).

II: Endurance exercise test

Cycling for 80 minutes at moderate intensity (45% W_{max}) on the braked upright bicycle ergometer was well tolerated by all healthy controls. In the patient group, however, 3 out of the 8 patients (subjects #1, 4, and 8) experienced signs of extreme fatigue and muscle pain in the upper leg during the endurance exercise. These three subjects had to prematurely end the endurance exercise, after respectively 65 (subject #1), 40 (subject #4), and 75 (subject #8) minutes of exercise (Figure 1A). Due

Table 2. Results of the CEPT (study session 1).

Subject no.	VO_2max (ml/kg/min)	VO_2max (ml/min)	W_{max}	AT (% pred. VO_2max)	AT (% own VO_2max)
1	35.9	2765	190	65%	52%
2	27.9	1912	162	52%	58%
3	33.9	2500	202	68%	62%
4	25.3	2350	178	70%	55%
5	42.4	3267	260	62%	61%
6	27.7	2719	322	47%	66%
7	n.d.	n.d.	218	n.d.	n.d.
8	38.6	2995	258	83%	63%
Patients	33.9 (25.3-42.2)	2719 (1912-3267)	210 (162-322)	65% (47-83%)	61% (52-66%)
Controls	36.9 (32.2-50.5)	2526 (2135-3740)	214 (178-290)	67% (57-118%)	66% (53-80%)

Characteristics of the patients are presented in separate rows. For the control group only medians and ranges are presented (bottom row). AT, anaerobic threshold.

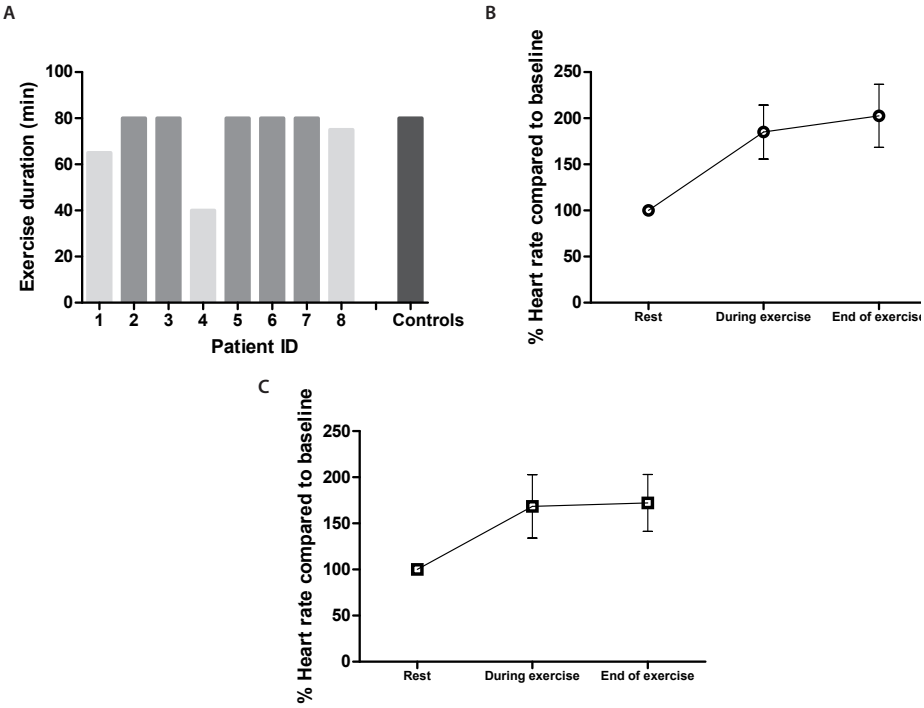

Figure 1. Exercise endurance in the group of MCAD deficient patients. Exercise endurance in minutes is depicted per patient (A). Black bars indicate patients who could complete the 80-minute exercise bout. Mean increase in heart rate during prolonged exercise in patients (B) and controls (C) is depicted. Error bars indicate the SD. Baseline heart rate is set at 100%. Heart rate during exercise is obtained after 45 minutes of exercise, heart rate at the end of exercise is obtained at termination of the exercise task.

to the premature termination of the prolonged exercise in these three patients, they were regarded patients with 'observed complaints of reduced exercise tolerance' during further analyses in the patient subgroups. The increase in heart rate during exercise was similar in the patient and the control group (p=0.15)(Figures 1B,C). The increase in heart rate in the patients with observed complaints of reduced exercise tolerance during the prolonged exercise task at moderate intensity did not differ from what was observed in the patient group without complaints.

Metabolic parameters during endurance exercise testing

I: Plasma metabolites

All subjects retained normoglycemia during the study sessions, and glucose concentrations could not be related to complaints during the 80-minute exercise bout. Glucose concentrations were similar in the patient and control group (Table 3). Concentrations of plasma FFA were comparable in both groups at all time points and increased upon exercise. In the patient group, concentrations of FFA were significantly higher post-exercise when compared to baseline (p<0.05, Table 3). Plasma TKB concentrations increased in both the patient and the control group upon exercise. After the prolonged moderate intensity exercise outside and inside the MR scanner, TKB concentrations were significantly lower in the patient group when compared to the control group (p<0.05, Table 3). Concentrations of 3-hydroxybutyrate were also significantly lower in the patient group (median MCAD deficient group 0.12 mmol/l, range 0.04 – 0.48 mmol/l and median control group 0.38 mmol/l, range 0.17 – 0.78

Table 3. Plasma metabolites during prolonged exercise test.

METABOLITE	SUBJECTS	TEST		
		Rest (t=0 min)	During exercise (t=40 min)	Post-exercise
Glucose	Controls	4.6 (4.0 – 5.4)	5.0 (4.2 – 7.4)	4.5 (3.9 – 4.8)
(mmol/l)	Patients	4.8 (4.5 – 5.3)	4.7 (5.2 – 4.1)	4.5 (3.7 – 5.5)
Free fatty acids	Controls	623 (228 – 938)	852 (666 – 1319)	773 (586 – 1183)
(µmol/l)	Patients	441 (103 – 1279)	888 (410 – 1829)	1038 (782 – 1371) **
Ketone bodies	Controls	0.08 (0.02 – 0.43)	0.17 (0.07 – 0.46)	0.50 (0.21 – 0.95) $
(mmol/l)	Patients	0.04 (0.02 – 0.36)	0.09 (0.03 – 0.33)	0.21 (0.05 – 0.69) $*
C_0-carnitine	Controls	32.0 (28.0 – 64.0)	29.0 (27.0 – 57.0) $	25.0 (22.0 – 41.0) $
(µmol/l)	Patients	12.5 (6.0 – 17.0) **	12.5 (9.0 – 24.0) **	15.0 (12.0 – 25.0) $**
C_2-carnitine	Controls	6.4 (3.0 – 7.3)	7.6 (5.6 – 9.6) $	9.5 (8.1 – 14.9) $
(µmol/l)	Patients	0.9 (0.5 – 2.3) **	1.8 (0.7 – 3.0) $**	3.6 (1.6 – 3.9) $**
Total carnitine	Controls	38.0 (33.0 – 73.0)*	40.0 (35.0 – 70.0)	37.0 (33.0 – 58.0)
(µmol/l)	Patients	20.0 (10.0 – 31.0)	28.5 (14.0 – 40.0) $*	25.0 (21.0 – 43.0) $*

Plasma metabolites at different time points during the prolonged exercise test. Medians and ranges are depicted. * Significantly different from control (p<0.05), ** Significantly different from control (p<0.01), $ significantly different from t=0 (p<0.05).

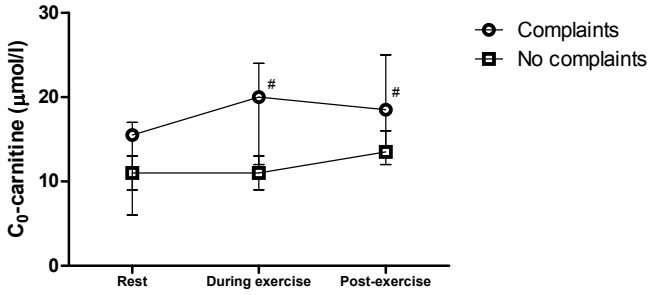

Figure 2: C_0-carnitine concentrations per patient group before, during and after prolonged moderate intensity exercise. Median C_0-concentrations in the patient group with complaints (open circles) and the patient group without complaints (open squares). Error bars indicate the range. # trend towards a difference from the patient group without subjective complaints (p<0.1).

mmol/l, respectively post-exercise, p<0.05). Concentrations of acetoacetate did not differ between the patient and control group.

At baseline, C_0-carnitine concentrations were significantly lower in the patient group compared to controls (p<0.01, Table 3). Concentrations of C_0-carnitine decreased significantly in the control group, but increased significantly in the patient group (Table 3). When C_0-carnitine concentrations in the patient group with complaints were compared to the patient group without complaints, there was a trend towards lower C_0-carnitine concentrations upon exercise and recovery in the latter group (p<0.1, Figure 2). Total carnitine concentrations were significantly lower in the patient group when compared to controls at all studied time points (p<0.05, Table 3). Total carnitine concentrations increased significantly upon exercise in the patient group (p<0.05, Table 3), but remained stable in time in the control group. Total carnitine concentrations correlated strongly to C_0-carnitine concentrations (Pearson R squared=0.94, p<0.001). Concentrations of C_2-carnitine were significantly lower in the patient group at all time points (p<0.01, Table 3).

II: Muscle

II.1. Quadriceps energy and proton balance during endurance exercise test

Intramuscular PCr and Pi concentrations in resting quadriceps muscle of the right leg of MCAD deficient patients were not significantly different from healthy controls (Figure 3). Figure 4A shows ^{31}P-MRS spectra acquired from the quadriceps muscle during supine in-magnet bicycling exercise at a workload equivalent to 45% W_{max} for a healthy control subject versus a patient. The amplitude of the Pi resonance compared to ATP appeared to be elevated in the patient compared to the healthy subject. This observation was objectified by the results of the spectral analysis: the average rise in intramuscular Pi concentration and concomitant decrease in intramuscular PCr concentration was almost threefold higher in the patient group compared to the control group (p<0.05, Figures 4B and C, respectively). The magnitude of these changes in Pi and PCr during exercise did not differ between

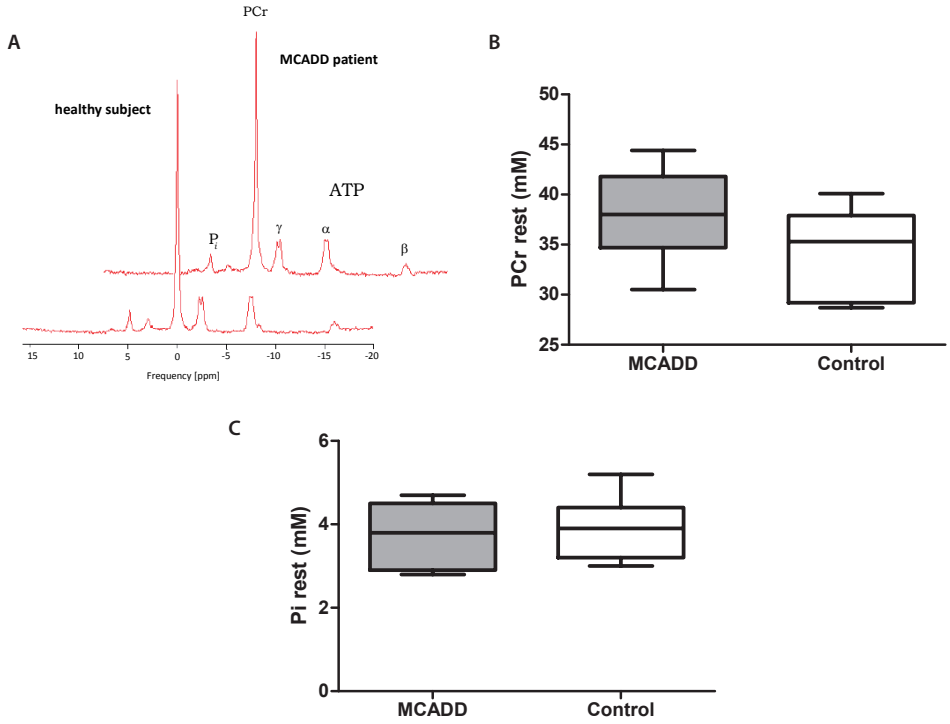

Figure 3. Stationary states of PCr and Pi in rest. Typical ^{31}P-spectra observed in patients and controls in rest (A), PCr (B) and Pi (C) in rest at baseline are depicted. Boxes represent the median and range in the patient (grey) and control (white) groups.

the group of patients with observed complaints of reduced exercise tolerance compared to the patients who could complete the 80-minute exercise test (data not shown).

Quadriceps muscle acidified moderately during the 10-minute supine bicycling exercise bout in all but one healthy control subjects (range 6.85-6.95; Figure 5). In the MCAD deficient patients, pH changes in exercising quadriceps muscle were more heterogeneous. In four out of seven patients quadriceps pH remained neutral (i.e. pH 6.99 – 7.06), versus mild acidification in two patients (-0.2 pH units) and a surprising alkalinization (+0.2 pH units) in one patient (Figure 5).

II.2. Metabolic recovery from exercise

Figures 6A and B show the time course of PCr and Pi levels, respectively, in quadriceps muscle of a patient during recovery from the 10-minute in-magnet exercise bout. Typically, the kinetics of metabolic recovery from exercise were best evaluated by tracking and analyzing the time course of recovery of Pi signal to basal levels. The Pi recovery rates (τPi) were not significantly different between the patient and control group (Figure 6C). Recovery rates of the Pi signal were also similar in the group of patients with observed complaints of reduced exercise tolerance compared to the group of patients

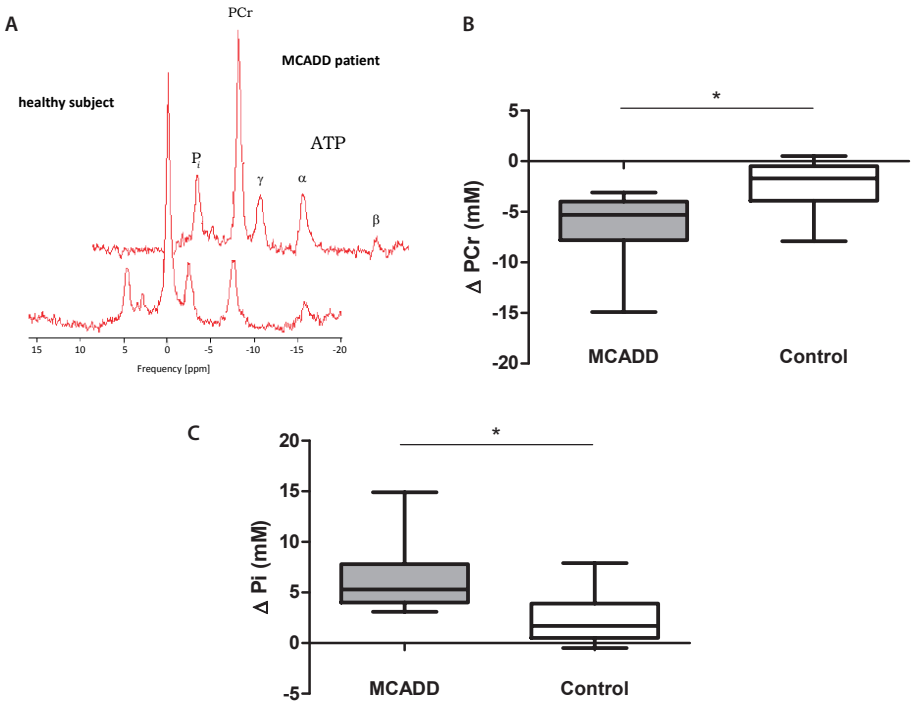

Figure 4. Intramuscular energy metabolites during in-magnet exercise. Typical ^{31}P-spectra observed in patients and controls during exercise (A). Figures B and C depict the changes in PCr (B) and Pi (C) concentrations upon exercise for the pooled group of patients and controls. Boxes represent the median and range in the patient (grey) and control (white) groups.

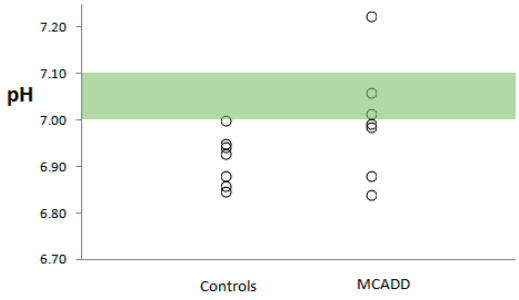

Figure 5. Quadriceps pH during exercise. Mean intramuscular pH during exercise in the patient and control groups is depicted. Normal pH range during rest is indicated in grey.

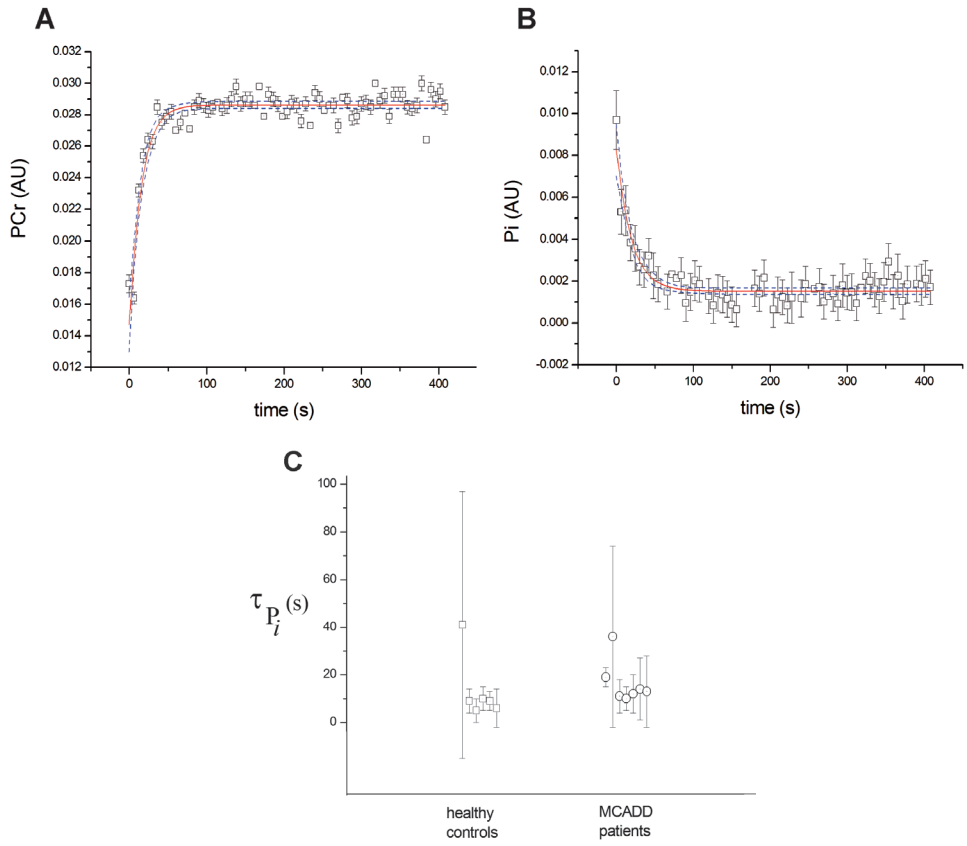

Figure 6. Recovery of PCr and Pi in a patient after in-magnet exercise. τPCr (A), and τPi (B) data of patient #8 are depicted as a typical example of the ^{31}P-spectra obtained in patients upon recovery. Comparison of τPi in patients and controls in depicted in Figure C. Recovery is depicted in seconds.

without observed complaints. With respect to the dynamics of quadriceps pH during recovery, no major differences were found between patients and healthy control subjects (data not shown). In patient #4 who developed a mildly alkaline muscle pH during exercise, pH recovered to the normal resting value upon recovery.

DISCUSSION

In this study, we addressed the question whether complaints of reduced exercise tolerance (e.g. muscle cramps or muscle fatigue during or after exercise) commonly reported in patients with MCAD deficiency could be objectified by finding a substrate underlying these complains. Eight MCAD de-

ficient patients with and without subjective complaints of reduced exercise tolerance were studied with ^{31}P-MRS during prolonged moderate intensity exercise and compared with age- and gender-matched healthy subjects. This is the first study that presents concrete evidence for abnormal energy metabolism in quadriceps muscle of patients with MCAD deficiency during prolonged exercise at moderate intensity. Three out of eight patients failed to complete the 80-minute exercise task at 45% W_{max}, of which two had reported subjective complaints of lowered exercise tolerance upon inclusion for this study. In contrast, all patients were able to complete the short-term exercise test at high intensity to estimate W_{max} and VO_2 max. In plasma, concentrations of FFA and of KB responded differently in patients when compared to controls. During a subsequent 10-minute bout of in-magnet exercise at a normalized submaximal workload, we observed a greater rise of Pi and concomitant greater decline in PCr concentration in patients compared to healthy controls. The kinetics of post-exercise metabolic recovery, assayed by the time constant of Pi recovery, did not differ between patients and healthy controls, ruling out dysfunctional mitochondrial ATP synthesis. Energy metabolism in muscle of patients with MCAD deficiency was abnormal during endurance exercise at moderate intensity. Below, the performance during the exercise tests will be discussed first, followed by a discussion of the observed changes in plasma metabolites concentrations and the MRS results.

Exercise performance in MCAD deficiency

In humans, striated muscle consists of two different types of muscle fibers: Type I 'slow-twitch' oxidative fibers, and type II 'fast-twitch' glycolytic fibers [14]. The type II fibers can be further subdivided in fast-twitch oxidative glycolytic fibers (type IIA), and fast-twitch anaerobic glycolytic fibers (type IIB). Type IIA fibers are relatively fatigue resistant, whereas the type IIB fibers are fast fatigable [15]. In human m. vastus lateralis, 50% of muscle fibers are type I fibers (slow oxidative), 35% are type IIA (fast oxidative), and ~15% type IIB fibers (fast glycolytic) [16]. During prolonged exercise at moderate intensity, such as applied in the present study, muscle power-output is produced mainly by type I oxidative fibers [15,17], for which fat is the predominant and most efficient source of energy (energy yield from 1 palmitate: 129 ATP) [18]. Type II fibers are recruited during any exercise at higher intensity [15,17]. Defect mFAO in case of MCAD deficiency can impair energy extraction from fat by type I and to a lesser extent by type IIA muscle fibers, leading to problems particularly during prolonged exercise at moderate intensity. All patients had the same *ACADM* genotype and all were able to complete the 10-minute incremental exercise cycling test, a short-term high-intensity test, without the development of any clinical symptoms. Three out of 8 patients were unable to complete the 80-minute prolonged exercise test at 45% of W_{max}, and showed altered but subject-dependent intramuscular pH dynamics. This indicates the importance of the type of test for evaluation of the exercise tolerance in these patients. Remarkably, not all patients with subjective complaints of reduced exercise tolerance failed to complete the prolonged exercise test. Two out of the four patients with subjective complaints of reduced exercise tolerance and one out of the four without subjective complaints failed the prolonged exercise test.

The groups of patients with subjective and objective complaints of lowered exercise tolerance consisted solely of women. This is in line with results that were described by Maher *et al.*, showing

that women have higher protein content of mFAO enzymes (including MCAD) in skeletal muscle, and oxidize more fat and less glucose during prolonged exercise when compared to men [19–21]. As a result, dependence on type I oxidative muscle fibers in order to be able to sustain prolonged exercise may be larger in women. Due to the low number of included patients, we were not able to objectify an effect of gender, however our observations of extreme fatigue in a subset of the women in our patient population during the endurance test may be indicative.

Plasma metabolite dynamics

In plasma of patients we observed higher concentrations of FFA and lower concentrations of KB compared to healthy controls during prolonged exercise at moderate intensity. MCAD deficiency results in impaired mFAO in all tissues. High FFA and low KB concentrations in plasma of patients with MCAD deficiency under fasting conditions in rest are generally assumed to result from impaired ability of the liver to oxidize fatty acids and produce KB. During prolonged moderate-intensity exercise muscle uses mainly mFAO for energy generation. In muscle of patients with MCAD deficiency mFAO is impaired and enhanced oxidation of KB may be able to compensate for this defect. The low concentrations of KB in MCAD deficiency during exercise observed here may thus result from low rates of production by the liver combined with high rates of consumption in exercising muscle. If this hypothesis is correct, supplementation of KB, might offer a possible therapy to improve exercise tolerance in these patients.

Muscle energy metabolism dynamics

The ^{31}P-MRS measurements during in-magnet exercise showed a larger decline in the intramuscular PCr levels, and a higher accumulation of intramuscular Pi in MCAD deficient patients when compared to healthy controls. These findings indicate altered energy balance in MCAD deficient quadriceps muscle during exercise. This altered energy balance may either result from a higher ATP turnover rate during the cyclic contraction and relaxation of muscle fibers during exercise, from a compromised capacity for ATP production due to mitochondrial impairment, or from a combination of both. Mitochondrial function was assayed independently by measuring the rate of recovery of PCr and Pi to resting levels following exercise [22]. In view of the small absolute changes in PCr and Pi during the preceding moderate exercise bout (Figure 4) together with the empirical fact that the Pi dynamics in exercising quadriceps muscle typically show an undershoot during recovery [23], these kinetics were determined in the present study using the Pi data rather than the PCr data. Since Pi is a direct substrate for the mitochondrial F1-ATPase the dynamics can be directly linked to mitochondrial function [18,24]. No evidence for mitochondrial impairment in MCAD patients was found in the recovery data (Figure 6). Therefore, the significantly different energy balance in quadriceps muscle that we observed in MCAD deficient patients exercising at the same normalized workload as healthy controls (i.e. 45% W_{max}; Figure 4) may be explained by a higher average ATP turnover rate in the active muscle mass of the quadriceps muscle in MCAD deficient patients than in healthy controls.

The average ATP turnover rate in quadriceps muscle during voluntary exercise at a particular power-output is determined by two variables: *(i)* the fiber-type composition of the muscle and

(ii) the number of quadriceps motor units recruited to produce the desired power-output. In the healthy control subjects, this resulted in a highly reproducible average change in steady-state Pi and PCr concentrations over the quadriceps muscle mass sampled by the surface coil (Figure 4). These concentrations represented the average of Pi and PCr concentrations in inactive fibers and active fibers recruited to produce the power-output corresponding to 45% of W_{max} determined during the CPET (median W_{max} 214 W in healthy subjects; Table 2). This particular workload was chosen under the assumption that it would only result in recruitment of the pool of type I motor units that make up 50% of the muscle. As such, the finding of threefold bigger changes in average Pi and PCr concentrations than control in quadriceps muscle of the MCAD deficient patients performing the same nominal exercise task suggests that in patients a significant fraction of type II motor units was additionally recruited to generate the required leg muscle power-output. Since the ATP cost of contraction of type II fibers is at least threefold higher than for type I fibers [22], this would thus explain our findings. The reason for any need to recruit type II fibers in addition to type I fibers in patients may be either: *(i)* the oxidative capacity of type I fibers deficient in MCAD is reduced and thereby their capacity to sustain a particular level of power-output without any mechanical fatigue, or *(ii)* the absolute pool size of type I fibers in MCAD deficient muscle is reduced as an adaptive response to the molecular defect. Indeed, evidence for the latter scenario has been found in the very long-chain acyl-CoA dehydrogenase knockout (VLCAD-KO) mouse [25]. Specifically, a slow (type I)-to-fast (type II) shift in fiber type composition of the quadriceps muscle was found in this mouse model [25]. Importantly, this slow-to-fast shift in muscle fiber type composition had occurred independently of any regular exercise tasking suggesting the molecular defect by itself underlies this alteration [25]. In case of MCAD deficiency, the mFAO defect by itself may thus likewise constitute a trigger for a switch in muscle fiber type. Observations of Madsen *et al*. in patients with MCAD deficiency support the hypothesis on a switch in fiber type composition in muscle of patients with mFAO defects [7]. Specifically, during prolonged moderate-intensity exercise (i.e. a 1-hour exercise task at a workload of 55% of VO_2 max) a decreased rate of palmitate oxidation and an increased rate of carbohydrate oxidation were observed in MCAD deficient patients when compared to healthy controls. Under resting conditions, no differences were observed.

One of the consequences of additional recruitment of type II fibers during exercise in patients with MCAD deficiency could perhaps be that muscle pH may be expected to undergo a greater extent of acidification than in healthy control subjects. This was however not observed in this study. On the contrary, quadriceps pH during the 10-minute in-magnet exercise bout remained neutral in four out of seven patients compared to one out of eight control subjects (Figure 5). Muscle pH during exercise is determined by a number of variables including CO_2 and lactate production rate, as well as wash-out of these metabolic end products [23]. As such, it is difficult to interpret these particular findings in the absence of any data on muscle perfusion for each subject. The surprising finding in a single patient of a remarkable rise of quadriceps pH during exercise (+0.2 pH units; Figure 5) further illustrates the highly heterogeneous and illusive phenotypical presentation of MCAD deficiency in humans. This female patient had the poorest performance in the endurance exercise test, and had to terminate the task prematurely. Interestingly, a similarly alkaline muscle pH was previously observed in resting muscle of a patient with VLCAD deficiency

[26]. However, in that patient muscle pH decreased during exercise [26]. Alkalinization of muscle during exercise has previously only been reported in patients with glycogen storage disease (e.g. glycogen storage disease type III and type V) [27–29]. While the physiological basis for this finding in these types of glycogen storage disease is well understood, its basis is unclear for the presented case with MCAD deficiency.

Finally, the results of the present study also warrant a discussion of the clinical utility of L-carnitine supplementation in MCAD deficiency. A subset of the included patients received L-carnitine supplementation prior to participation in this study, and reported negative effects on exercise tolerance after termination of supplementation despite normal plasma C_0-carnitine concentrations. It is important to realize that plasma concentrations of C_0-carnitine are no reflection of intramuscular C_0-carnitine status, and that intramuscular C_0-carnitine concentrations can decrease considerably upon exercise, while plasma concentrations may remain unchanged. This has been reported in VLCAD-KO mice during exercise [30]. L-carnitine supplementation has not been found to upregulate total fatty acid oxidation upon exercise in MCAD deficient patients [7]. However, Lee *et al.* described that VO_2 max increased after 4 weeks of L-carnitine supplementation [5–7]. At this moment there are no evidence-based indications for L-carnitine supplementation in patients with MCAD deficiency.

CONCLUSIONS

In conclusion, this study shows for the first time an altered intramuscular energy balance in patients with MCAD deficiency during in-magnet exercise. During exercise, the decrease in intramuscular concentrations of PCr was larger, as was the increase in Pi concentrations in quadriceps muscle of MCAD deficient patients when compared to healthy controls. However, recovery of PCr and Pi occurred at a normal rate in MCAD deficient patients. Based on these results, we hypothesize that in MCAD deficiency intramuscular mitochondrial energy production is maintained, whereas substrate and product concentrations are changed in order to maintain the flux needed for energy production. Moreover, a switch in fiber type composition in muscle of MCAD deficient patients might add to this. So far, there is no evidence that L-carnitine supplementation can enhance exercise performance in general, and in particular in patients with MCAD deficiency. Supplementation of KB might offer an alternative to prevent complaints of fatigue in MCAD deficient patients.

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Chapter 5

Experimental evidence for protein oxidative damage and altered antioxidant defense in patients with medium-chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

The objective of this study was to test whether macromolecule oxidative damage and altered enzymatic antioxidative defenses occur in patients with medium-chain acyl coenzyme A dehydrogenase (MCAD) deficiency. We performed a cross-sectional observational study of *in vivo* parameters of lipid and protein oxidative damage and antioxidant defenses in asymptomatic, non-stressed MCAD-deficient patients and controls. Patients were subdivided into three groups, based on therapy: patients without prescribed supplementation, patients with L-carnitine supplementation, and patients with L-carnitine plus riboflavin supplementation.

Compared to healthy controls, non-supplemented MCAD-deficient patients and patients receiving L-carnitine supplementation displayed decreased plasma sulfhydryl content (indicating protein oxidative damage). Increased erythrocyte superoxide dismutase activity in patients receiving L-carnitine supplementation probably reflects a compensatory mechanism to scavenge reactive species formation. The combination of L-carnitine plus riboflavin was not associated with oxidative damage.

These are the first indications that MCAD-deficient patients are subjected to protein oxidative damage and that combined supplementation of L-carnitine and riboflavin may prevent these biochemical alterations. The results suggest involvement of free radicals in the pathophysiology of MCAD deficiency. The underlying mechanisms behind the increased SOD activity upon L-carnitine supplementation need to be determined. Further studies are necessary to determine the clinical relevance of oxidative stress, including the possibility of antioxidant therapy.

INTRODUCTION

Medium-chain acyl coenzyme A dehydrogenase (MCAD) deficiency [OMIM 201450] is the most common inherited disorder of mitochondrial fatty acid oxidation (mFAO) [1]. Acute clinical presentation classically involves infants in the first five years of life [2]. During periods with increased metabolic stress, fasting can precipitate acute symptoms, such as drowsiness or lethargy, sometimes progressing into coma or sudden death. Pathognomonic metabolite profiles in plasma or whole blood consist of increased concentrations of medium-chain fatty acids and their carnitine derivatives [3]. Treatment consists of prevention of prolonged fasting, an emergency regimen and may include supplementation of vitamins (L-carnitine and/or riboflavin), but evidence for any treatment is limited [1]. The prognosis is very good after diagnosis, to which neonatal bloodspot screening (NBS) programs have made important contributions [4,5].

Oxidative stress can be defined as a state where the production of reactive species (RS) cannot be compensated for by the antioxidant defense, i.e. RS removal capacity, leading to macromolecule damage [6]. The intimate relationship between mFAO, electron transport and oxidative phosphorylation justifies the study of oxidative stress in human disorders of mFAO [1]. Macromolecule oxidative damage, impaired antioxidant defense and defect mitochondrial energy homeostasis have been demonstrated after incubation of rat brain, liver and skeletal muscle preparations with medium-chain fatty acid metabolites [7–10]. Additionally, evidence for oxidative stress in mFAO defects was reported in various genetic mouse models [11,12].

The objective of this study was to substantiate whether macromolecule oxidative damage and altered antioxidative defense occur in patients with MCAD deficiency. We determined parameters of lipid and protein oxidative damage and enzymatic antioxidant defenses in blood samples that were obtained from MCAD-deficient patients during routine hospital visits under normal, unstressed conditions.

METHODS

Patients

The study was approved by the Ethics Committee of the University Medical Center Groningen. Written informed consent was obtained from all patients and/or their parents.

MCAD-deficient patients were recruited from the Section of Metabolic Diseases, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, The Netherlands. Blood samples from 27 MCAD-deficient patients (9 males, 18 females; median age 5.3 years, age range 0-27 years) were obtained during normal hospital visits. The complete group of patients was subdivided into three groups, based on the treatment provided. In our practice, we aim to normalize of plasma free carnitine concentrations (i.e. >16 µmol/l) in case we prescribe L-carnitine (30-50 mg/kg/d). Although riboflavin supplementation is not routinely prescribed, some older patients in our cohort still use this

supplementation, which they are familiar with. In those patients, riboflavin was dosed 50-150 mg/d, according to Duran *et al.* [13].

Biological sample collection and preparation

Blood samples from patients and controls were processed and stored by the same procedure. Blood samples from 12 age- and gender-matched control subjects (6 males and 6 females; median age 10.0 years, age range 3-27 years) were recruited anonymously from the laboratory of the general practitioners' service in Groningen, The Netherlands. At the time of sampling, no blood samples from younger children were available.

After venous puncture and collection in heparinized vials, plasma and cells were separated by centrifugation at 3000 x g for 10 minutes at 4°C. Plasma was removed by aspiration and stored at -80°C until further analysis. After plasma separation, erythrocytes samples were washed 3 times with cold NaCl 0.9% in equal volumes of erythrocytes and NaCl, and centrifuged for 10 minutes at 800 x g, after each washing. Lysates were prepared by addition of 1 ml of distilled water to 100 µL of washed erythrocytes and subsequent storage at −80 °C until determination of the antioxidant enzyme activities.

Plasma analysis

Thiobarbituric acid reactive species (TBA-RS), a measure for malondialdehyde which is a parameter of lipid oxidative damage, were determined spectrophotometrically in plasma according to the method described by Esterbauer and Cheeseman [14]. TBA-RS were expressed in nmol/mg protein.

Sulfhydryl content, a parameter of protein oxidative damage, was determined in plasma spectrophotometrically based on the reduction of 5.5'dithio-bis (2-nitrobenzoic acid) by thiols, yielding a yellow derivative [15]. The sulfhydryl content is inversely correlated to oxidative damage to proteins and results were reported as µmol/L. Carbonyl content, a parameter of protein oxidative damage, was determined in plasma spectrophotometrically as previously described by Levine *et al.*[16]. Plasma carbonyl content is expressed as nmol/mg protein.

Plasma protein concentration was determined according to the Biuret method using a commercial kit (Labtest Kit®, Lagoa Santa, Minas Gerais, Brazil).

Erythrocyte antioxidant analysis

Antioxidant enzyme activities were determined spectrophotometrically in erythrocyte lysates. Superoxide dismutase (SOD) converts superoxide radicals into hydrogen peroxide. Cytosolic SOD activity was determined using a kit (RANSOD®, Randox Laboratories, Antrim, United Kingdom), and isrepresented as units/mg protein. Catalase (CAT) catalyzes the degradation of hydrogen peroxide into molecular oxygen and water. CAT activity was determined according to the method described by Aebi [17]. Glutathione peroxidase (GPx) catalyzes the conversion of glutathione (GSH) and hydrogen peroxide into glutathione disulfide and water. GPx activity was measured using a kit (RANSEL®, Randox Laboratories, Antrim, United Kingdom). Erythrocyte GSH is a parameter of the non-enzymatic antioxidant defense. GSH concentrations were measured according to Browne and Armstrong [18] and

expressed as nmol/mg protein. Total antioxidant status (TAS) represents the quantity of tissue antioxidants and was measured using a kit (Randox, Antrim, United Kingdom). The results are expressed in mmol/l. Protein content of erythrocyte lysates was determined by the method of Lowry. The final enzymatic activities are presented as percentage of control values.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and SPSS version 20 (International Business Machines Corp., Armonk New York, USA). Data were tested for statistically significant outliers using the Grubbs' test with a conservative approach (alpha = 0.01) (http://graphpad.com/quickcalcs/Grubbs1.cfm). Normality of the data was assessed using the Shapiro-Wilk normality test. Differences between the complete group of patients and controls were tested using either the two-tailed unpaired Student's t-test or Mann-Whitney test. Differences between patient subgroups and the control group were tested by the Kruskal-Wallis test and followed by the Dunn's multiple comparison test when the *p*-value was significant. Correlations were analyzed with either Pearson's analysis or Spearman's analysis. In case of a correlation between outcome parameter and age, multiple linear regression was performed. Non-parametric data were log-transformed before linear regression. Differences were considered statistically significant at $p<0.05$.

RESULTS

Table 1 summarizes the patient characteristics. All patients had an *ACADM* genotype that has been associated with clinical symptoms. In all patients, plasma acylcarnitine profiles and urinary organic acid analysis were severely abnormal, demonstrating the classical biomarkers of the disease at the time of the study (data not shown).

Parameters of macromolecule oxidative damage and antioxidant defense in controls and MCAD-deficient patients were not influenced by the *ACADM* genotype (Table 2). Plasma TBA-RS values appeared significantly increased in MCAD-deficient patients, and correlated negatively to age in the patient group only (r^2 0.25, $p<0.05$). Subdivision of the patient group according to treatment showed no significant differences in TBA-RS concentrations between the different groups.

We observed that plasma sulfhydryl content, which is inversely related to protein oxidative damage, was significantly decreased in MCAD-deficient patients when compared to controls ($p < 0.05$, Table 2). After correction for age, data in both the patient group without supplementation (median 541.4 µmol/L, range 445.9-641.9 µmol/L, $p<0.05$), and the patient group with L-carnitine supplementation (median 536.5 µmol/L, range 531.6-565.9 µmol/L, $p<0.05$) indicated oxidation of sulfhydryl groups (Figure 1). Addition of riboflavin to L-carnitine supplementation appeared to be effective in preventing protein oxidative damage. In contrast, carbonyl levels, which are products of protein oxidation, were similar in MCAD-deficient patients and controls.

Table 1: Characteristics of the MCAD-deficient patients and control group

Patient	Gender	Age	NBS	Supplementation	Allele 1	Allele 2
(no.)	*(m / f)*	*(years)*	*(yes / no)*	*(no / C / C+R)*		
1	m	5.6	yes	no	c.985A>G	c.985A>G
2	f	1.5	yes	no	c.985A>G	c.985A>G
3	f	2.1	yes	no	c.233T>C	c.233T>C
4	m	18.7	no	no	c.985A>G	c.157C>T
5	f	5.7	yes	no	c.985A>G	c.985A>G
6	f	3.6	yes	no	c.985A>G	c.985A>G
7	f	1.5	yes	no	c.985A>G	c.233T>G
8	f	0.0	yes	no	c.985A>G	c.985A>G
9	f	4.3	yes	no	c.985A>G	c.985A>G
10	f	1.7	yes	no	c.985A>G	c.985A>G
11	m	3.1	yes	no	c.985A>G	c.985A>G
12	m	2.9	yes	no	c.985A>G	c.789A>C
13	m	0.8	yes	no	c.985A>G	c.789A>C
14	m	5.5	yes	no	c.985A>G	c.789A>C
15	f	2.9	yes	C	c.985A>G	c.985A>G
16	f	3.4	yes	C	c.985A>G	c.985A>G
17	f	26.0	no	C	c.985A>G	c.985A>G
18	f	11.3	no	C	c.985A>G	c.985A>G
19	f	4.9	yes	C	c.985A>G	c.985A>G
20	m	5.9	yes	C	c.985A>G	c.985A>G
21	m	5.3	yes	C	c.985A>G	c.985A>G
22	m	8.4	no	C + R	c.985A>G	c.985A>G
23	f	8.9	no	C + R	c.985A>G	c.985A>G
24	f	12.7	no	C + R	c.985A>G	c.985A>G
25	f	18.3	no	C + R	c.985A>G	c.985A>G
26	f	21.4	no	C + R	c.985A>G	c.985A>G
27	f	23.6	no	C + R	c.985A>G	c.985A>G

Legend: No, no supplementation; C, carnitine supplementation; C+R, carnitine and riboflavin supplementation; NBS, identified by the Dutch population NBS program. Median age and range are depicted for the control group.

Plasma TAS was significantly increased in MCAD-deficient patients when compared to controls (Table 2). Subdivision according to treatment showed that this effect was mostly attributable to the non-supplemented patient group (p=0.08) however, treatment groups did not differ significantly from the control group. Erythrocyte activities of SOD and CAT were significantly increased in the complete group of MCAD-deficient patients, compared to controls (Table 2). L-carnitine supplementation was associated with significantly higher SOD activities (median 325%, range 126-540%, p<0.05, Figure 2), and plasma free carnitine concentrations correlated with SOD activities in MCAD-deficient patients

(Pearson r 0.398; $p<0.05$). Riboflavin supplementation had no additive effect (Figure 2). Associations between supplementation and CAT activities were not found. The anti-oxidative parameters did not correlate with age.

Table 2. Parameters of oxidative damage and antioxidant defense in plasma and erythrocyte lysates from controls and MCAD-deficient patients.

Parameter	Controls			Patients			*p* value
	median	*range*	*n*	*median*	*range*	*n*	
TBA-RS (nmol/mg protein)	0.018	0.007-0.071	12	0.037	0.010-0.092	26	0.040#
Sulfhydryl (µmol/L)	613.7	526.7-654.1	12	548.8	445.9-641.9	27	0.000*
Carbonyl (nmol/mg protein)	0.22	0.12-0.26	12	0.21	0.11-0.28	27	0.737
SOD (%)	91.7	9.3-194.7	11	194.2	22.7-540.0	26	0.015*
CAT (%)	94.5	79.7-136.6	12	129.8	68.6-353.6	25	0.014#
GPx (%)	98.0	64.5-164.9	12	107.5	20.0-200.0	26	0.376
GSH (nmol/mg protein)	3.46	2.05-6.56	12	3.30	0.23-6.20	25	0.224
TAS (mmol/l)	0.50	0.39-1.12	12	0.65	0.46-1.11	27	0.046#

Legend: No, no supplementation; C, carnitine supplementation; C+R, carnitine and riboflavin supplementation. TBA-RS: Thiobarbituric acid reactive species; SOD: superoxide dismutase; CAT: catalase activity: GPx: glutathione peroxidase; GSH: glutathione; TAS: total antioxidant status. *, tested by Student's t-test; #, tested by Mann-Whitney test.

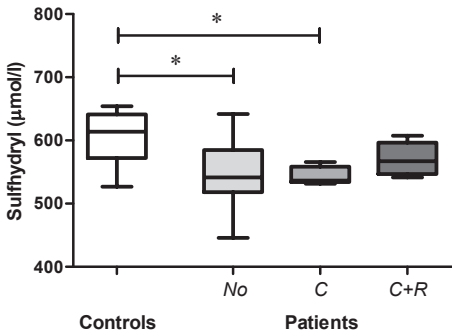

Figure 1. Plasma sulfhydryl content in controls and MCAD-deficient patients, according to their treatment.
Legend: No, no supplementation; C, carnitine supplementation; C+R, carnitine and riboflavin supplementation. * $p <$ 0.05. Boxes represent interquartile ranges with median, whiskers indicate ranges.

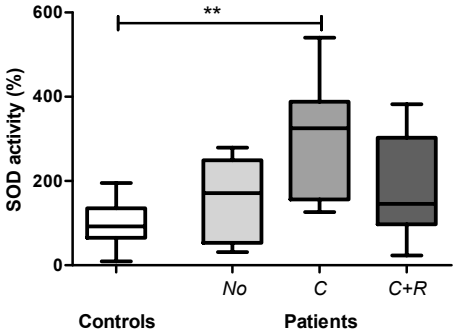

Figure 2. SOD activities in controls and MCAD-deficient patients, according to their treatment.
Legend: No, no supplementation; C, carnitine supplementation; C+R, carnitine and riboflavin supplementation. * $p <$ 0.05. Boxes represent interquartile ranges with median, whiskers indicate ranges.

DISCUSSION

Macromolecule oxidative damage and impaired antioxidant defenses have been reported to play a role in the pathophysiology of several inherited metabolic disorders and the parameters have been suggested as biomarkers for follow up [19,20]. Previous experimental studies in rat tissue preparations incubated with medium-chain fatty acid metabolites [7–10] and genetic mouse models [11,12] demonstrated a relationship between macromolecule oxidative damage, impaired antioxidant defense and defective mFAO. MCAD-deficient patients display abnormal medium-chain fatty acid metabolite profiles in plasma and urine under normal, unstressed circumstances, even though they are clinically asymptomatic. Therefore, this study evaluated lipid and protein oxidative damage and the antioxidant status in MCAD-deficient patients under these normal, unstressed circumstances.

This study demonstrates decreased sulfhydryl contents and altered antioxidant defense in MCAD-deficient patients. Decreased sulfhydryl indicates protein oxidative damage. Two thirds of the sulfhydryl groups are protein bound and the remaining is a component of small molecules, such as GSH, a well-known effective antioxidant [15]. Sulfhydryl groups also act as free radical scavengers preventing lipid oxidative damage [15]. Several *in vitro* studies reported mitochondrial dysfunction and oxidative damage after incubation with metabolites accumulating in MCAD deficiency [7–10]. In rat brain, octanoic and decanoic acid caused uncoupling of mitochondrial oxidative phosphorylation, mitochondrial cytochrome c release, increased lipid and protein oxidation damage, and decreased GSH levels [8]. Scaini *et al.* recently demonstrated similar effects in preparations of rat liver and skeletal muscle cells, where TBA-RS (lipid peroxidation) and carbonyl content (protein oxidation) were increased, and muscle GSH was decreased [9].

The theoretical rationale *in favor of* L-carnitine supplementation in MCAD deficiency is to reduce the number and severity of metabolic decompensations by (*a*) correcting the secondary carnitine deficiency, (*b*) removing, conjugating and increasing urinary excretion of toxic metabolites, (*c*) restoring

intramitochondrial acyl-CoA/CoA ratios, and (*d*) the antioxidant effects of carnitine [21]. Nevertheless, there is no systematic clinical study supporting *routine* L-carnitine supplementation in most inherited metabolic diseases, including MCAD deficiency [22]. The role of L-carnitine supplementation in MCAD deficiency therefore remains a matter of debate.

Interestingly, our study may support the rationale *against* L-carnitine supplementation, as sulfhydryl content was decreased and SOD activity was increased in patients with MCAD deficiency receiving L-carnitine. L-carnitine supplementation increases (acyl)carnitine concentrations in blood and also stimulates the carnitine cycle, transporting acyl-CoA from the cytosol into the mitochondrial matrix, thereby increasing intramitochondrial acyl-CoA concentrations [1]. Tonin *et al.* recently reported the effect of incubating cerebral cortical cells from developing rats with high concentrations of medium-chain acylcarnitines (hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine and cis-4-decenoylcarnitine, 0.01 - 1.0 mM) [10]. In particular, decanoylcarnitine was responsible for lipid and protein oxidative damage. Sauer *et al.* found no negative effect of medium-chain acylcarnitines (with chain-lengths until octanoylcarnitine) on mitochondrial respiratory chain enzyme activities in bovine heart mitochondria [23]. Nevertheless, octanoyl-CoA was found to inhibit complex III, unlike any other acyl-CoA ester. Octanoyl-CoA is the most important substrate of MCAD and hence, although speculative, may accumulate even more in case of L-carnitine supplementation in combination with MCAD deficiency. Koeth *et al.* recently demonstrated in studies in humans and mice that free carnitine concentrations can promote artherosclerosis, via the formation of trimethylamine-*N*-oxide from carnitine by intestinal microbiota [24].

Riboflavin is the precursor of flavin adenine dinucleotide (FAD), an essential cofactor of many intramitochondrial enzymes, including MCAD. Duran *et al.* studied the *in vitro* MCAD enzyme activity in lymphocytes from five MCAD-deficient patients before and after three weeks of riboflavin supplementation at a dose of 50-150 mg/day [13]. Octanoyl-CoA dehydrogenase activity at least doubled in these patients after supplementation. Although this study did not discriminate between a *specific* increase of residual MCAD enzyme activity, a *general* increase of octanoyl-CoA dehydrogenase activity, or a combination, this has been the theoretical basis behind riboflavin supplementation in a subset of our older MCAD-deficient patients. In our study, patients receiving L-carnitine and riboflavin displayed normal sulfhydryl concentrations and SOD activities in contrast to patients with only L-carnitine supplementation. Our observations suggest an antioxidant role of riboflavin as described by Depeint *et al.*[25], which could be explained by the chaperone function enhancing folding efficiency of MCAD protein in patients carrying a missense mutation in the *ACADM* gene [26].

Several methodological aspects of this study need to be discussed. First, it was difficult to obtain age- and gender-matched control samples, especially for the younger group of patients. Second, this is an observational cross-sectional study in samples that were obtained during normal hospital visits, which only enables us to speculate about causal relations. Third, the studied parameters showed large ranges, which can be partially explained by the inter-assay variability. In addition, we studied parameters in biological samples (blood) from our patients. It is important to realize that blood carnitine concentrations do not necessarily reflect tissue homeostasis, and blood oxidative stress

parameters may thus be no reflection of oxidative stress in the liver [27]. The MCAD-deficient mouse model is characterized incompletely [28,29] and would facilitate mechanistic studies under acute and chronic conditions, discriminating contributions of different oxygen-consuming organs such as the brain, liver, heart and muscle.

Summarizing, we demonstrate oxidative damage and altered antioxidant status in asymptomatic MCAD-deficient patients. Population NBS programs for the disorder identify increasing numbers of patients, whose metabolite profiles never normalize. Although *in vitro* studies suggest that these metabolites may contribute to oxidative damage, their increased concentrations have never been associated with any clinical phenotype. Interesting experimental data originate from animal studies. Ibdah *et al.* studied ageing heterozygotes of a knock-out mouse model of long-chain mFAO, in which hepatic steatosis and insulin resistance were associated with reduced liver GSH and increased antioxidant enzyme activities [11]. These observations suggest that oxidative damage, hepatic steatosis and insulin resistance are intimately related under circumstances of perturbed mFAO. Data from large follow-up cohort studies of MCAD-deficient patients until adulthood are not yet available.

CONCLUSIONS

MCAD-deficient patients display oxidative damage and altered antioxidant defense. Due to the intimate relationships between mFAO, electron transport and oxidative phosphorylation, RS may be generated by different simultaneous actions. Patients receiving both L-carnitine and riboflavin did not display oxidative damage. L-carnitine alone was associated with protein oxidative damage, and altered antioxidant defense. Patients receiving L-carnitine showed similar results to non-supplemented patients with regards to oxidative stress. Future mechanistic studies on the role of oxidative stress in MCAD deficiency are required.

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Chapter 5

Gaining insight in the pathophysiology of medium-chain acyl-CoA dehydrogenase deficiency by studying a mouse model

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In preparation

ABSTRACT

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most common mitochondrial fatty acid oxidation defect. Nevertheless, the pathophysiological mechanism underlying the hypoketotic hypoglycemia that is infrequently seen in patients with the disease remains to be determined. In 2005, a mouse model for MCAD deficiency was described for the first time, on a 129P2xC57BL6/J background. We subsequently crossed this mouse model back to a C57BL6/J background. This new MCAD-KO mouse model showed a considerable phenylpropionyl-CoA oxidative activity in liver homogenate and cultured skin fibroblasts, which has not been observed in cultured skin fibroblasts of patients with MCAD deficiency. However, *Acadm* gene expression and MCAD protein were absent. Prolonged fasting alone did not trigger a phenotype. Simulating MCAD deficiency in a recently published computational model of mitochondrial fatty acid oxidation showed an increased sensitivity to substrate overload as compared to the wild type condition. Differences in mitochondrial fatty acid oxidation between man and mouse make it difficult to study the pathophysiological mechanism underlying the clinical phenotype as is seen in patients with MCAD deficiency. However, by combining the knowledge that we have obtained thus far from the studies in both MCAD-KO mouse models with the latest advances in systems biology we have been able to generate a new hypothesis on a pathophysiological mechanism that may contribute to the development of the clinical phenotypes that can be seen in patients with MCAD deficiency.

INTRODUCTION

The most common mitochondrial fatty acid oxidation (mFAO) defect is medium-chain acyl-CoA dehydrogenase (MCAD) deficiency [1]. Patients with the disease can present with a life-threatening hypoketotic hypoglycemia, which can progress into coma and even sudden death. Metabolic crises occur infrequently, typically after an episode with prolonged fasting and increased metabolic stress, such as during intercurrent illness. The clinical spectrum varies considerably, ranging from neonatal death to remaining asymptomatic throughout life. Even within a group of patients with the same *ACADM* genotype, this complete clinical spectrum can be seen. As the pathophysiology of the disease remains unclear, it is currently impossible to predict which patient is at risk for the development of clinical symptoms and who is not.

In 2005, Tolwani *et al.* first described a mouse model for MCAD deficiency [2]. This mouse model was generated by inserting a vector leading to duplication of exons 8, 9, and 10, and was created on a mixed background of 129P2 and C57BL6/J strains. As a result of the inserted duplication a stop codon is introduced upon translation, leading to truncation of the MCAD monomer after exon 10. No MCAD mRNA or protein could be detected in tissues of the MCAD knock-out mice. Biochemically and clinically, this mouse model shared some characteristics with patients with the disease such as neonatal

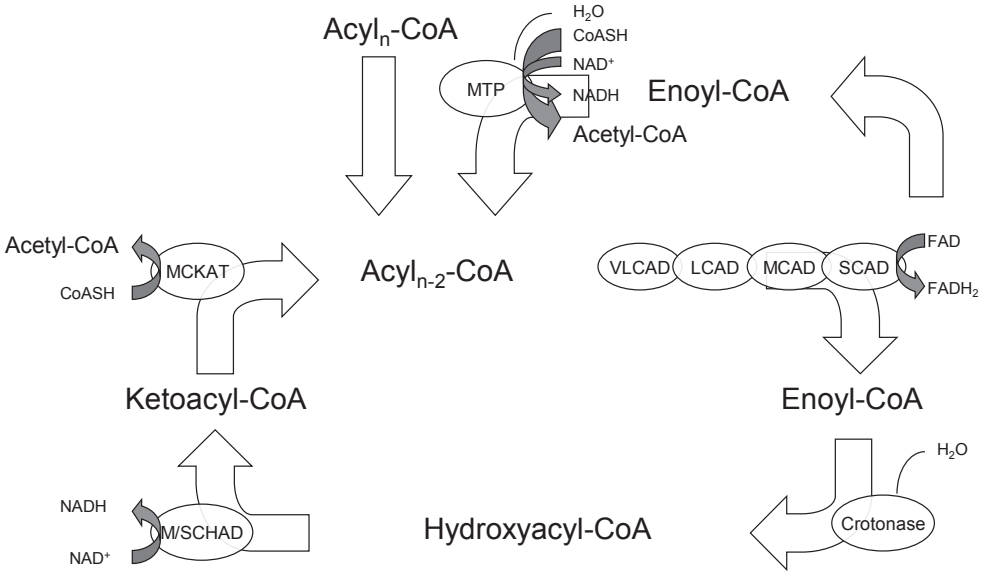

Figure 1. Schematic overview of the cyclic mFAO structure.
CoASH: Free CoA; FAD: Flavin Adenine Dinucleotide; NAD: Nicotinamide adenine dinucleotide; MCAD: Medium-chain acyl-coA dehydrogenase; MCKAT: Medium-chain ketoacyl-CoA thiolase; MSCHAD: Medium-chain hydroxyacyl-CoA dehydrogenase; MTP: Mitochondrial trifunctional protein; LCAD: Long-chain acyl-CoA dehydrogenase; LCHAD: Long-chain hydroxyacyl-CoA dehydrogenase; SCAD: Short-chain acyl-CoA dehydrogenase; SCHAD: Short-chain acyl-CoA dehydrogenase; VLCAD: Very long-chain acyl-CoA dehydrogenase.

deaths, abnormal acylcarnitine profiles, and fatty liver upon prolonged fasting. Hypoglycemia upon prolonged fasting was not observed in this mouse model. Irrespective of the absence of MCAD protein, considerable aspecific oxidative capacity has been reported in different MCAD enzyme assays: with hexanoyl-CoA as a substrate the aspecific activity was 51%, with the highly specific substrate phenylpropionyl-CoA (PP-CoA) it was 7% and in an electron transfer flavoprotein reduction assay with octanoyl-CoA the activity was 25% [2,3]. For each of these assays, observed oxidative activities were considerably higher in the mouse model than in patients [4].

Recently, we published a computational model of mFAO, which takes into account the cyclic structure of mFAO (Figure 1) and the consequent competition of acyl-CoA intermediates for the same enzymes as the original acyl-CoA substrate upon re-entering the mFAO [5]. The model was developed based on published values for the kinetic parameters of the individual enzymes for rat liver. With this model, we were able to calculate the mFAO rate, which was very close to the values measured during experimental validation in isolated rat liver mitochondria. We used this model to simulate the consequences of MCAD deficiency.

In this chapter, we describe the first results of the characterization of the backcrossed C57BL6/J MCAD-KO mouse model. Additionally, the results of the first simulations of MCAD deficiency in the computational model for mFAO will be presented.

METHODS

Mice

Regular backcrossing of MCAD-KO mice on the mixed 129P2xC57BL6/J background with the C57BL6/J strain was performed. Mice were backcrossed for 5 generations, before a subpopulation with heterozygous breeding pairs was started for the generation of MCAD-KO and wild type (WT) mice that could be used for experiments.

Genotyping of the mice occurred after weaning, and was performed with real time PCR in ear tissue, testing for a SNP only present in the inserted vector, using the following primers and probes:

Primer sequences:	Mcad-GT-Fwd: TTT GTG GTT TTC AGC GAC TAG G; Mcad-GT-Rev: GTG AAA GAT GAA CTA CAC ACA GGA CA.
Probe sequences:	Mcad-GT-WT-FAM: caa{T}{C}tc{C}{T}{C}tcca{C}c (5' 6-FAM / 3'-BHQ1); Mcad-GT-KO-YY: caa t {C}tc{C}{C}{C}tcca{C}c (5'-YY / 3'-BHQ1)).

Male mice were used for experiments at the age of 2-6 months, age variation within one experiment was never >1 month. Mice were fed commercially available laboratory chow (ABDiets, Woerden, The Netherlands).

For experiments under fasted conditions, mice were placed in a clean cage at 9 p.m. and terminated and dissected 12 hours later by cardiac puncture under isoflurane anesthesia.

All experiments were approved by the local review board for animal experiments at the University of Groningen, The Netherlands.

Metabolite analysis

Blood obtained by cardiac puncture was collected in heparinized vials. Samples were centrifuged for 10 minutes at 1200 g and 4C°. Plasma was transferred to a fresh tube and stored at -20 °C for later analysis. Free fatty acids (FFA) and β-hydroxybutyrate were determined with the Vitalab E Selectra using standard laboratory methods (reagents: DiaSys, Holzheim, Germany). Blood glucose was determined with glucose strips (One Touch® Ultra Easy, Tilburg, The Netherlands) after tail bleeding under fed and fasted conditions. Bloodspots for acylcarnitine analysis were obtained from blood obtained by cardiac puncture.

After termination, livers were dissected rapidly. One lobe of the liver was freeze clamped in liquid nitrogen and stored at -80°C until further analysis. The other part of the liver was kept fresh in sucrose buffer for oxygen uptake experiments in isolated mitochondria.

Acylcarnitines were determined in blood spots and liver homogenates under fed and fasted conditions, according to Derks *et al*.[4]

Cell culture

Skin biopsies were obtained from the ears of the mice for the isolation of fibroblasts for cell culture. Ear tags were added to culture flasks with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 20% fetal bovine serum and 1.8% penicillin/streptomycin, and incubated in a 37°C 5% CO2 incubator (Steri-cycle, Thermo). Culture medium was refreshed twice a week, and the cultured skin fibroblasts were transferred to a larger culture flask when confluency was reached.

Enzyme analysis

MCAD enzyme analysis was performed in liver homogenate (n=3/group) and cultured skin fibroblasts (n=5/group). Tissues were disrupted by sonication in phosphate-buffered saline (PBS). MCAD enzyme activity was determined using phenylpropionyl-CoA as a substrate, the product cinnamoyl-CoA was measured on an HPLC system with UV detection (Waters, Milford, MA, USA) [4,6]. Residual MCAD enzyme activity was expressed as percentage of control.

Western blot analysis

The SCAD, MCAD, LCAD, and VLCAD protein levels were measured by Western blot analysis in isolated liver mitochondria (n=6/group). Isolated liver mitochondria were re-suspended in MiR05 buffer for oxygen consumption analysis (see below), and the remainder was disrupted by freezing and thawing for Western blot analysis. Protein determination was performed with a Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Inc.). For Western blot analysis, 10 µg protein was denatured, separated on a 12% SDS-page gel, and transferred onto a nitrocellulose membrane (Trans-Blot Transfer pack (Bio-Rad), 10 minutes, 25V). Blots were blocked for 1 hour with 2% ELK (Campina) powder, 0.5% BSA in PBS with 0.1% Tween-20.

The nitrocellulose membrane was immunoblotted overnight at 4°C with 1:2000 dilutions of either Anti-MCAD (Abcam, ab94261, spanning amino acid 240-290 on exon 9 and 10); Anti-SCAD (Abcam, ab156571); Anti-LCAD (Abcam, ab82853); Anti-VLCAD (Abcam, ab155138); and 1:5000 Anti-Citrate Synthase (Abcam, ab129095) for Western blot analysis on isolated liver mitochondria. Blots were then incubated for 1 hour at room temperature with 1:2000 dilution of a goat anti-rabbit polyclonal HRP secondary antibody (DakoCytomation, P0448). The Western blots were developed with SuperSignal Wester Dura Substrate (Thermo Scientific).

Quantitative PCR

Total RNA was isolated from homogenates of freeze-clamped liver tissue of adult male MCAD-KO (n=6) and WT C57BL6/J (n=6) mice using Trizol (Invitrogen, Carlsbad, CA). Quantification of total mRNA was done by NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 1 µg of total RNA according to manufacturer protocols (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using an ABI-Prism 7700 fast PCR system (Applied Biosystems, Foster City, CA, USA). *Acadm* (spans exons 3-4, TaqMan gene expression assays, Mm01323360_g1), *Acads* (Mm00431617_m1), *Acadl* (Mm00599660_m1), and *Acadvl* (Mm00444293_m1) primers were commercially obtained (Applied Biosystems, Foster City, CA, USA). Obtained mRNA expression levels were calculated relative to the housekeeping gene 36B4, and normalized according to the mean expression levels that were obtained in the control group.

Oxygen consumption analysis

Mitochondria were isolated from livers of adult male MCAD-KO (n=6) and WT C57BL6/J mice (n=6), according to Mildaziene *et al.* [7]. The rate of oxygen consumption was measured in isolated mitochondria with palmitoyl-carnitine (C_{16}-carnitine) or octanoyl-carnitine (C_8-carnitine) as a substrate at 37°C in a stirred Oroboros oxygraph-2k (Oroboros, Innsbruck, Austria). All analyses were performed in 2 ml MiR05 buffer according to the Oroboros Instruments protocols (www.oroboros.at) [8], with 0.5 mg/ml mitochondrial protein, 2 mM malate, 1 mM ADP, 500 µM L-carnitine, 0.2 µM FCCP (uncoupler), and 25 µM of substrate.

Calculation of rate of oxygen consumption due to the mFAO

The measured oxygen consumption is not directly related to C_{16}-carnitine oxidation in the presence of malate. Malate was added to the medium to enable regeneration of CoASH from acetyl-CoA by reaction (1):

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O \longleftrightarrow \text{Citrate} + \text{CoASH} + H^+ \qquad \text{(reaction 1)}$$

In this reaction oxaloacetate is generated by malate oxidation. As a consequence, the oxygen consumption during C_{16}-carnitine oxidation is the sum of malate oxidation (1/3 of measured oxygen consumption) and β-oxidation (2/3 of measured oxygen consumption).

Modeling MCAD deficiency

MCAD deficiency was simulated in the dynamic model of the mFAO as previously described by van Eunen *et al.* [5]. In our model we used experimentally determined kinetic parameters of the involved enzymes published for rat liver. The model outcome was experimentally validated in isolated rat liver mitochondria, and was found to qualitatively predict metabolite concentrations and rates in time without prior parameter estimation. In contrast to the two other computational models that had previously been reported for mFAO, our model takes into account the complex interactions and competitions that take place in the pathway as a consequence of its cyclic nature and the overlap in substrate specificity of the enzymes involved [5,9,10]. In order to model MCAD deficiency, the V_{max} for liver MCAD was adapted to 10% of control values, which corresponded to the average aspecific oxidative activity for PP-CoA oxidation observed in the MCAD-KO mouse model.

Statistical analysis

Dichotomous data were analyzed with a chi-squared test. Differences between normally distributed continuous data were analyzed using parametric tests, and data that were not normally distributed were analyzed using non-parametric tests. The significance level was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., version 5.00, 2007).

RESULTS

Mouse characteristics

Neonatal deaths as seen in the 129P2xC57BL6/J MCAD-KO strain were not observed in the heterozygous breeding program of the C57BL6/J MCAD+/- mice (upon weaning 339 pups: 73 MCAD-KO (22%), 176 MCAD+/- (51%), 90 WT (27%); chi-square with Mendelian inheritance as null hypothesis, $p=0.45$). Body weight under fed and fasted conditions was similar in the MCAD-KO and WT group. Loss of body weight and the liver/body weight ratio upon fasting were also similar in both groups (Table 1). Concentrations of blood glucose, free fatty acids, and β-hydroxybutyrate were similar in the MCAD-KO group and the control group after 12 hours of fasting (Table 1). C_6-, C_8-, and $C_{10:1}$-carnitine, and the C_8/C_{10} ratio were significantly higher in bloodspots of the C57BL6/J MCAD-KO mice under these same conditions ($p<0.01$, Table 1).

Medium-chain acylcarnitine concentrations in liver homogenate fed and fasted

Acylcarnitine profiles in liver homogenates of fed and 12-hour fasted mice showed remarkable results. Under fed conditions, concentrations of C_6-, C_8-, C_{10}-, and $C_{10:1}$-carnitine were similar in the MCAD-KO and the WT group. Upon a 12-hour fast C_6- and C_8-carnitine remained similar in the MCAD-KO group when compared to fed conditions, whereas concentrations of C_{10}- and $C_{10:1}$-carnitine increased (Table 2). In the WT group, concentrations of C_6-carnitine appeared to decrease; however, this effect did not reach statistical significance. Concentrations of C_{10}-carnitine increased significantly. Concentrations of

Table 1: Mouse characteristics after a 12-hour fast

	MCAD-KO	WT
Fasted body weight (g)	23.0 (20.7-27.1)	23.9 (17.8-27.1)
Body weight loss upon fasting (%)	11.2 (10.0-13.8)	12.4 (7.8-19.9)
Body weight/liver ratio	0.050 (0.041-0.053)	0.047 (0.043-0.052)
Fasted glucose (mmol/l)	5.3 (4.6-6.8)	5.8 (4.6-7.8)
Fasted β-OH-butyrate (mmol/l)‡	1.35 (1.29 – 1.99)	2.30 (1.59 – 2.60)
Free fatty acids (mmol/l) ‡	583 (516 – 598)	432 (410 – 714)
C_6-carnitine bloodspot (µmol/l)	0.14 (0.09 – 0.30)**	0.06 (0.04 – 0.07)
C_8-carnitine bloodspot (µmol/l)	0.49 (0.46 – 0.56)**	0.07 (0.06 – 0.42)
C_8/C_{10} ratio bloodspot	7.6 (5.3 – 9.3)**	3.2 (2.4 – 6.9)
C_{10}-carnitine bloodspot (µmol/l) ‡	0.07 (0.05 – 0.09)	0.03 (0.02 – 0.06)
$C_{10:1}$-carnitine bloodspot (µmol/l) ‡	0.22 (0.10 – 0.26)**	0.02 (0.01 – 0.02)

Median and range are depicted. ‡ Blood samples of two mice were pooled for this analysis. Three pooled plasma samples were analyzed per genotype. ** $p<0.01$.

the other medium-chain acylcarnitines did not change in this group upon fasting. Fasting concentrations of C_6-, C_{10}- and $C_{10:1}$-carnitine were significantly higher in the C57BL6/J MCAD-KO group when compared to the WT group upon fasting.

Enzyme activity

Analysis of the MCAD enzyme activity with PP-CoA in liver homogenate resulted in a median PP-CoA oxidation activity of 9.3% (n=3, range 9.0-11.2%) when compared to control. These results were comparable to activities that were previously measured in the 129P2xC57BL6/J MCAD-KO strain [3]. In cultured skin fibroblasts of the same mouse strains a median PP-CoA oxidation activity of 10.9% (n=5, range 9.8-19.6%) of control was found.

Western blot analysis

No MCAD protein was detected upon Western blot analysis in isolated liver mitochondria of MCAD-KO mice (Figure 2A, B). The amount of SCAD, LCAD, and VLCAD protein detected upon prolonged fasting was similar in the MCAD-KO and WT group.

Table 2. Concentrations of medium-chain acylcarnitines in liver homogenate under fed and fasted conditions.

		FED	FASTED
		n=5	n=6
MCAD-KO	C_6	0.52 (0.42 – 0.54)	0.52 # (0.48 – 0.64)
	C_8	0.06 (0.04 – 0.10)	0.07 (0.06 – 0.08)
	C_{10}	0.06 (0.04 – 0.08)	0.29 *# (0.10 – 0.38)
	$C_{10:1}$	0.00 (0.00 – 0.02)	0.04 *# (0.02 – 0.06)
		n=6	n=5
WT	C_6	0.50 (0.40 – 0.64)	0.40 # (0.40 – 0.50)
	C_8	0.06 (0.04 – 0.08)	0.08 (0.04 – 0.12)
	C_{10}	0.06 (0.02 – 0.06)	0.12 *# (0.08 – 0.14)
	$C_{10:1}$	0.00 (0.00 – 0.02)	0.02 # (0.00 – 0.04)

Concentrations in µmol/l. * significantly different from fed conditions (p<0.05); # significantly different from other fasted group (p<0.05). Mice in the 'fasting' group were fasted for 12 hours.

Quantitative PCR

Acadm mRNA was almost absent in liver homogenate of MCAD-KO mice. Expression of *Acads, Acadl,* and *Acadvl* upon prolonged fasting was similar in the MCAD-KO and WT group (Figure 3).

Oxygen consumption rate

Analysis of the rate of oxygen consumption in isolated liver mitochondria of fed mice determined similar oxygen consumption rates in the MCAD-KO mouse when compared to the WT group (median 43.8 nmol/min/mg protein vs. 55.0 nmol/min/mg protein respectively) when C_{16}-carnitine was used as a substrate (Figure 4A). When C_8-carnitine was used as a substrate, median maximum rate in the MCAD-KO mice was 50% of the median maximum rate that was observed in the WT group (median 29.0 nmol/min/mg protein vs. 58.4 nmol/min/mg protein respectively, p<0.001)(Figure 4B).

Modeling MCAD deficiency

Our computational model of mFAO [5] predicted that the cyclic structure and the overlapping substrate specificity of the enzymes involved makes mFAO susceptible for substrate overload. After one cycle of mFAO the shortened acyl-CoAs will re-enter the mFAO cycle for further oxidation. They thereby compete with other intermediates and newly entering acyl-CoAs for the same enzymes in the oxidative pathway (Figure 1). This competition leads to feedforward inhibition, with subsequent accumulation

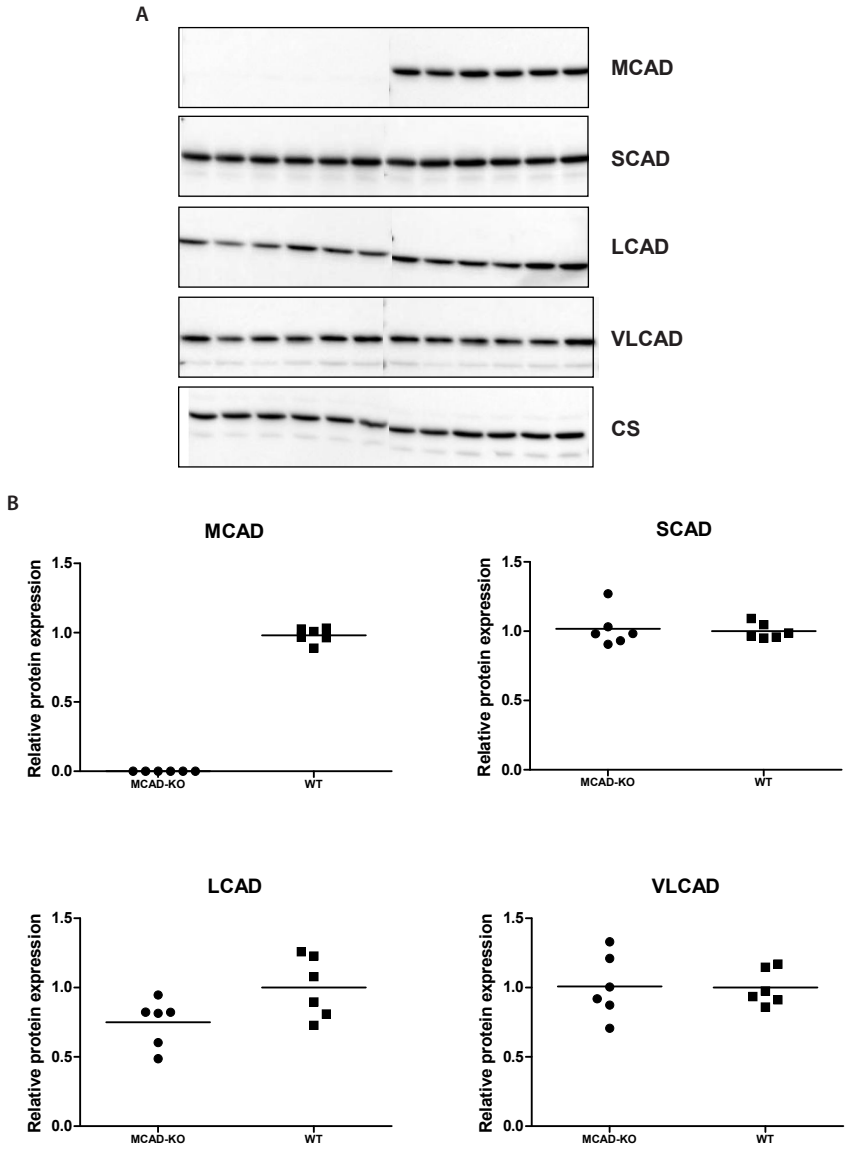

Figure 2. Western blot of MCAD, SCAD, LCAD, VLCAD, and citrate synthase protein in isolated liver mitochondria of fasted mice (A). CS: Citrate synthase. Quantification occurred according to the housekeeping gene. Mean relative protein expression in the WT group was set at 1. Relative protein expression in all animals was related to the mean relative protein expression in the WT group (B).

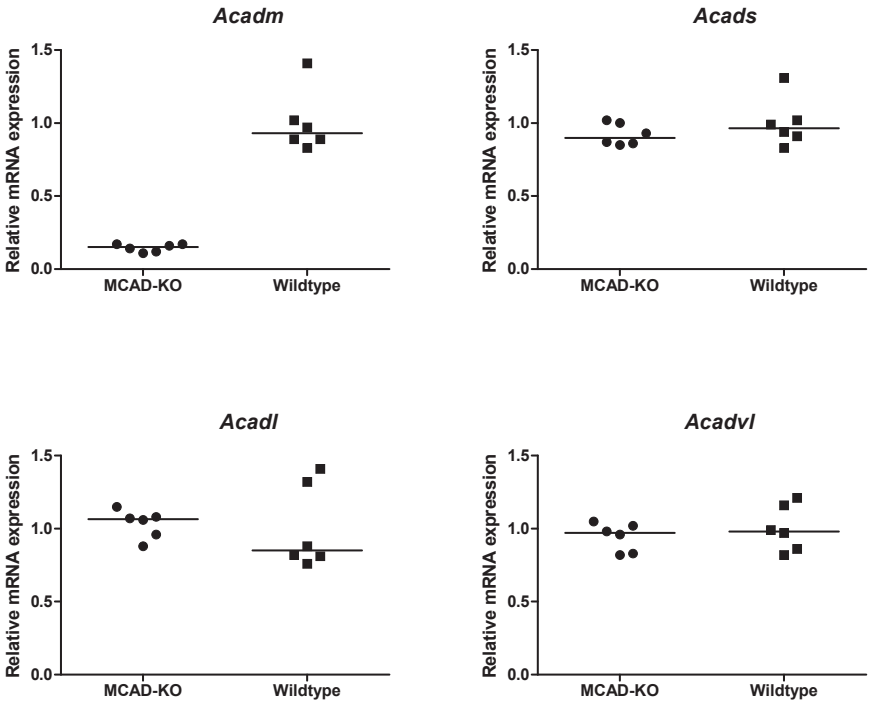

Figure 3. Relative mRNA expression of *Acadm, Acads, Acadl,* and *Acadvl* in liver homogenate of fasted C57BL6/J MCAD-KO and WT mice. *Acadm*: Gene encoding MCAD; *Acads*: Gene encoding SCAD; *Acadl*: Gene encoding LCAD; *Acadvl*: Gene encoding VLCAD. Mean gene expression per group is indicated.

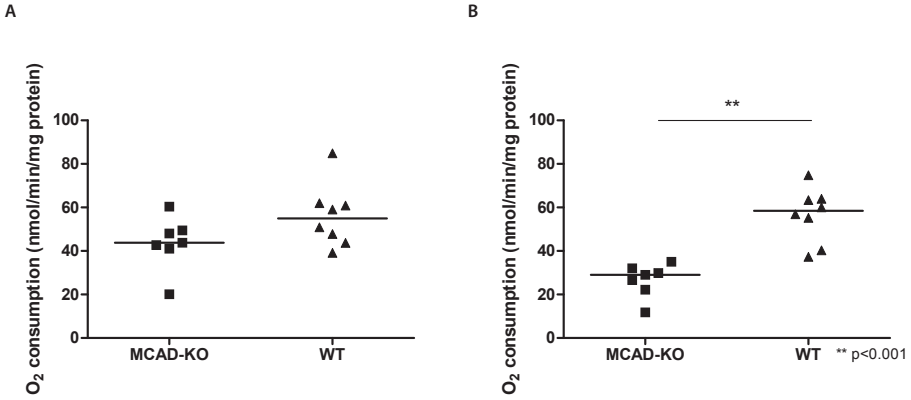

Figure 4. Oxygen consumption rate measured in oxygraph in isolated liver mitochondria, with C_{16}-carnitine (A) and C_8-carnitine (B) as substrates. The total oxygen consumption (of which 1/3 can be attributed to malate dehydrogenase activity, and 2/3 to the β-oxidation) is depicted. Oxygen consumption in nmol/min/mg protein. ** $p<0.001$.

of acyl-CoA esters and depletion of CoASH [5]. Based on the outcome of these model simulations we wondered if MCAD deficiency makes our model of mFAO even more vulnerable to substrate overload. To simulate MCAD deficiency, V_{max} of MCAD was set at 10% of normal. The MCAD deficient model showed accumulation of medium-chain acylcarnitines upon simulation of C_{16}-CoA oxidation, which showed similarities to the acylcarnitine profiles that were observed in liver homogenate of the fed MCAD-KO mouse model. C_6-carnitine was the predominant metabolite, followed by respectively C_8-carnitine and C_{10}-carnitine (Figure 5A). We then performed a series of simulations to mimic substrate overload (Figures 5B, C). In the unperturbed model, in which the V_{max} of MCAD was kept at 100% of normal (WT), we increased the concentrations of C_{16}-CoA in a stepwise manner and calculated the steady-state rate (i.e. flow) through mFAO. At a concentration of 50 μM of C_{16}-CoA the WT rate through the β-oxidation dropped abruptly (Figure 5B) with a concomitant rapid increase in the concentration of various acyl-CoA intermediates and a pronounced decrease in CoASH concentration (Figure 5C). When the same series of simulations were performed in the MCAD deficient model, a sudden drop in rate occurred already at a much lower concentration of C_{16}-CoA (approximately 10 μM), accompanied by comparable changes in the concentration of mFAO intermediates and CoASH as observed in the WT simulations, albeit at lower concentrations of C_{16}-CoA (Figure 5B). Quite strikingly, close inspection

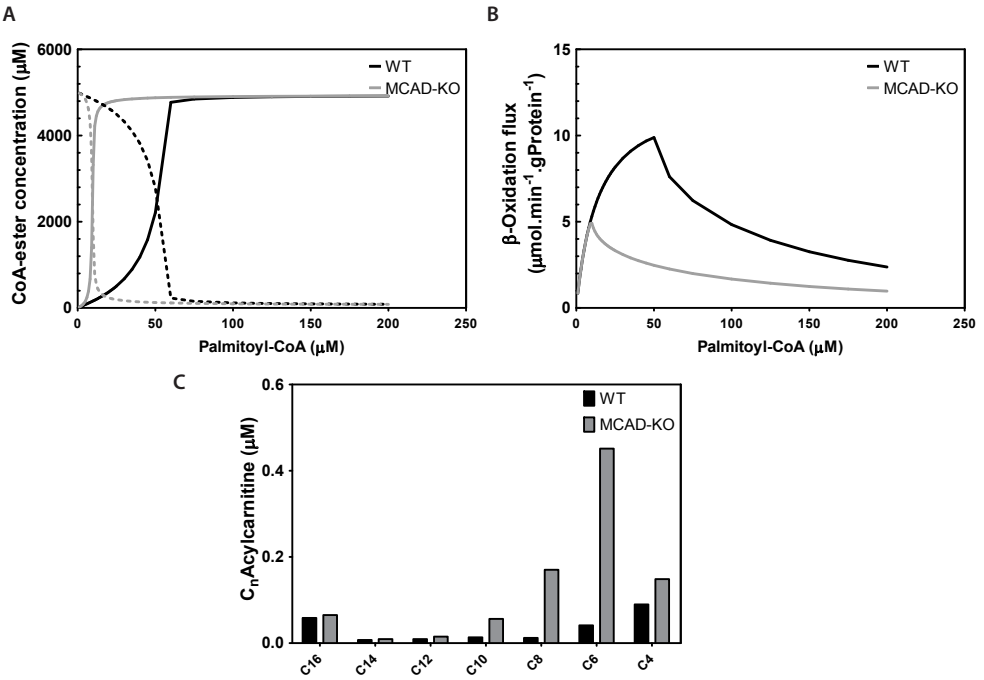

Figure 5. Acylcarnitine concentrations (A), steady-state oxygen consumption rate through the β-oxidation (B), and acyl-CoA ester concentrations (C) in a computational model for MCAD deficiency. Black bars and lines: Wild type situation; Grey bars and lines: MCAD-KO situation (V_{max} 10% of control). Figure A: Solid lines, Acyl-CoA ester concentrations; dotted lines, CoASH concentrations.

of the outcome of these model simulations revealed that the predicted rates of oxidation of C_{16}-CoA were the same in both models up to the point where substrate overload became manifest in the MCAD deficient model. Our simulations predicted that the decreased ability to oxidize fatty acids in the case of MCAD deficiency was due to substrate overload instead of a proportionally decreased rate of oxidation at all concentrations of C_{16}-CoA.

DISCUSSION

We present a first characterization of the MCAD-KO mouse model on a C57BL6/J background. Furthermore, we applied our dynamic model of mFAO to simulate MCAD deficiency. Characterization of the MCAD-KO C57BL6/J mouse led to similar results as were reported in the MCAD-KO 129P2xC57BL6/J mouse model [2,3]. On a C57BL6/J background, the MCAD-KO mice showed acylcarnitine profiles in bloodspots similar to those observed in MCAD deficient patients, even though profiles were slightly different in liver homogenate, where C_6-carnitine was the predominant metabolite. Aspecific oxidative capacity measured in frozen liver homogenate of C57BL6/J MCAD-KO mice with PP-CoA showed almost 10% of capacities observed in C57BL6/J WT mice, considerably higher than what is generally observed in MCAD deficient patients (<1% of control). Oxygen consumption upon administration of C_8- and C_{16}-carnitine showed characteristics of MCAD deficiency. Introducing MCAD deficiency in our dynamic model of mFAO predicted almost equal C_{16}-CoA steady-state rate in the MCAD deficiency model when compared to the unperturbed WT model at low concentrations of the substrate. However, the outcome of our predictions showed an increased vulnerability for substrate overload in the case of the MCAD deficiency model.

Phenotypically the mouse model did not show the full clinical spectrum that has been described in human patients. We did not observe neonatal deaths when breeding these mice heterozygously, and these mice did not develop hypoketotic hypoglycemia upon prolonged fasting alone. Although remaining asymptomatic is a 'phenotype' that we often observe in patients with MCAD deficiency, in the C57BL6/J MCAD-KO mouse model we did not observe the other, potentially lethal, end of the spectrum as can be seen in patients. This may correspond to the considerable PP-CoA oxidation activity that was observed in cultured skin fibroblasts of this mouse model (~10% of WT activity), but not in patients with classical *ACADM* genotypes (<1% of normal). However, the origin of this PP-CoA oxidative activity in the MCAD-KO mouse is unclear, as PP-CoA is thought to be specifically oxidized by MCAD, at least in rat and human liver tissue [11]. Moreover, this aspecific oxidative activity was not found to be liver specific.

Besides residual PP-CoA oxidative activities, observed activities for C_6-CoA and C_8-CoA oxidation in C57BL6/J MCAD-KO mice were considerably higher than activities measured in patients with *ACADM* genotypes that are associated with the risk to develop a metabolic crisis [2,3]. These differences may be attributable to differences in chain length specificities and kinetic parameters of the various mFAO enzymes between humans and rodents. For instance, in rodents long-chain acyl-CoA dehydrogenase

(LCAD) participates in mFAO, whereas in humans LCAD plays only a minor role in saturated fatty acid oxidation, and is hardly expressed [12]. As rodent LCAD displays a considerable activity towards C_8-CoA, this enzyme may play an important role in substrate dehydrogenation and thereby partially compensate for the absent MCAD enzyme in MCAD-KO mice [13]. Adaptations to defective mFAO by upregulation of other acyl-CoA dehydrogenases has been described in the mouse model for very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency [14]. However, analysis of LCAD protein and mRNA expression in both liver homogenate and cultured skin fibroblasts showed no differences between MCAD-KO mice and WT mice. Compensation for the defect by LCAD could therefore not be established in the C57BL6/J MCAD-KO mouse model, although we did not measure LCAD activity, which is modified by post-translational modification [15]. The origin of the aspecific oxidative capacity of C_6-CoA, C_8-CoA, and PP-CoA remains unknown.

Remarkable acylcarnitine profiles were observed in liver homogenates of MCAD-KO mice under both fed and fasted conditions. The acylcarnitine profiles that we observed in liver homogenate of MCAD-KO and WT mice, and its response to fasting, may include activated lipogenesis under fed conditions [16]. It has previously been shown that lipogenetic fatty acids can be recovered in acylcarnitines in the circulation. ^{13}C-label of short-chain fatty acids could be recovered in C_{16}-carnitine [17]. These findings may indicate that in MCAD-KO mice, a similar phenomenon occurs under fed conditions. However, under fasted conditions, concentrations of medium-chain acylcarnitines were significantly higher in MCAD-KO mice when compared to WT mice. Upon prolonged fasting, high concentrations of triacylglycerols are released from adipose tissue, for the generation of free fatty acids that can undergo β-oxidation in the liver [18]. As mFAO is activated during fasting, the load of medium-chain length acyl-CoA esters and acylcarnitines increases under MCAD-deficient conditions. As lipogenesis is inhibited upon fasting [19], it may not be sufficient for the prevention of accumulation of medium-chain length acylcarnitines.

When the oxidation of C_{16}-carnitine was measured in isolated liver mitochondria of MCAD-KO mice we observed an almost normal rate of oxygen uptake. Oxidation of C_8-carnitine was 50% of normal, by itself a remarkable observation, because of the complete absence of the MCAD enzyme. Apparently, other enzymes are also active in oxidizing C_8-carnitine. If we look more closely at the way β-oxidation proceeds, an almost normal oxidation rate of C_{16}-carnitine in isolated liver mitochondria of MCAD-KO mice can be explained. Oxidation of C_{16}-carnitine to C_8-CoA in MCAD deficiency leads to the production of 4 acetyl-CoA units at a rate which can be normal, when intermediate acyl-CoAs do not perturb the rate through the β-oxidation. When it is assumed that the next 4 acetyl-CoA units, from C_8-CoA to acetyl-CoA, will be produced at 50% of the normal rate, irrespective of the actual concentration of C_8-CoA during C_{16}-carnitine oxidation, the overall rate of oxygen consumption during C_{16}-carnitine oxidation would only be decreased to 75% of the value observed in isolated liver mitochondria of WT mice.

In the past decade, the application of systems biology in medical research has grown steadily with the development of computational models for glucose metabolism [20,21], the TCA cycle [22], and mFAO [5]. We recently published a new dynamic model for mFAO incorporating competition between short-

ened products and the original substrate acyl-CoAs due to the cyclic nature of mFAO. We predicted that this competition would make mFAO vulnerable to substrate overload. This led us to hypothesize that mFAO defects may increase this vulnerability even further. We simulated mFAO of C_{16}-CoA when the V_{max} for MCAD enzyme activity was reduced to 10% of the value we normally used in the model for rat liver. The simulation showed an enhanced vulnerability to substrate overload of mFAO when MCAD activity was impaired when compared to the WT situation. Already at low concentrations of C_{16}-CoA the simulations predicted an abrupt drop in the rate through mFAO, indicative for substrate overload. The model also predicted that until the point of substrate overload was reached, the rate through mFAO in the presence of MCAD insufficiency was almost indistinguishable from the rate in the presence of a normal value for the V_{max} of MCAD. This might indicate that the rate through mFAO with an MCAD V_{max} of 10% of WT might be normal, irrespective of impaired MCAD activity. If mFAO in MCAD-KO mice truly has an almost normal activity, acute inhibition of mFAO during metabolic crises in MCAD deficient patients might be the consequence of substrate overload only. When it occurs depends on the capacity of oxidative phosphorylation, and the ability to dissipate the excess acyl-CoAs into pathways such as lipogenesis and acylcarnitine formation, thereby preventing substrate overload.

Administration of L-carnitine may both stimulate and inhibit the development of substrate overload. First of all, it can aid in the disposal of acyl-CoAs by conversion to acylcarnitines. On the other hand, it may also drive the mitochondrial entry of acyl-CoAs, especially in case of increased energy demands, thereby increasing the susceptibility of mFAO for substrate overload. Studies hereon are lacking in patients with mFAO defects.

Patients might also become more vulnerable to metabolic crises when delivery of dicarboxylic acids to the liver by gut microbial metabolism is combined with heightened energy demand from mFAO. This may occur during intercurrent illness and in particular during a gastrointestinal infection. Many of the dicarboxylic acids are oxidized by the concerted action of a ligase followed by MCAD. As a result, sensitivity to substrate overload may even be further increased. If this is the case, and substrate overload is a purely acute hepatic metabolic process, it indicates that no trace of the decompensation of the patient is left after the metabolic crises has resolved. It should however be noted that this outcome is based on simulations in an isolated model of mFAO in rat liver. Adaptations to render this model useful for studying mouse metabolism and integration of mFAO in total mitochondrial metabolism in detail are ongoing. Finally, the translation to the human situation has to be made.

CONCLUSIONS

In conclusion, differences in mFAO between mouse and human make it difficult to study pathophysiological mechanisms contributing to the development of the clinical phenotype as is infrequently seen in patients with MCAD deficiency. Not only is the residual activity towards PP-CoA, C_6-CoA, and C_8-CoA oxidation higher in the mouse model than in patients, concentrations of accumulating inter-

mediates and their chain-length also differ slightly from the acylcarnitine profiles that are usually seen in patients. The possible role of peroxisomes and microsomes in compensating for the MCAD defect remains to be elucidated [23]. Additionally, approaches studying the possibility of substrate overload, as predicted by our dynamic model of mFAO are required to obtain insight in factors that determine the risk for a metabolic crisis. Once the pathophysiological mechanisms contributing to the development of a hypoketotic hypoglycemia have been unraveled, studies on therapeutic options can be initiated.

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Chapter 7

From genome to phenome – Simple inborn errors of metabolism as complex traits

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ABSTRACT

Sporadically, patients with a proven defect in either mFAO or OXPHOS are described presenting with a metabolic profile and clinical phenotype expressing concurrent defects in both pathways. Biochemical linkages between both processes are tight. Therefore, it is striking that concurrent dysfunction of both systems occurs so infrequently. In this review, the linkages between OXPHOS and mFAO and the hypothesized processes responsible for concurrent problems in both systems are reviewed, both from the point of view of primary biochemical connections and secondary cellular responses, i.e. signaling pathways constituting nutrient-sensing networks. We propose that affected signaling pathways may play an important role in the phenomenon of concurrent defects. Recent data indicate that interference in the affected signaling pathways may resolve the pathological phenotype even though the primary enzyme deficiency persists. This offers new (unexpected) prospects for treatment of these inborn errors of metabolism.

INTRODUCTION

Inborn errors of metabolism arise from single enzyme deficiencies but behave as complex traits [1]. The broad spectrum of clinical presentations that can be observed in patients with the same enzyme deficiency is a clear example of this. Apparently, cellular metabolism and physiology have many ways to adapt to perturbations in biochemical pathways, and each of these (mal-) adaptations comes with their own set of clinical symptoms. Unbiased '–omics' approaches i.e. transcriptomics and metabolomics, can be of great help to go beyond textbook biochemistry and generate hypotheses concerning possible mechanisms that account for these adaptations. Only recently application ofthese approaches to the field of inborn errors of metabolism has started in the field of mitochondrial diseases.

In the mitochondrion, mFAO, OXPHOS, and the tricarboxylic acid (TCA) cycle function together in the formation of adenosine triphosphate (ATP). Mitochondrial FAO takes care of the repetitive shortening of acyl-CoA esters for the generation of acetyl-CoA, NADH, and $FADH_2$. OXPHOS is the process of ATP synthesis via the transfer of electrons from NADH and $FADH_2$, which have been generated in mFAO or the TCA cycle, to oxygen over the electron transport chain (ETC). According to textbook biochemistry, this continuous regeneration of NAD^+ and FAD by the ETC and oxidation of the acetyl-residue of acetyl-CoA in the Krebs cycle to release free CoA (CoASH) are essential for the

Table 1. Clinical characteristics of OXPHOS and mFAO defects.

OXPHOS defects	mFAO defects
Metabolic profile	
Lactic acidemia, worsening upon glucose administration	(Hypo)ketotic hypoglycemia Abnormal acylcarnitine profile Secondary free carnitine deficiency Organic aciduria
Muscle	
Muscle weakness, atrophy, hypotonia, myoglobinuria, hypertrophic cardiomyopathy	Musculoskeletal or cardiac muscle weakness (VLCAD, LCHAD, MTP, SCAD)
Gastro-intestinal	
Pancreatic dysfunction, diabetes mellitus; Renal failure and tubulopathy; Diarrhea, villous atrophy	Hepatomegaly, fatty liver
Hematology	
Anemia, neutropenia, trombopenia, myelodysplasia	HELLP syndrome in mothers carrying a fetus with LCHAD deficiency
Nervous system	
Hypotonia, cerebellar ataxia, leukodystrophy, hereditary spastic paraplegia, peripheral neuropathy, sensorineural deafness, optic/retinal atrophy, ptosis	
Hormonal	
Hypothyroidism, hypoparathyroidism, hypothalamic hypocorticism, GH deficiency	

Adapted from figure 99-2 in [2], and [3].

mFAO to proceed. It is inherent to this that dysfunctional OXPHOS should accompany impairment of mFAO. However, the opposite is observed. In humans, deficiencies in many of the enzymes important in mFAO and OXPHOS have been described [2,3]. When defects and resulting clinical symptoms are considered, mFAO and OXPHOS usually behave as independently functioning systems (for reviews on individual mFAO and OXPHOS defects and their respective clinical presentation we refer to Table 1 and [3–8]). It is sporadically observed that patients with an established OXPHOS or mFAO defect present with clinical symptoms or metabolite patterns that imply concurrent defects in both systems. The question then arises what molecular mechanisms determine this sporadic occurrence of signs of a combined defect.

In this review we like to distinguish between two major responses of the body to defects in mFAO and OXPHOS; the 'primary biochemical linkages' and 'secondary cellular response'. In Figure 1 we depict several levels of integration. At the metabolic level the conversion of substrates into biomass, energy, and products takes place. At this level all biochemical reactions take place, as well as the metabolite-driven regulation of enzyme activity. Until now, the concurrence of mFAO and OXPHOS defects has mainly been discussed at the level of 'primary biochemical linkages', i.e. at this metabolic level. Indirectly, metabolism is regulated via the other 3 levels, which are collectively considered the 'secondary cellular responses'. The second level represents the nutrient-sensing signaling networks, which influence the biochemical reaction by changing enzyme activities by post-translational modification like phosphorylation. The third level represents the level in which nutrient-sensing transcription factors are active. Since these factors are proteins themselves, they can also undergo post-translational modifications. The reactions in the second and third level are driven by changes in concentration of metabolites, i.e. AMP, NAD^+, fatty acids or phosphorylated sugars. The fourth level is

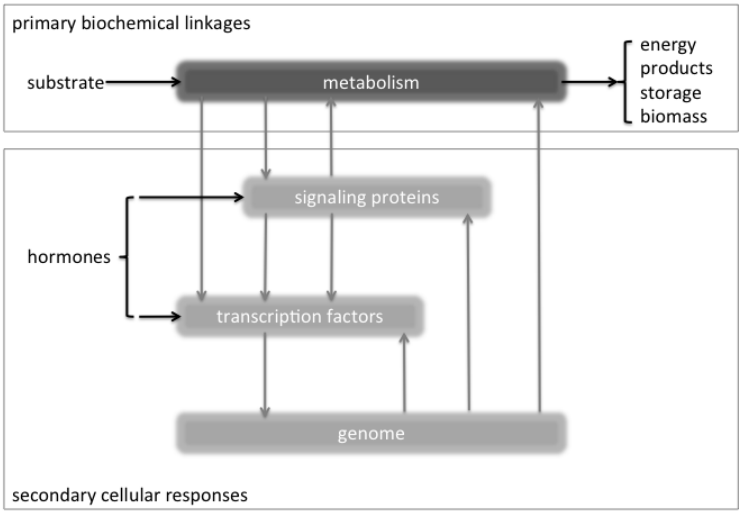

Figure 1. General scheme of metabolic regulation.
Legends: PTM: post-translational modification

the genome, in which transcription of proteins feeds back on biochemically active enzymes, signaling proteins and transcription factors.

In the next paragraphs, we will first discuss the known primary biochemical and structural linkages between OXPHOS and mFAO, the reported clinical cases and related *in vitro* studies on presentation of concurrent OXPHOS and mFAO dysfunction. Next, we will discuss the emerging literature on 'secondary cellular responses', and their role in mitochondrial metabolism. Moreover, we will suggest possible interventions in the pathological signaling as new treatment options.

PRIMARY BIOCHEMICAL LINKAGES BETWEEN OXPHOS AND MFAO

Biochemically, mFAO and OXPHOS are coupled by the TCA cycle, complex I of the ETC and electron transfer flavoprotein (ETF) (Figure 2). During each cycle of mFAO, one molecule of acetyl-CoA, one NADH, and one $FADH_2$ are formed. Acetyl-CoA enters the TCA cycle, generating more NADH and $FADH_2$, or is alternatively used for the production of ketone bodies (KB) or lipogenesis. NADH donates

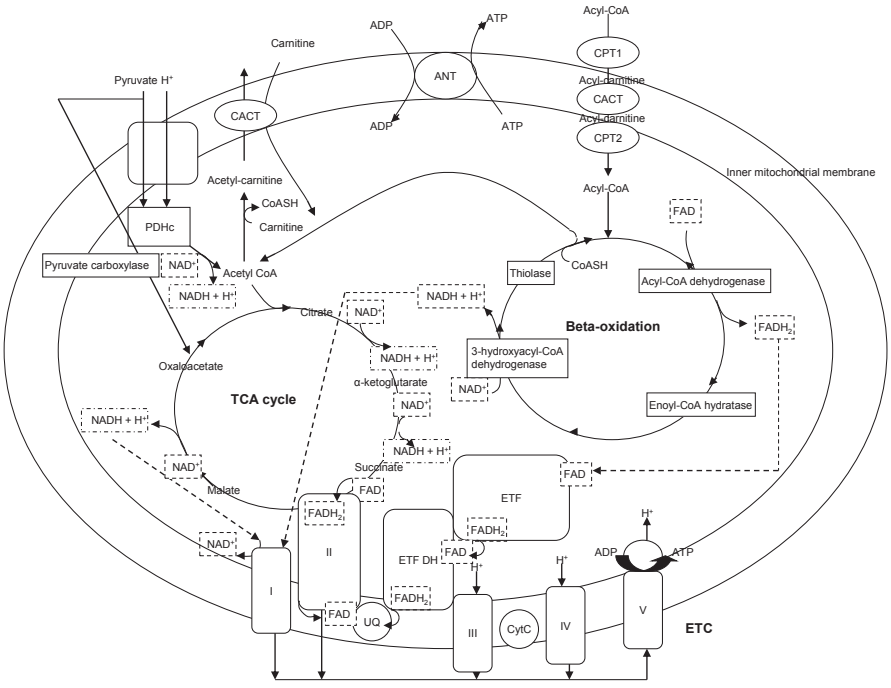

Figure 2. Simplified version of the metabolic pathways that take place in the human mitochondrion. ANT: Adenine nucleotide transporter; CPT: Carnitine palmitoyltransferase; CytC: cytochrome c; ETC: Electron transport chain; ETF: Electron transfer flavoprotein; ETF-DH: ETF-dehydrogenase; FAD: Flavin adenine dinucleotide; mFAO: Mitochondrial fatty acid oxidation; PDHc: Pyruvate dehydrogenase complex; TCA: Citric acid cycle; UQ: Ubiquinone.

its electrons directly to complex I of the ETC. $FADH_2$ is oxidized by transfer of electrons to ETF. Reduced ETF subsequently transfers its electrons to ETF-ubiquinone oxidoreductase (ETF-QO), which donates the electrons to the ETC via ubiquinone (UQ) [9] (Figure 2).

During electron transfer through the ETC, an electrochemical proton gradient over the inner mitochondrial membrane is generated, and subsequently dissipated while ATP is formed by complex V (Figure 2) [2]. The formed ATP can be exported out of the mitochondria in exchange for ADP by the adenine nucleotide transporter (ANT).

The mitochondrial NADH/NAD^+ ratio is important for proper functioning of mitochondrial metabolism, as the NAD^+-linked 3-hydroxyacyl-CoA dehydrogenases (HADHs: respectively short- and long-chain 3-hydroxyacyl-CoA dehydrogenase, SCHAD and LCHAD) are extremely sensitive to changes in the NADH/NAD^+ ratio [10]. To a lesser extent, NAD^+ is required for functioning of the TCA cycle (isocitrate dehydrogenase-3, α-ketoglutarate dehydrogenase complex, and malate dehydrogenase), and pyruvate dehydrogenase complex (PDHc) [3,11–13]. When an altered mitochondrial NADH/NAD^+ ratio results in an altered cytosolic NADH/NAD^+ ratio, the lactate/pyruvate ratio will change [2,11,14,15]. Furthermore, the $FADH_2$/FAD ratio is important for proper functioning of the group of ACADs in mFAO and complex II in the TCA cycle and ETC [16].

Proteins involved in OXPHOS and mFAO are not only linked kinetically via metabolite concentrations, but they also associate into physical complexes. In humans, attachment of VLCAD to the inner mitochondrial membrane enables a direct interaction between mFAO and OXPHOS [17,18]. ETF-QO is also attached to the inner mitochondrial membrane [19], and has in pig liver been found to form a so-called "supercomplex" with MCAD, ETF, UQ, and complex III [9]. Furthermore, "supercomplexes" between complex I of the ETC and HADH, and complex I and VLCAD were identified in pig heart mitochondria [20,21]. The acyl-CoA dehydrogenase (ACAD) proteins have also been found associated with OXPHOS "supercomplexes", in which various ETC complexes are assembled in one large complex for presumably more efficient electron transport and limiting reactive oxygen species formation [9,22–25].

DEFECTS IN OXPHOS ASSOCIATED WITH SECONDARY FATTY ACID OXIDATION DEFECTS

The complex interrelationships between OXPHOS and mFAO have been underlined in studies showing that defects in OXPHOS sporadically lead to secondary mFAO inhibition. Most commonly, primary biochemical links and altered ratios are put forward as an explanation for these observations [16,26–29]. We will present the reported clinical symptoms according to the primary defect.

Complex I – NADH dehydrogenase defects

The clinical phenotype in patients with complex I deficiency is highly variable (Table 1) [30]. Only a few patients have been reported with signs of a combined complex I and secondary HADH dysfunction, presenting with hepatosplenomegaly, cardiomyopathy, and muscle weakness. Biochemically, lactic acidosis, increasing blood lactate after an oral glucose bolus, and a low KB/FFA ratio corresponding to

hypoketosis during hypoglycemia have been reported [31]. Organic acids in the urine showed elevated concentrations of lactate and 3-hydroxydicarboxylic acids during hypoglycemia. LCHAD and SCHAD activities were normal, whereas complex I activity measured in lymphoblasts was severely lowered [27,29,32–34].

In vitro studies underlined these clinical findings, as oxidation of labeled hexadecanoic acid ($C_{16:0}$) was found to be decreased in cultured skin fibroblasts of a group of patients with a complex I deficiency when tritium release of [9,10*(n)*-$^3H_2$]-$C_{16:0}$ was measured [35,36]. Acylcarnitine analysis in cultured skin fibroblasts of two patients with identified mtDNA mutations causing complex I deficiency showed multiple acyl-CoA dehydrogenase deficiency (MADD)-like profiles with mildly elevated concentrations of C_4-carnitine, and high concentrations of C_6- and C_8-carnitine [37].

Complex II – Succinate dehydrogenase defects

Two patients with a primary defect in complex II have been described presenting with lactic acidosis, cardiomyopathy, hypotonia, and hepatomegaly. *In vitro* [$^2H_3$]-$C_{16:0}$ oxidation in cultured skin fibroblasts of these patients again led to elevated concentrations of [$^2H_3$]-C_4, -C_6, -C_8, -C_{10}, and -C_{16} acylcarnitines, resembling acylcarnitine profiles seen in MADD [16,37]. Selective SCAD and MCAD deficiency were excluded. Besides lowered complex II activities, activities of complexes III and IV were also mildly reduced [16].

Complex III – Cytochrome bc_1 complex defects

Only one patient with an isolated complex III deficiency has been described presenting with a hypoglycemia, lactic acidosis and moderate hepatomegaly after prolonged fasting with gastro-enteritis [38]. The hypoglycemia responded well to glucose infusion. Upon a fasting tolerance test again hypoglycemia and lactic acidosis developed. KB concentrations and organic acid profiles were normal. FAO in cultured skin fibroblasts was normal, and complex III deficiency was identified by enzyme and mutation analysis [38].

In vitro studies in rat heart mitochondria acutely inhibited with myxothiazol, an inhibitor of complex III, underlined the reported clinical findings. Upon hexadecanoyl-CoA (C_{16}-CoA) oxidation, titration with myxothiazol led to accumulation of metabolites that typically accumulate in HADH dysfunction. Direct effects of myxothiazol on MTP enzyme activity were excluded by enzyme assays. When the concentration of myxothiazol was further increased above 0.2 μM, concentrations of the abovementioned intermediates decreased and concentrations of C_{16}-CoA and –carnitine increased, indicating impaired flux through VLCAD [26,36]. That mild inhibition of complex III affects mFAO primarily through HADH, fits with the very low equilibrium constant of HADH, which makes the mFAO more sensitive to the NADH/NAD^+ than to the $FADH_2$/FAD ratio [39].

Complex IV – Cytochrome c oxidase defects

Various clinical presentations indicating secondary mFAO inhibition resulting from complex IV deficiency have been described. Similar to what has been described for complex I and III deficiencies, two patients

presented with a hypoglycemia. One of these patients presented with hypoglycemia, elevated lactate concentrations and high KB concentrations already after a short-term fast (4 hours). Mitochondrial FAO was not studied in cultured skin fibroblasts of this patient. In contrast, the second patient presented with a hypoketotic hypoglycemia with decreased KB/FFA ratio, moderate hepatomegaly, and normal lactate levels upon prolonged fasting (15 hours), with normal mFAO in cultured skin fibroblasts [38].

A combination of progressive neurological complaints, Reye-like syndrome with convulsions, and abnormal organic aciduria (i.e. ethylmalonic acid (EMA), glutaric acid, and *N*-acylglycine excretion, resembling MADD), with normal [9,10*(n)*-$^{3}H_{2}$]-tetradecanoic acid oxidation in cultured skin fibroblasts has also been described in complex IV deficient patients [40,41].

In vitro studies in cultured skin fibroblasts from patients with an isolated complex IV deficiency underlined the reported clinical symptoms, as $C_{16:0}$-oxidation was lowered when measured by tritium release from [9,10*(n)*-$^{3}H_{2}$]-$C_{16:0}$ [36]. Acylcarnitine profiles in cultured skin fibroblasts were MADD-like [37].

Overall, MADD-like acylcarnitine profiles and hypoglycemia were most commonly seen in patients with an OXPHOS defect and concurrent mFAO dysfunction (Figure 3).

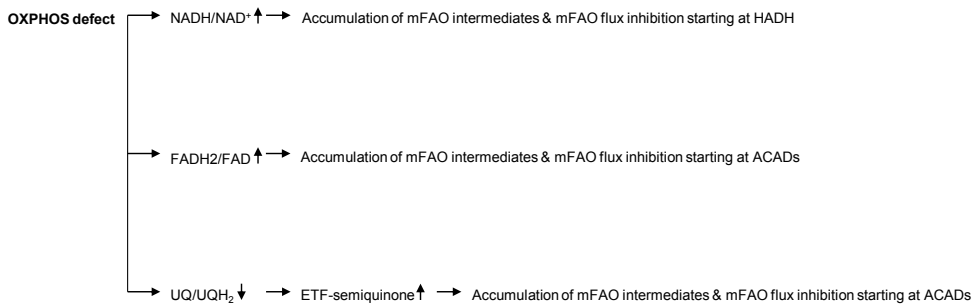

Figure 3. Schematic representation of proposed hypotheses on how OXPHOS affects mFAO
ACAD: Acyl-CoA dehydrogenase; ETF: electron transfer flavoprotein; HADH: Hydroxyacyl-CoA dehydrogenase; mFAO: mitochondrial fatty acid oxidation; UQ: ubiquinone

DEFECTS IN MFAO ASSOCIATED WITH SECONDARY OXPHOS DYSFUNCTION

When considering the close biochemical interrelations in mitochondrial metabolism, primary mFAO defects may cause secondary dysfunctional OXPHOS (Figure 4). The experimental focus of most studies was on the "intoxication" phenotype resulting from accumulation of mFAO intermediates, the severity of which largely depends on the chain-length of the accumulating mFAO intermediates [42].

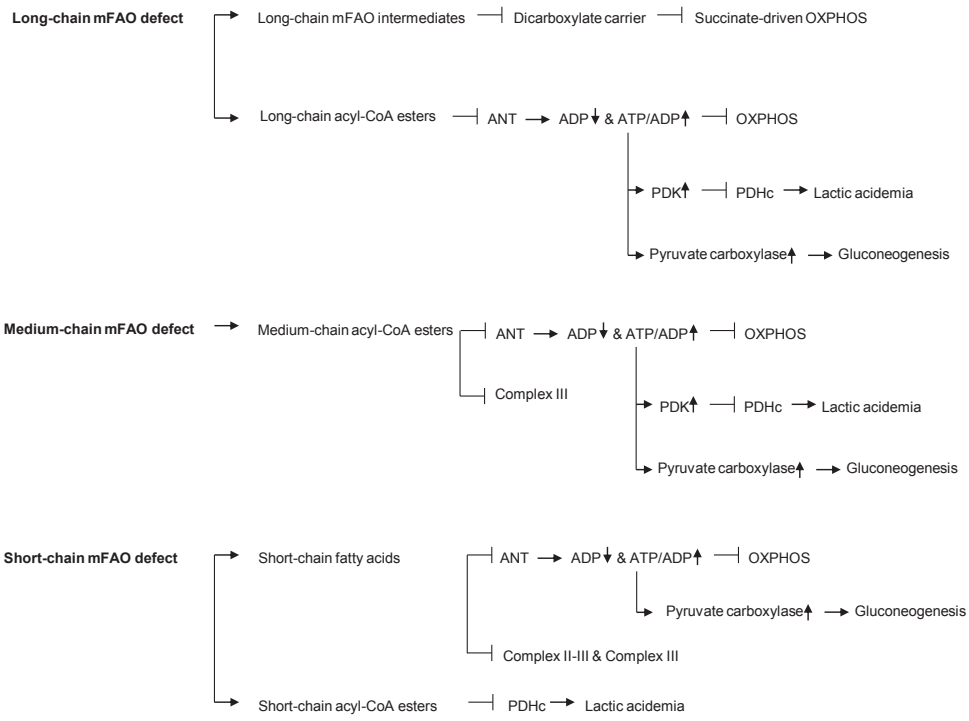

Figure 4. Schematic representation of proposed hypotheses on how mFAO affects OXPHOS.
ANT: Adenine nucleotide transporte; OXPHOS: oxidative phosphorylation; PDK: pyruvate phosphate dikinase; PDHc: pyruvate dehydrogenase complex. : ⊣ Inhibition

Long-chain mFAO defects

With regards to long-chain mFAO defects, only patients with isolated LCHAD deficiency or with MTP deficiency have been described to present clinically with symptoms such as lactic acidosis, retinopathy, cardiomyopathy, muscle hypotonia, hepatosteatosis, and hypoglycemia, combined with elevated plasma long-chain hydroxyacylcarnitines, low free carnitine concentrations, and abnormal organic aciduria (with elevated concentrations of C_6-C_{10} dicarboxylic acids, and both saturated and unsaturated adipic and suberic acids) [43–46]. One case report described a patient who presented with unresponsiveness to intravenous glucose during gastroenteritis with hypoglycemia, increasing lactate concentrations and lactate/pyruvate ratio upon glucose administration, and improvement upon administration of vitamins B_1, C, K_1, and riboflavin. These symptoms are highly indicative for defective OXPHOS. In a skeletal muscle biopsy enzyme activities of complexes I, II, III, and IV were hardly detectable [43,45]. Similar findings were reported in post-mortem studies in muscle tissue of LCHAD deficient patients (all homozygous for the G1528C mutation) [46].

When digitonin-permeabilized skin fibroblasts from healthy volunteers were incubated with C_{16}-CoA and intermediates of C_{16}-CoA oxidation (hexadec-2-enoyl-CoA, 3-hydroxyhexadecanoyl-CoA, 3-ketohexadecanoyl-CoA, and 3-hydroxytetradecanoic acid), succinate- and glutamate-driven OXPHOS were inhibited in a concentration-dependent manner [47–51]. Decreased formation of malate from succinate most likely resulted from direct inhibition of the dicarboxylate carrier by C_{16}-CoA or its β-oxidation intermediates [48]. Additionally, ADP-induced respiration was found to be inhibited, most likely due to direct inhibition of the mitochondrial adenine nucleotide transporter (ANT) by long-chain acyl-CoA esters.[50,52].

Finally, *in vitro* studies showed uncoupling effects of the long-chain mFAO fatty acids $C_{16:0}$, 3-hydroxydodecanoic acid, 3-hydroxytetradecanoic acid and 3–hydroxyhexadecanoic acid in rat liver, brain and heart mitochondria [51,53,54]. In uncoupled mitochondria, the proton gradient is not entirely used for ATP production, but generates heat [55].

Medium-chain mFAO defects

Cases of MCAD deficiency leading to clinical presentations corresponding to secondary OXPHOS defects have not been described in literature, whereas *in vitro* studies showed various associations between accumulation of medium-chain mFAO intermediates and hampered OXPHOS. These associations, however, were observed mostly at non-physiological concentrations of these intermediates that are rarely seen in patients with MCAD deficiency.

In vitro studies in homogenates of rat cerebral cortex revealed an uncoupling effect of octanoic acid (OA), decanoic acid (DA), and *cis*-4-decenoic acid (cDA) on mitochondrial function [56,57]. Only cDA inhibited 2,6-dichloroindophenol reduction by succinate (complex II), cytochrome *c* reduction by succinate (complex II + CoQ + complex III), and oxidation of cytochrome *c* (complex IV) at concentrations that can sporadically be found in plasma of MCAD deficient patients during metabolic crisis (0.1 – 0.25 mM) [58]. However, synergism between these three metabolites could not be excluded [58]. OA has further been described to affect the intramitochondrial ATP/ADP ratio and lower the P/O ratio (amount of ATP generated per reduced oxygen atom in OXPHOS) [59,60]. *In vitro* studies in isolated bovine heart cells reported complex III inhibition by octanoyl-CoA in a concentration-dependent manner, possibly due to prevention of reduction of cytochrome b [61]. Octanoyl-CoA was furthermore found to inhibit PDHc activity [61]. Octanoyl-carnitine, which most commonly accumulates in blood of patients with MCAD deficiency, did not exert these effects [59,61].

Short-chain mFAO defects

As for MCAD deficiency, no case reports presenting patients with SCAD deficiency and associated OXPHOS dysfunction have been described in literature. However, it should be noted that the phenotypical spectrum varies considerably. Due to the position of SCAD in mFAO, where MCAD can largely compensate for deficient SCAD, it is difficult to understand the observed symptoms in these patients [3]. Consequently, it is even questionable whether SCAD deficiency can be considered an inborn error of metabolism. OXPHOS impairment by short-chain mFAO intermediates has only been observed

in vitro at concentrations that were considerably higher than the concentrations that have been observed in subjects with SCAD deficiency.

Subcutaneous injection of the main metabolite in SCAD deficiency, EMA (3 times 4 µmol/g body weight) in young rats did not affect OXPHOS [62]. However, *in vitro* studies showed evidence for inhibiting effects of short-chain mFAO intermediates. Incubation of normal human muscle cells with EMA resulted in inhibition of cytochrome *c* reduction by NADH (complex I + CoQ + complex III) at concentrations of 0.25 mM [63]. Cytochrome *c* reduction by succinate (complex II + CoQ + complex III) and 2,6-dichloroindophenol reduction by succinate (complex II) were inhibited at higher concentrations [64]. In rat liver mitochondria, short-chain fatty acids have been reported to decrease the P/O ratio in a concentration-dependent manner upon incubation. However, the effect of butyrate was weaker than the effect of medium-chain fatty acids at equal concentrations [60]. PDHc was inhibited by acyl-CoA esters in a chain-length specific manner, with short-chain monocarboxylic acyl-CoAs exerting the strongest effect. This latter finding has a physiological explanation, as acetyl-CoA is a product of PDHc, which allosterically inhibits PDHc and thereby prevents overload of the TCA cycle. As for medium-chain length metabolites, short-chain dicarboxylic acyl-CoAs or acylcarnitines were not found to inhibit OXPHOS [28,61].

Multiple acyl-CoA dehydrogenase deficiency

Patients with MADD were defined as patients with biochemical characteristics of MADD due to impairment of ETFα, ETFβ or ETF-DH function. A subset of the patients carrying mutations in ETF-DH has been described to have milder riboflavin-responsive forms of MADD [7]. Several studies have described cases of riboflavin-responsive MADD (not due to a riboflavin-transporter defect) with lowered activities of complexes I and II of the ETC and secondary UQ deficiency [65–68]. Riboflavin supplementation was found to induce complete recovery of the clinical symptoms, with improved activities of the ACADs, and normalized activities of complexes I and II. Decreased availability of FAD was found to be the main cause for the accompanying OXPHOS dysfunction in MADD [65–67].

In summary, inhibition of ANT due to the accumulating intermediates, with secondary dicarboxylate carrier and PDHc dysfunction were most commonly reported in studies on concurrent OXPHOS dysfunction in response to an mFAO defect.

THE ROLE OF SECONDARY CELLULAR RESPONSES

In general, clinical presentations of defects in OXPHOS or mFAO are distinguishable separate entities. However, based on the close biochemical interrelations, one would expect to regularly see clinical presentations of associations between OXPHOS and mFAO defects. As the concurrence of OXPHOS and mFAO is sporadically displayed in a clinical presentation, and the observed clinical presentations are very heterogeneous, these episodes cannot be solely explained by primary biochemical linkages.

We hypothesize that the enzyme insufficiencies or deficiencies that were discussed in this review may lead to improper activation of signaling pathways due to alterations in metabolite concentrations, and ultimately to an insurmountable obstacle causing failure to reach normal homeostasis. Even though a new steady state will eventually be reached, it will be pathologic in nature. It should be realized that metabolic functioning is tightly linked to metabolic regulation. Metabolic diseases result in abnormal intracellular steady-state concentrations of essential metabolites. Often normal oxygen uptake rates have been described in patients with mitochondriopathies [69]. As a result, supply of redox equivalents to reduce oxygen from NADH and $FADH_2$ via ETC will remain normal, as will the flux through the TCA cycle. The partially deficient enzyme, however, can only work at normal speed if its reduced catalytic capacity is compensated by increased substrate and/or decreased product concentrations. The cell will recognize the new steady-state as a disrupted homeostasis and a counter-regulatory response may be mounted via signal transduction and/or gene expression. We therefore hypothesize that these secondary cellular responses to changes in the metabolome are an important, and currently under-exposed, contributor to the development of a clinical phenotype.

Data underlining this hypothesis were recently presented in a study on secondary cellular responses in tissue samples of patients with a confirmed complex I deficiency. The data were not linked yet to mFAO impairment. Zhang *et al*. studied the transcriptome by microarray analysis in muscle biopsies and cultured skin fibroblasts [69]. By means of this unbiased approach a highly tissue-specific response was detected, with alterations at the level of the global transcriptome and in parallel in the activity of nutrient-sensing signaling networks of i.e. mTORC1, AMPK, SIRT, FOXO and PPAR. Alterations in these networks resulted in conflicting anabolic and catabolic responses occurring simultaneously. Most strikingly, intervening in these secondary cellular responses by treating complex I deficient cultured skin fibroblasts with nicotinic acid, a precursor for NAD^+, activated the NAD^+–dependent protein deacetylase SIRT, PPAR, mTORC1 and AMPK signaling. This led to normalization of the activity of these networks, and improved cellular respiratory capacity, without affecting the severity of the OXPHOS defect. Similar effects were seen in mice with cardiac complex I deficiency (due to knock-out of cardiac *Ndufs4*) after treatment with nicotinamide mononucleotide, another NAD^+ precursor [70]. It should however be noted that these effects may in part be due to alterations in the primary biochemical reactions, because changes in NADH/NAD^+ ratio will directly affect metabolism as well. However, this possibility does not apply to experiments performed by Johnson *et al.* in *Ndufds4*$^{-/-}$ mice, a mouse model for Leigh syndrome, a mitochondrial disease [71]. These mice suffer from a progressive neurodegenerative phenotype characterized by lethargy, ataxia, weight loss, and ultimately death at a median age of 50 days. When these mice were treated daily with rapamycin, an inhibitor of the protein kinase complex mTORC1, the symptoms alleviated dramatically, with a striking extension of median and maximum lifespan (maximum 269 days). Apparently, normalization of the nutrient-sensing networks caused the gene-expression profiles to revert to what was observed in unaffected cells, despite unaltered severity of the primary defect. Moreover, determination of residual respiratory capacity in cultured skin fibroblasts indicated that a limiting respiratory capacity *per se* was not the main reason for the secondary cellular responses [69,70].

Similarly, conflicting anabolic (i.e. mTORC1) and catabolic (i.e. AMP-activated protein kinase) signaling was observed in studies in the Twinkle mouse, which suffers from multiple mtDNA deletions. These mtDNA lesions induced OXPHOS defects and late-onset mitochondrial myopathy. Tyynismaa *et al.* determined a starvation-like response to the OXPHOS defects, with induced Akt signaling and fibroblast growth factor 21 expression, a hormone related to fasting. Muscle protein degradation was upregulated, and lipid synthesis was enhanced in this mouse model [72]. When these mice were treated with a ketogenic or high-fat diet, mitochondrial biogenesis was induced and fibroblast growth factor 21 expression normalized, without affecting mtDNA deletion load. Collectively, these data clearly show that the clinical phenotype is not linked to the enzyme deficiency *per se* but results from aberrant responses in signaling networks.

With regards to primary mFAO defects, altered mTORC1 signaling was found to determine phenotype severity in zebrafish with MADD due to inactivation of the *etfa* gene, coding for ETFα protein. *Etfa* deficient zebrafish showed similarities with MADD-patients both biochemically and clinically. Acylcarnitine profiles were abnormal, and fatty liver was observed. Additionally, hyperplastic neural progenitor cells, hepatocytes and kidney tubule cells were observed. Increased mTORC1 signaling was observed with cellular enlargement and proliferation, which was abolished by rapamycin [73]. The authors speculate that the persistent increase of leucine, activator of mTORC1 and substrate of isovaleryl-CoA dehydrogenase, which needs ETFα for its activity, might be central in eliciting the secondary cellular response in MADD [73].

Circumstantial evidence on a potential role for secondary cellular responses in determining the clinical phenotype in mFAO defects was also found in LCAD-deficient mice [74]. In this mouse model, amino acid metabolism was intrinsically disturbed, possibly also due to altered mTORC activation resulting from the mFAO dysfunction.

In OXPHOS and mFAO defects, dietary treatment forms the basis of therapy. Administration of high doses of intravenous glucose is necessary in patients with a defect in mFAO who are in a metabolic crisis, whereas this treatment may lead to an undesired increased lactate concentration and aggravation of symptoms in patients with an OXPHOS defect. Patients with an OXPHOS defect, in particular complex I defects, require a high lipid, low-carbohydrate diet supplemented with vitamins (e.g. riboflavin). Glucose oxidation largely occurs aerobically in the liver and requires NAD^+. As the NADH/NAD^+ ratio is disturbed due to the OXPHOS defect, glucose oxidation will provide more substrate for lactate formation, and can thereby lead to further increased lactate concentrations in patients [2]. Vitamin supplementation is used as a treatment to increase mitochondrial supply of $FADH_2$ as compensation for the diminished mitochondrial oxidation of NADH [2,8,43,75]. It should however be realized that all of the traditional treatment modalities are based on interfering in the primary biochemical reactions, generating product but preventing excessive metabolite accumulation. It remains to be determined if the negative effects of glucose supply in case of OXPHOS defects truly result from the enzymatic defect, or whether they result from aberrant signaling through nutrient sensing networks. The recently published studies on the role of secondary cellular responses [69–72] point to new and exciting treatment modalities in relation to mitochondriopathies [76]. Restoration of the secondary cellular response may

not only be able to prevent the clinical phenotype, but can possibly also normalize the primary biochemical reactions. Various examples of NAD^+ precursors (i.e. niacin and nicotinamide) have already been approved by the U.S. Food and Drug Administration and may be interesting compounds to be studied in relation to at least OXPHOS defects, but possibly also mFAO defects.

CONCLUSION AND FUTURE PERSPECTIVES

In this review we addressed the question why defects in OXPHOS only sporadically behave as mFAO defects and *vice versa*. Inborn errors of metabolism are complex traits [1]. The sporadic nature of clinical phenotypes displaying the concurrence of OXPHOS and mFAO defects is an illustration of this principle. In this review, we discussed the published reports on clinical presentations associated with combined defects in both OXPHOS and mFAO. Based on the discussed results we can conclude, albeit preliminary, that instead of the primary biochemical links, secondary cellular responses may well be the criterion to determine whether an individual will be at-risk for a metabolic crisis involving both OXPHOS and mFAO. Since nutrient-sensing signaling networks integrate signals from many sources, outside and inside the cell by complex feed-forward and feed-backward responses, changes in the metabolic state of affected cells or conditions in the environment of these cells may well explain the accidental nature of these metabolic crises. This problem asks for studies from a cellular point of view with an unbiased approach i.e. an –omics approach (entailing respectively, metabolomics, proteomics, and genomics at the level of the secondary signaling pathways) and careful histology [77]. From the point of view of OXPHOS defects, application of these unbiased approaches has resulted in identification of new inborn errors of phospholipid metabolism affecting OXPHOS [see [78] for a review]. Exome sequencing followed by Sanger sequencing identified mutations in the gene coding for an enzyme involved in phospholipid remodeling (SERAC1), which resulted in OXPHOS defects [79]. Integrative analysis of the results of various –omics technologies will be the next step. Although not yet applied in clinical practice, combinations of metabolomics and genomics have been used in studies of population cohorts with promising results [80,81]. What is however missing are 'phenomes': thorough, coherent and exact descriptions of patient clinical characteristics [82,83]. The availability of structured phenotypic information in genomic databases will improve clinical care [84].

In conclusion, the study of secondary cellular responses that results from primary OXPHOS defects has now started. Most strikingly, these studies hold promise to genuinely new approaches for diagnosing and treating patients with OXPHOS and mFAO defects. It calls for the more integrative approach of systems medicine with implementation of medical practice driven by clinical investigations, –omics technologies and computational models.

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Chapter ∞

General discussion

MCAD deficiency: Where do we go from here?

In this thesis, we aimed at stratifying and predicting the disease burden (i.e. the clinical phenotype) in the group of patients with a positive newborn screening (NBS) for MCAD deficiency, using two approaches. First of all, we studied metabolic parameters and the outcomes of molecular studies and enzymatic analyses in subjects with a positive screening test for MCAD deficiency, and related them to the disease burden. Secondly, we studied the pathophysiology behind the clinical phenoptypes in MCAD deficiency. In this thesis, we globally distinguished two types of clinical presentations in MCAD deficiency: the risk to abruptly develop an acute life-threatening event with severe hypoketotic hypoglycemia, and the development of a more chronic phenotype with muscular complaints. Not all patients develop either of these phenotypes. The question arises whether a common etiology exists for both presentations, and how the observed variation in clinical phenotype can be explained.

In this general discussion we would like to explore the possibility that the two phenotypes result from essentially different consequences of the deficiency of MCAD function. Therefore, we will discuss the relevant biochemical models of mitochondrial fatty acid oxidation (mFAO) and use this knowledge in an attempt to elucidate the underlying mechanisms in both the risks of a life-threatening metabolic crisis and the development of a chronic phenotype. Then we return to the clinical phenotype and discuss the therapeutic consequences of our pathophysiological model.

ASSOCIATIONS IN MCAD DEFICIENCY

Introduction of MCAD deficiency into the nationwide NBS program has led to a considerable reduction in mortality and morbidity. However, it has also posed new questions, as we now identify subjects with variant *ACADM* genotypes of which the clinical consequences remain questionable. By early identification of patients with MCAD deficiency upon NBS and early advent of treatment, the development of a clinical phenotype can largely be prevented. However, even after diagnosis acute hypoglycemia during intercurrent illness can be observed. As a result, the question arises who is at risk for the development of these metabolic crises. Resulting from this, there is discussion on what treatment regimen should be followed, and how this can be tailored to the individual patient. In order to be able to tailor treatment to the patient, identification of factors that contribute to the development of a clinical phenotype is of utmost importance.

By searching for associations between clinical symptoms and metabolite concentrations, molecular studies, and enzymatic analyses, we determined that residual MCAD enzyme activity is an important indicator for the risk of a subject after positive NBS (**Chapter 2**). Clinical symptoms and the need to prescribe L-carnitine supplementation have thus far not been seen in subjects with residual MCAD enzyme activities ≥10% when measured with the phenylpropionyl-CoA (PP-CoA) enzyme assay [1], and with residual *in vivo* MCAD enzyme activity determined with a phenylpropionic acid (PPA) loading test [2]. Additionally, the *ACADM* genotypes associated with residual MCAD enzyme activities ≥10% have only been identified since introduction of NBS. The C_8/C_{10} ratio determined upon NBS correlated strongly with residual MCAD enzyme activity, and could therefore be considered an important early

marker for risk-stratification. Although the C_8-carnitine concentration alone also correlated, it showed considerable overlap between patients with <1% residual MCAD enzyme activity and subjects with residual MCAD enzyme activities ≥10%.

A recent study by Sturm *et al.* proposed a cut-off percentage of 20% using C_8-CoA as a substrate for determination of residual MCAD enzyme activity [3]. This study described residual MCAD enzyme activities as high as 16% in subjects with classical *ACADM* genotypes, which is considerably higher than the residual MCAD enzyme activities that we measured in this group of patients with PP-CoA [3]. No correlations between residual MCAD enzyme activities measured with C_8-CoA and C_8-carnitine concentrations upon NBS were reported in this study [3].

The question is whether we can explain the observed associations from a biochemical point of view.

BIOCHEMISTRY

As was discussed in previous chapters, the mitochondrial fatty acid oxidation (mFAO) is a cyclic pathway in which acyl-CoA esters of different chain-lengths undergo stepwise shortening into acetyl-CoA and an acyl-CoA that is shortened by 2 carbon atoms. The resulting acyl-CoA esters of various chain lengths and the newly entering acyl-CoA esters compete for the different acyl-CoA dehydrogenases (ACADs). Eventually, this can lead to feed-forward inhibition when the concentration of substrate increases, rendering the system sensitive to substrate overload [4].

In humans, defects in the group of ACADs can occur at 3 different positions: at the level of very long-chain acyl-CoA dehydrogenase (VLCAD), medium-chain acyl-CoA dehydrogenase (MCAD), or short-chain acyl-CoA dehydrogenase (SCAD). In rodents, a fourth ACAD participates in mFAO: long-chain acyl-CoA dehydrogenase (LCAD). Dysfunction of one of these enzymes can come in gradations, as was described in the previous paragraph. Each of the ACADs has substrate specificity for acyl-CoA esters with different chain-lengths, but their substrate specificities overlap. When a deficiency in one of the ACADs occurs, flux through the mFAO can only be kept constant when the substrate concentration increases and/or the product concentrations decrease. When the concentration of substrates is increased in the cyclic mFAO, the flux through the system will initially be kept constant. However, as was previously predicted by a dynamic computational model of rat liver mFAO [4], eventually extensive feed-forward inhibition will occur due to the competition of acyl-CoA intermediates and newly entering acyl-CoA esters for the different ACADs, leading to further accumulation of intermediates and subsequent depletion of free CoA (CoASH). The flux through the system was predicted to drop suddenly upon CoASH depletion [4].

Besides possibly hampering mFAO, depletion of CoASH can also impede glucose oxidation, as the conversion of pyruvate to acetyl-CoA is CoA-dependent. Additionally, gluconeogenesis and TCA cycle functioning depend on CoASH, as this is an essential co-factor in the formation of succinyl-CoA out of 2-ketoglutarate. Upregulated acyl-CoA ester formation, and a subsequent depletion of CoASH,

may thus eventually lead to complete mitochondrial dysfunction. Resulting from this, defective urea cycle functioning may occur, as both the TCA cycle and CoASH contribute to the formation of N-acetylglutamate which is required for activation of carbamoyl phosphate synthase, the enzyme that catalyzes the first step in the urea cycle. As a result of urea cycle dysfunction, ammonia concentrations may increase, as has been described in patients during an acute metabolic crisis [5].

From these predictions we propose that MCAD deficiency will increase the sensitivity of mFAO to substrate overload. In **Chapter 6** we have shown that this is indeed the case when the V_{max} for MCAD was adapted to 10% of wild type (WT). CoASH was depleted more rapidly and the breaking point was reached earlier than in the WT situation. Remarkably, flux through the system was normal until the breaking point was reached. It can be hypothesized that the extent of the enzyme deficiency can determine the rate at which abrupt CoASH depletion and substrate overload will occur.

RELEVANCE FOR THE PATIENT – THE ACUTE PHENOTYPE

Until now we have not been able to predict the occurrence of a metabolic crisis in patients based on biochemical profile or *ACADM* genotype [6,7]. If substrate overload underlies the hypoketotic hypoglycemia in MCAD deficiency, the absence of a genotype-phenotype or a biochemical phenotype-clinical phenotype correlation may be expected. Even though we know from some *ACADM* genotypes that they are associated with residual MCAD enzyme activities <1%, residual MCAD enzyme activity alone does not completely determine the sensitivity to substrate overload. Upon simulations in the dynamic model for rat liver mFAO substrate overload was predicted to depend on acyl-CoA and CoASH concentrations, parameters that can currently not be measured in plasma of patients. The amount of substrate that is administered to mFAO thus seems to be far more important, even though this may largely relate to residual MCAD enzyme activity. Besides acyl-CoA esters and acylcarnitines, other metabolites (e.g. carboxylic acids derived from microbial metabolism in the gut) require metabolization by mFAO and may thus be able to negatively affect sensitivity to substrate overload [8].

Substrate overload may be a purely hepatic process. From studies in the mouse model for VLCAD deficiency it is known that responses to the mFAO defect are tissue-specific, with different adaptive mechanisms in liver, heart, and muscle respectively [9]. Liver concentrations of long-chain acylcarnitines increased particularly upon prolonged fasting in the cold, but not upon exercise. In muscle, the opposite was seen, and the effects in blood were 8-10 times lower than what was observed in liver and muscle [9]. It is therefore possible that substrate overload upon an acute metabolic crisis is only expressed in the liver, but cannot be observed in bloodspots or plasma. If this is the case, prediction of a metabolic crisis in a patient will be impossible. This problem is further hampered by the fact that acyl-CoA esters cannot be measured in bloodspots or plasma.

Fasting tolerance test

The acute character of the drop in flux upon substrate overload may be able to explain the acute metabolic crisis with hypoketotic hypoglycemia that can be seen in MCAD deficient patients. These clinical phenotypes generally become manifest upon prolonged fasting with intercurrent illness [10]. In **Chapter 3** we used a fasting tolerance test to study the effect of metabolic stress on metabolite profiles and fasting tolerance. In the population of subjects with classical *ACADM* genotypes and residual MCAD enzyme activities <1%, the response to prolonged fasting was found to vary widely (personal observation). Hypoglycemia upon prolonged fasting alone was only sporadically observed, and an elevated FFA/KB ratio (indicating hypoketosis) was not always present. This indicates that the response to prolonged fasting is an individual process in MCAD deficiency.

In subjects over 6 months of age with variant *ACADM* genotypes and residual MCAD enzyme activities ≥10% we determined that fasting tolerance and ketone body production were normal upon prolonged fasting (>15 hours) under healthy conditions. However, in this study, concentrations of medium-chain length acylcarnitines (and in some cases also longer-chain acylcarnitines) increased with duration of fasting, indicating dysfunctional mFAO. Final acylcarnitine concentrations varied per person, even within subjects with the same *ACADM* genotype, again showing an individual response to prolonged fasting. When considering the proposed hypotheses behind the clinical phenotype in MCAD deficiency on substrate overload (**Chapter 6**), or toxicity of accumulating intermediates (**Chapter 7**), the observed increase in medium- and long-chain acylcarnitines upon prolonged fasting may have clinical relevance. It should be noted that direct toxic effects have *in vitro* predominantly been attributed to long-chain mFAO intermediates, whereas medium-chain acylcarnitines were the predominant accumulating metabolites in the subjects that were included in our study [11–14]. Currently no reference values exist for acylcarnitine concentrations upon prolonged fasting where we can refer to with regards to our patients.

If the acute phenotype with hypoglycemia can be attributed to substrate overload, then it remains unresolved why the responses to prolonged fasting vary and are individual when analyzing a group of MCAD deficient patients with the same *ACADM* genotype or residual MCAD enzyme activity. First of all, peroxisomal function may play a role in this individual sensitivity, as it has been shown to be important in medium-chain fatty acid metabolism. A study in an *L-bpe* (a peroxisomal enzyme) knock-out mouse model showed that peroxisomal metabolism is important in detoxification of dietary medium-chain fatty acids via dicarboxylic acid formation and degradation. *L-bpe* knock-out mice excreted very high concentrations of dicarboxylic acids and dicarboxyl-carnitines in the urine, and experienced liver failure upon administration of coconut-oil, which is rich in medium-chain fatty acids [15]. As metabolism of medium-chain fatty acids is hampered in patients with MCAD deficiency, there may be an important role for the peroxisomes in maintaining homeostasis. In MCAD deficient patients, high urinary excretion of dicarboxylic acids has been observed, predominantly upon prolonged fasting or during a metabolic crisis. The extent of the urinary dicarboxylic acid excretion may be related to individual peroxisomal capacity, which may in turn add to the individual sensitivity to substrate overload.

Secondly, the secondary cellular responses (i.e. nutrient-sensing networks) in mitochondrial metabolism that were discussed in **Chapter 7** may provide some answers. The persisting elevated concentrations of mFAO intermediates due to the enzymatic defect may affect nutrient-sensing networks, as was described in the zebrafish model of multiple acyl-CoA dehydrogenase deficiency (MADD) [16]. The alterations in these nutrient-sensing networks may lead to the expression of a clinical phenotype. Additionally, as the nutrient-sensing networks are complex and function via extensive feed-forward and feed-back mechanisms, individual characteristics of these networks may determine the individual response to prolonged fasting, and thereby the sensitivity to substrate overload. The individual effect of MCAD deficiency on secondary cellular responses may then aid in explaining the variable expression of an acute phenotype upon intercurrent illness.

Fever and intercurrent illness

Fever may increase the sensitivity to substrate overload by affecting residual MCAD enzyme activity. As metabolic crises generally occur during intercurrent illness, studies on the effect of fever may be even more important to determine potential risks for patients than fasting tolerance tests. MCAD enzyme activity has been found to be temperature-sensitive, a property that may be of particular interest in case of variant *ACADM* genotypes associated with considerable residual MCAD enzyme activities [17–19]. Residual MCAD enzyme activities have not been sufficiently studied under conditions of increased temperature, especially not *in vivo* [18–20]. A decrease in residual MCAD enzyme activity in response to fever may increase the load of metabolites, not only by increasing the concentrations of medium-chain acyl-CoAs and acylcarnitines, but also the concentrations of microbial carboxylic acids that require metabolization in the β-oxidation [21]. Particularly during episodes with fever or gastrointestinal infection, concentrations of these metabolites can be significantly elevated when elevated body temperature increases the energy requirements, and the increased permeability of the intestinal wall due to gastrointestinal infection enhances the supply of microbial monocarboxylic acids competing with a high supply of long-chain fatty acids [8,22]. Especially in young children an increased body temperature can seriously affect glucose requirement, rendering a properly functioning mFAO of major importance [22,23].

On top of this, L-carnitine has been described to have an inhibitive effect on excretion of glycine conjugates, which may further aggravate mFAO substrate load by hampering the disposal of mFAO mediates [24]. As a large proportion of the patients with MCAD deficiency receive L-carnitine supplementation, and even in higher concentrations during fever according to the protocol of various metabolic centers, this is an extra point of caution when the hypothesized sensitivity to substrate overload is the underlying problem. With the increasing substrate load, the breaking point for overload may be reached already in an earlier stage. Adding to this, individual alterations in secondary cellular responses resulting from the permanently elevated concentrations of mFAO intermediates may further determine the sensitivity to substrate overload upon intercurrent illness and prolonged fasting.

RELEVANCE FOR THE PATIENT – THE CHRONIC PHENOTYPE

As was described in **Chapter 4**, lowered exercise tolerance and muscular complaints are among the most frequently heard chronic complaints in MCAD deficiency [5]. Not all patients suffer from these complaints. Subjective muscular complaints have thus far only been observed in a subset of the patients with classical *ACADM* genotypes, which are associated with residual MCAD enzyme activities <1% (personal observation). As for the acute phenotype, complaints related to exercise tolerance thus also seem to be associated with residual MCAD enzyme activity. However, due to the low mean age in the group of subjects with variant *ACADM* genotypes and residual MCAD enzyme activities ≥10% we may not have a complete picture on this. Exercise tolerance does not seem to be related to acylcarnitine profiles, free carnitine concentrations in plasma, and L-carnitine supplementation [25–27], even though subjectively patients report beneficial effects of L-carnitine supplementation.

In **Chapter 4** we describe a study using magnetic resonance spectroscopy (MRS) for analysis of intramuscular energy metabolism during exercise in patients with MCAD deficiency. With this non-invasive method, we were able to determine altered intramuscular energy metabolism in MCAD deficiency, most likely resulting from a shift in predominant muscle fiber type from type I oxidative fibers to type II glycolytic fibers. Mitochondrial function was found to be normal in muscle of patients. The response to prolonged moderate intensity exercise varied considerably within the group of included patients, even though all patients had the same *ACADM* genotype. Both the proposed shift in muscle fiber type and the variation in exercise tolerance may be attributed to alterations such as single nucleotide polymorphisms in nutrient-sensing networks. As discussed before, the only evidence for effects of an mFAO defect on nutrient-sensing networks has been reported in zebrafish with MADD, in which mTORC1 signaling was altered [16]. This was not studied in relation to muscle function, however, mTORC1 is known to play a role in muscle metabolism [28,29].

Besides alterations in the nutrient-sensing networks, resulting from the enzymatic defect, oxidative stress may also participate in the development of the muscular phenotype, and in particular to the subjective aggravation of the muscular symptoms with age. Oxidative stress has been proposed as an important pathogenic feature of various metabolic diseases [30], and various oxidative stress parameters have already been proposed as markers for disease progression [30]. Studies on oxidative stress in MCAD deficiency follow logically from the close links between mFAO and oxidative phosphorylation (OXPHOS), and previous reports on the association between disturbed mitochondrial function and increased concentrations of oxidative stress [31–33].

In **Chapter 5** we describe protein oxidative damage and an altered enzymatic antioxidative defense in an asymptomatic non-stressed population of MCAD deficient patients with classical *ACADM* genotypes. L-carnitine and riboflavin supplementation appeared to have different effects on oxidative stress. In the group of patients receiving L-carnitine supplementation signs of protein oxidative damage and altered antioxidative defense were found, whereas no signs of oxidative stress were observed in the group of patients receiving a combination of L-carnitine and riboflavin. A hypothesis for these findings is that L-carnitine supplementation increased the mitochondrial entry of acyl-CoA

esters, thereby influencing substrate load and the effects of increased concentrations of mFAO intermediates on nutrient-sensing networks. A case report has described potential harmful effects of L-carnitine on the clinical phenotype in a patient with MADD [34], however most reports in literature attribute beneficial effects to L-carnitine supplementation with regards to oxidative stress [35–38].

It should be noted that the clinical relevance of the oxidative stress that we reported in MCAD deficient patients needs to be determined before conclusions on its role in disease progression can be drawn. As oxidative stress could already be observed under non-stressed conditions, it is well possible that alterations in secondary cellular responses and their effects on OXPHOS underlie these findings. Besides, it can be hypothesized that metabolic stress or exercise can negatively affect the oxidative stress burden in MCAD deficiency by increasing concentrations of mFAO intermediates. It is known that muscle contraction leads to the generation of reactive oxygen species [39]. The other way around, oxidative stress may affect muscle energy metabolism as it is known to be able to affect posttranslational modification of proteins, thereby inhibiting for example insulin signal transduction [40,41]. As metabolism in MCAD deficiency under normal conditions already has to deal with a burden of reactive oxygen species that exceeds the antioxidative capacity, it is well possible that the extra load that is generated in response to exercise may cause additional oxidative damage. Oxidative stress may for example cause damage to mitochondrial membrane integrity, leading to uncoupling of OXPHOS and impaired mitochondrial function [42], which may consequently lead to a lowered exercise tolerance.

FUTURE PERSPECTIVES

In this thesis we have proposed various hypotheses on the pathophysiology behind the clinical phenotypes that can be observed in MCAD deficiency. Currently, the process towards identification of the trigger that leads to both the acute and the chronic clinical phenotype is hampered by the limited sources for information about metabolic processes. At present, we can only obtain information from the metabolome and the genome, and even with regards to the metabolome we are limited in the type of metabolites that can be detected (e.g. acyl-CoA esters cannot be determined). From previous studies it is known that *ACADM* genotype alone cannot determine the risk on a clinical phenotype. Whereas determination of residual MCAD enzyme activity can aid in stratification, more information about the metabolome is needed in order to improve the stratification process. Analyses in the *in silico* model for MCAD deficiency have been found to be useful in providing information on metabolites that cannot be studied in plasma or urine of patients, thereby enabling insights for new hypotheses. Optimization of the *in silico* model for mouse mFAO, and in the future also for human mFAO, is therefore required. As human and rodent mFAO differ, predominantly in the presence and function of LCAD, hypotheses generated for the rat or mouse situation may not be applicable to the human situation. Besides, the predictions that were described in this chapter for the *in silico* model only regard simulations with long-chain mFAO intermediates. For MCAD deficiency, *in vitro* studies have reported possible harmful effects of medium-chain mFAO intermediates [43–45]. Simulations with

medium-chain mFAO intermediates are therefore required to analyze the possible intoxicating and CoASH depleting effects of these intermediates in time.

Exploring the possible role of substrate overload in the generation of the acute clinical phenotype in patients can start by analyzing routes of substrate disposal. An important route is urinary excretion of acylcarnitines, dicarboxylic acids and glycine conjugates [24]. A first step can be elucidation of the role of peroxisomes in medium-chain fatty acid metabolism in patients with MCAD deficiency. It may be interesting to generate MCAD-KO / *L-bpe* knock-out mice to obtain more insight in this problem. Furthermore, retrospective analysis of urine samples of MCAD deficient patients obtained under various circumstances (e.g. under fed, fasted, or stressed conditions) may aid in extending the formula of known risk factors (that currently only consists of *ACADM* genotype and residual MCAD enzyme activity). Residual MCAD enzyme activity determined with PP-CoA as a substrate was found to be useful for risk stratification. As PP-glycine is a typical product of PP-CoA metabolism in MCAD deficiency, analysis of urinary excretion of this metabolite may in particular provide important information.

As sensitivity to substrate overload has only been predicted in an isolated *in silico* model, linking the *in silico* mFAO model to other models of energy metabolism is required. Adaptive mechanisms in for example glucose metabolism or TCA cycle functioning may add to the individual sensitivity to substrate overload.

Determining the role of secondary cellular responses in the pathophysiology of mFAO defects is of major importance as this information may aid in resolving some of the questions that remain unanswered with regards to the individual sensitivity to developing a clinical phenotype, and in determining the proper treatment for mFAO defects. Besides the studies in the zebrafish model of MADD, studies in tissues of mice and humans with OXPHOS defects also showed alterations in nutrient-sensing networks. Disease could be modified by interfering in the affected nutrient-sensing networks (e.g. by correcting a distorted NAD^+/NADH ratio or by inhibiting the mammalian target of rapamycin (mTORC) pathway), and in some cases the clinical phenotype could largely be reverted [16,46–49]. Studies on the different levels of metabolic regulation, combining proteomics, metabolomics, and genomics at the level of nutrient-sensing networks, are required in order to study the possible contribution of these networks in the pathophysiology of mFAO defects other than MADD. As metabolic defects affect multiple layers of metabolic regulation, studies on interactions between these levels are also necessary in order to understand the complete picture. Over the past years, it has become possible to reprogram various cell lines, to generate induced pluripotent stem cells (iPSCs). These stem cells can then be used for the creation of cell lineages with different properties than the cell type that was originally isolated from the patient, and may thereby aid in unraveling tissue-specific responses in MCAD deficiency [50]. Reports have described the creation of hepatocyte-like cells from human skin fibroblasts [51], and metabolic disorders have successfully been modeled using iPSCs [52]. It should however be noted that iPSCs are not completely similar to embryonic stem cells, which may affect the characteristics of the cell lineages that are generated [50]. Insight in the role of nutrient-sensing networks in the development of clinical phenotypes associated with mFAO defects opens the door

to new and previously unexpected treatment modalities. This type of treatment should in particular be considered for interfering in networks that are associated with a chronic phenotype or individual sensitivity to the development of a metabolic crisis.

The role of L-carnitine supplementation in mFAO defects remains a point of discussion. The potential benefits and doubts of L-carnitine supplementation in MCAD deficiency have been repeatedly discussed in this thesis. It remains questionable whether administration of L-carnitine leads to better disease outcome, especially when looking at the data that were described in **Chapter 5** on oxidative stress and in **Chapter 6** on a possible role of L-carnitine in CoASH depletion. The potential drawbacks of supplementation are particularly alarming, as a large percentage of the patients currently uses L-carnitine supplementation. Proper cross-over studies evaluating effects of L-carnitine supplementation on clinical outcome (i.e. the number of clinical derailments), oxidative stress parameters and muscle metabolism (analyzed by MRS) in larger study populations should therefore be considered.

Long-term effects of MCAD deficiency have insufficiently been elucidated due to the low mean age of the current patient population. Oxidative stress may participate in disease progression, in particular with regards to the chronic phenotype, and the development of clinical symptoms later in life. As the proportion of adult patients with the disease increases, knowledge on long-term effects of MCAD deficiency becomes more and more important. The usefulness of oxidative stress parameters in risk stratification of true-positive subjects after NBS should therefore be explored.

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Chapter 9

Summary of the thesis

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most prevalent defect in mitochondrial fatty acid oxidation (mFAO). Inclusion of MCAD deficiency in national newborn screening (NBS) programs worldwide has decreased morbidity and mortality considerably. Clear genotype-phenotype correlations do not exist and the pathophysiology of the disease has not been fully elucidated. In this thesis we aimed at obtaining insight in pathophysiological mechanisms contributing to the development of a clinical phenotype and disease progression. Additionally, we aimed at risk stratification of patients with MCAD deficiency after positive NBS. This has led to some new insights on how to approach MCAD deficiency.

IN VITRO ANALYSES IN MCAD DEFICIENCY

In **Chapter 2** we determined that risk stratification can be performed after positive NBS by analyzing residual MCAD enzyme activity. In our study, residual MCAD enzyme activity determined with phenylpropionyl-CoA (PP-CoA) was found to be a useful parameter for risk stratification. Residual MCAD enzyme activities ≥10% measured with PP-CoA have never been associated with clinical symptoms or L-carnitine supplementation during follow-up. Besides, all subjects with classical *ACADM* genotypes that are known to be associated with metabolic crises showed residual MCAD enzyme activities that were considerably lower than 10%. The C_8/C_{10} correlated strongly with residual MCAD enzyme activity, and can therefore be used as an early marker for risk stratification.

IN VIVO ANALYSES IN MCAD DEFICIENCY

In **Chapter 3** we determined that subjects with residual MCAD enzyme activities ≥10% when compared to control have a normal fasting tolerance, and considerable *in vivo* residual MCAD enzyme activity as determined with a phenylpropionic acid (PPA) loading test. We integrated the results of *in vitro* tests mentioned in Chapter 2 with additional enzyme assays (using C_6-CoA as a substrate) and two *in vivo* tests: the PPA loading test, and the fasting tolerance test. The PPA loading test is based on the *in vivo* conversion of PPA in hippuric acid by the MCAD enzyme. In subjects with variant *ACADM* genotypes and residual *in vitro* MCAD activities ≥10% either no PP-glycine excretion, or trace amounts of PP-glycine were observed in the urine, indicating considerable *in vivo* residual MCAD enzyme activity.

A normal fasting tolerance and ketone body production were observed upon prolonged fasting (>15 hours) under healthy conditions. Concentrations of medium-chain length acylcarnitines, and in some cases also of long-chain acylcarnitines, increased in time, showing a large variation in the various included subjects. As no reference values exist for acylcarnitines during prolonged fasting, the clinical relevance of these findings remains to be determined.

As the effect of intercurrent illness and fever on *in vivo* MCAD enzyme activities remains to be determined, treatment regimens can currently not be altered in subjects with variant *ACADM* genotypes.

In **Chapter 4** we used magnetic resonance spectroscopy (MRS) and described an abnormal energy balance in exercising quadriceps muscle of patients with MCAD deficiency. We determined that patients with MCAD deficiency could tolerate short-term high-intensity exercise without problems. However, tolerability of prolonged moderate intensity exercise varied widely. Mitochondrial function was not hampered in muscle of patients with MCAD deficiency. Exercise tolerance could not be attributed to C_0-carnitine concentrations, glucose concentrations or L-carnitine supplementation. The shifted energy balance in exercising quadriceps muscle of MCAD deficient patients may be attributed to a moderate shift in the predominant muscle fiber type from type I oxidative fibers to type II glycolytic fibers.

OXIDATIVE STRESS AND MCAD DEFICIENCY

In **Chapter 5** we determined signs of protein oxidative damage and an altered antioxidant defense in plasma and erythrocytes of MCAD deficient patients. L-carnitine supplementation appeared to aggravate oxidative damage, whereas riboflavin appeared to have protective effects. L-carnitine is believed to be beneficial in MCAD deficiency, as it can aid in scavenging the toxic acyl-CoA esters by converting them into acylcarnitines that can be excreted in the urine. However, large cross-over studies analyzing possible beneficial or harmful effects of L-carnitine have not been described. Further studies are required to determine the clinical relevance of the observed results, and of the role of L-carnitine in generating oxidative stress and disease progression in MCAD deficiency.

STUDIES IN OTHER MODELS FOR MCAD DEFICIENCY

In **Chapter 6** we characterized the mouse model for MCAD deficiency and discussed how this model may aid in unraveling the pathophysiology behind the clinical phenotype. When MCAD deficiency was modeled in the recently published *in silico* model of rat liver mFAO by adapting V_{max} for MCAD to 10% of control (based on the PP-CoA oxidative activities that were observed in MCAD-KO mouse liver) an increased sensitivity to substrate overload was found when compared to the wild type situation. Even though this model requires adaptations in order to be useful for modeling mouse mFAO, and in a later stage also for the human situation, these findings may be useful for the generation of hypotheses for further studies on the pathophysiology behind the acute metabolic crisis in MCAD deficiency.

In the review article described in **Chapter 7** we discussed how mitochondrial metabolism is regulated by complex feed-forward and feedback pathways, and how metabolic diseases may affect secondary cellular responses, i.e. nutrient-sensing networks, thereby affecting mitochondrial metabolism and exerting a chronic clinical phenotype. Studies in a zebrafish model for MADD and studies in tissues of mice and humans with OXPHOS defects have shown that alterations in nutrient-sensing networks resulting from the genetic defect play a role in phenotype determination. Disease could be modified by interfering in these nutrient-sensing networks. Individual characteristics of these networks possibly determine whether a patient is at risk for the development of a clinical phenotype. Studies on different levels of metabolic regulation, combining proteomics, metabolomics, and genomics, are required to further unravel the role of nutrient-sensing networks in the pathophysiology of mFAO defects.

Chapter 10

Nederlandse samenvatting voor de niet-medicus

INLEIDING

Ons lichaam is opgebouwd uit cellen. Elke cel bestaat uit verschillende onderdelen, waaronder de mitochondriën, de energiefabriekjes van de cel. In de mitochondriën vinden vele processen plaats die bijdragen aan het produceren van energie die nodig is voor verschillende processen in het lichaam. Verschillende 'enzymen' dragen bij aan het produceren van deze energie. Enzymen kunnen stof A op een efficiënte manier omzetten in product B. Vele verschillende enzymen werken samen in het proces van energieproductie. De benodigde voedingsstoffen voor het produceren van energie kan het lichaam halen uit de voeding.

In de voeding zitten verschillende brandstoffen: suikers, eiwitten en vetten. Suikers zijn de meest gebruikte energievorm onder normale omstandigheden. De suikers die we uit de voeding halen worden in de uren na de maaltijd verbruikt. Als er een teveel aan suikers via de voeding binnen is gekomen dan worden deze tijdelijk opgeslagen in de vorm van glycogeen in de lever. Wanneer er gedurende enkele uren niet gegeten wordt en de suikers uit de voeding opgebruikt zijn, dan kan de glycogeenvoorraad aangesproken worden voor het vrijmaken van energie. Na enige tijd raakt ook deze voorraad op en is een alternatieve brandstof nodig. Voornamelijk tijdens langdurig vasten, sporten of bij ziekte is dit het geval en wordt vetverbranding (ook wel 'vetzuuroxidatie' genoemd) een belangrijke bron van energie.

Tijdens de vetzuuroxidatie worden vetzuren stapsgewijs afgebroken. Het MCAD enzym speelt een belangrijke rol in de vetzuuroxidatie en draagt bij aan de stapsgewijze afbraak van middellange keten vetzuren. MCAD deficiëntie is een erfelijke stofwisselingsziekte waarbij de werking van het MCAD enzym verstoord is. Als gevolg van deze verstoring kan de omzetting van middellange keten vetzuren naar energie niet goed plaatsvinden. Aangezien we onder normale omstandigheden voornamelijk energie uit suikers halen, hebben patiënten met MCAD deficiëntie meestal geen klachten. De problemen ontstaan pas op het moment dat het lichaam afhankelijk wordt van de vetzuuroxidatie voor het vrijmaken van energie, zoals bijvoorbeeld het geval is tijdens langdurig vasten, ziekte of sporten. Wanneer er onder dergelijke omstandigheden onvoldoende energie vrijgemaakt kan worden kan er een energietekort in de vorm van een lage bloedsuiker optreden. Kenmerken van een lage bloedsuiker zijn zweten en sufheid, maar ook ernstigere symptomen zoals stuipen en coma kunnen optreden. Als er niet op tijd ingegrepen wordt kunnen patiënten met MCAD deficiëntie overlijden als gevolg van de lage bloedsuiker. Voordat dit ziektebeeld in 2007 opgenomen werd in de hielprikscreening overleed 25% van de kinderen met MCAD deficiëntie tijdens een acute presentatie voordat de diagnose gesteld was. Naast symptomen van een lage bloedsuiker zijn spierklachten en klachten van een verminderde mogelijkheid tot sporten (een verminderde inspanningstolerantie) regelmatig gehoorde klachten.

Zodra de diagnose gesteld is, is de prognose goed. Dit komt doordat een acute presentatie bij dit ziektebeeld vrij eenvoudig voorkomen kan worden door langdurig vasten te vermijden. Op jonge leeftijd houdt dit in dat patiënten ook 's nachts gevoed worden. Verder wordt geadviseerd om vaker te eten tijdens sporten en ziekte en zullen patiënten bij ziekte laagdrempelig opgenomen worden in het

ziekenhuis voor een glucose infuus. Naast het voorkomen van langdurig vasten wordt ook regelmatig vrije carnitine bijgegeven, omdat de carnitine-waarden in het bloed van patiënten laag kunnen zijn. Te lage vrije carnitine-waarden kunnen leiden tot spierpijn en vermoeidheid. In het verleden werd ook regelmatig riboflavine voorgeschreven. De gedachte achter deze medicatie was dat het de overgebleven functie van het MCAD enzym zou kunnen verbeteren. Voor zowel het voorschrijven van carnitine als riboflavine is weinig bewijs in de literatuur.

STAPELING VAN TUSSENPRODUCTEN

De diagnose MCAD deficiëntie kan gesteld worden aan de hand van de stapeling van bepaalde stoffen (ook wel tussenproducten genoemd) die kenmerkend zijn voor dit ziektebeeld. Kenmerkend voor MCAD deficiëntie is de stapeling van middellange acylcarnitines in bloed. Deze acylcarnitines worden gevormd door binding van de middellange keten vetzuren die niet omgezet kunnen worden door het defecte MCAD enzym, aan vrije carnitine. Gebonden aan carnitine kunnen de vetzuren uit de mitochondriën getransporteerd worden. Tijdens metabole stress wordt het lichaam in toenemende mate afhankelijk van vetzuuroxidatie, waardoor de concentratie van de acylcarnitines in het bloed van patiënten met MCAD deficiëntie kan stijgen. Maar ook onder gezonde omstandigheden zijn deze tussenproducten in verhoogde mate aanwezig vergeleken met gezonde personen zonder MCAD deficiëntie. Wanneer vorming van acylcarnitines in hoge mate plaatsvindt, kunnen de concentraties van vrije carnitine in het bloed dalen en kan aanvulling van vrije carnitine door middel van medicatie nodig zijn.

HIELPRIKSCREENING VOOR MCAD DEFICIËNTIE

Sinds een aantal jaar is het mogelijk om betrouwbaar en snel acylcarnitine profielen te bepalen in bloedspots. Door deze ontwikkeling is het mogelijk geworden om op grote schaal te screenen op de aanwezigheid van tussenproducten die typisch zijn voor verschillende vetzuuroxidatie defecten. Sinds 1 januari 2007 is MCAD deficiëntie daarom in heel Nederland opgenomen in de landelijke hielprikscreening. In de eerste week van het leven wordt bloed van pasgeborenen gescreend op onder anderen de aanwezigheid van C_8-carnitine (een middellange acylcarnitine). C_8-carnitine stapelt specifiek in het geval van MCAD deficiëntie. Wanneer een afwijkende hielprikuitslag gevonden wordt, wordt het bloedonderzoek herhaald in het ziekenhuis en kan de diagnose MCAD deficiëntie vervolgens gesteld worden aan de hand van enzymonderzoek (het meten van de functie van het MCAD enzym in bloed) en DNA onderzoek (onderzoek naar foutjes in het erfelijke materiaal dat codeert voor het MCAD enzym, het *ACADM* gen).

Sinds de hielprik is de prevalentie van MCAD deficiëntie aanzienlijk gestegen. Voor de hielprikscreening werden veel patiënten gemist omdat zij geen klinische symptomen passend bij het ziek-

tebeeld ontwikkelden. Tegenwoordig wordt er vroeg in het leven gescreend, waardoor de diagnose bijna alleen nog maar in pasgeborenen zonder symptomen gesteld wordt. Naast het diagnosticeren van patiënten zonder klachten zijn er sinds de hielprikscreening ook een aantal nieuwe mutaties in het DNA gevonden die een mildere vorm van MCAD deficiëntie lijken te veroorzaken. Deze nieuwe mutaties noemen wij 'variante mutaties'. Om MCAD deficiëntie te ontwikkelen zijn twee mutaties in het *ACADM* gen nodig. Een combinatie van 2 mutaties die wij niet eerder gezien hebben voor de hielprikscreening wordt een 'variant *ACADM* genotype' genoemd. Het is niet bekend of kinderen met deze variante *ACADM* genotypen als patiënt beschouwd moeten worden, omdat we nog nooit een lage bloedsuiker in deze kinderen gezien hebben.

HET ZIEKTEBEELD SINDS HIELPRIKSCREENING

In **Hoofdstuk 2** beschrijven we dat het bepalen van de restactiviteit van het MCAD enzym kan helpen in het maken van een individuele risico-inschatting na een positieve hielprik voor MCAD deficiëntie. Klinische symptomen en gebruik van vrije carnitine medicatie werden niet gezien in de groep patiënten met MCAD restactiviteiten van meer dan 10% van normaal. Daarnaast hadden alle kinderen met *ACADM* genotypen waarvan bekend is dat zij risico geven op een acute presentatie MCAD restactiviteiten van ver onder de 10%. De ratio tussen twee middellange acylcarnitines die tijdens de hielprik bepaald worden (de C_8/C_{10} ratio) correleerde goed met de MCAD restactiviteit en zou daarom gebruikt kunnen worden als een vroege marker in het maken van een risico-inschatting.

In **Hoofdstuk 3** beschrijven we dat kinderen met MCAD restactiviteiten van meer dan 10% van normaal een normale periode kunnen vasten en een aanzienlijke *in vivo* MCAD restactiviteit hebben. In dit hoofdstuk combineerden we de resultaten uit hoofdstuk 2 met een andere enzymtest en twee functietesten. Deze functietesten zijn zogenaamde *in vivo* onderzoeken, die niet in bloed van de patiënt uitgevoerd kunnen worden, maar die in de patiënt zelf plaatsvinden. Voorbeelden zijn een vastentest of een belastingstest met de stof fenylpropionzuur dat specifiek door het MCAD enzym omgezet wordt. Bij de fenylpropionzuur belastingstest drinkt de patiënt een oplossing met fenylpropionzuur. Vervolgens wordt er in de urine gekeken of het fenylpropionzuur omgezet is door het MCAD enzym (in dat geval wordt er hippuurzuur in de urine gevonden), of niet (dan wordt er fenylpropionylglycine in de urine gevonden). Hieruit bleek dat de groep kinderen met variante *ACADM* genotypen zich zowel tijdens de enzymtesten als tijdens de *in vivo* testen duidelijk onderscheidt van de patiënten met genotypen waarvan we weten dat deze tot klinische problemen kunnen leiden. De MCAD restactiviteiten in de kinderen met variante *ACADM* genotypen zijn hoger en zij konden zonder problemen langdurig vasten en fenylpropionzuur omzetten in hippuurzuur. Wel bleken de concentraties van middellange acylcarnitines te stijgen tijdens de vastentest. De relevantie hiervan moet nog uitgezocht worden.

Uit de resultaten die beschreven worden in hoofdstukken 2 en 3 kunnen wij concluderen dat kinderen met variante *ACADM* genotypen en MCAD restactiviteit van meer dan 10% onder normale omstandigheden waarschijnlijk geen of een laag risico lopen op het ontwikkelen van klinische symptomen. Hierbij moet wel opgemerkt worden dat er tot nu toe onvoldoende bekend is over het effect van ziekte en koorts op de MCAD restactiviteit en het bijkomende risico op klinische symptomen bij deze kinderen onder deze omstandigheden.

Naast acute klinische symptomen zijn chronische klachten ook een regelmatig gehoord probleem. Met name in de groep met volwassen patiënten zijn spierklachten (bijvoorbeeld spierkrampen of verminderde inspanningstolerantie) een regelmatig gehoorde klacht. Een duidelijke oorzaak voor deze klachten is tot nu toe nog niet beschreven. In **Hoofdstuk 4** beschrijven wij dat de energiebalans in spier van patienten met MCAD deficiëntie verstoord is tijdens inspanning. Met behulp van een nieuwe techniek (magnetische resonantie spectroscopie, MRS) kan de spierstofwisseling onder verschillende omstandigheden (zoals in rust, of tijdens inspanning) bekeken worden, zonder dat hiervoor weefsel uit de patiënt gehaald hoeft te worden. MRS maakt onderzoek naar processen in het lichaam mogelijk op een manier die weinig belastend is voor de patient. Met MRS hebben wij aan kunnen tonen dat de mitochondriële functie normaal was in spier van patiënten met MCAD deficiëntie. De energiebalans in de spier was verstoord tijdens inspanning. Dit fenomeen is mogelijk toe te schrijven aan een verandering in het spiervezeltype dat gebruikt wordt tijdens de inspanning. In de spier zijn hoofdzakelijk twee typen spiervezels aanwezig: 1) Spiervezels die zeer snel, maar kortdurend een bijdrage aan activiteit kunnen leveren en voornamelijk werken op suikerafbraak (type II glycolytische spiervezels) en 2) spiervezels die tijdens langdurige inspanning een bijdrage leveren en voornamelijk op vetverbranding functioneren (type I oxidatieve spiervezels). De hypothese is dat het spiervezeltype dat hoofdzakelijk gebruikt wordt tijdens inspanning in de spieren van patiënten met MCAD deficiëntie verschoven is van type I oxidatieve spiervezels naar type II glycolytische spiervezels in reactie op het defecte MCAD enzym.

OXIDATIEVE STRESS EN MCAD DEFICIËNTIE

In **Hoofdstuk 5** wordt besproken dat er onder gezonde omstandigheden in bloed van patiënten met MCAD deficiëntie aanwijzingen gevonden worden voor oxidatieve stress. Tijdens normale stofwisselingsprocessen in de cel worden reactieve zuurstofverbindingen (ROS: Reactive Oxygen Species) gevormd. Deze verbindingen kunnen schade toebrengen aan de cel en worden daarom meestal onschadelijk gemaakt door antioxidatieve mechanismen. Wanneer de antioxidatieve capaciteit onvoldoende is voor de gevormde reactieve zuurstofverbindingen, dan kan oxidatieve stress optreden. In bloed van patiënten met MCAD deficiëntie werden aanwijzingen voor oxidatieve stress en een veranderde antioxidatieve capaciteit gevonden. Riboflavine en vrije carnitine medicatie leken verschillende effecten te hebben op de oxidatieve stress. Riboflavine leek een gunstig effect te hebben,

terwijl vrije carnitine mogelijk leidt tot meer oxidatieve stress. Aanvullende studies in grotere groepen patiënten waarin verschillende behandelstrategieën getest worden zijn nodig om hier meer inzicht in te krijgen.

STUDIES IN ANDERE MODELLEN VOOR MCAD DEFICIËNTIE

In **Hoofdstuk 6** worden de resultaten van de karakterisering van een muismodel voor MCAD deficiëntie besproken. In het bloed van muizen met MCAD deficiëntie wordt stapeling van de kenmerkende middellange acylcarnitines gezien en de functie van het MCAD enzym is verminderd. Echter, met de methode waarmee de MCAD restactiviteit in mensen getest wordt, worden hogere activiteiten gevonden in de muis (~10% in leverweefsel en gekweekte huidcellen) dan in de mens (<1% in gekweekte huidcellen). De meest voor de hand liggende verklaring hiervoor is dat de vetzuuroxidatie in de muis net iets anders functioneert dan in de mens, waardoor de muis mogelijk aanpassingsmogelijkheden in de vetzuuroxidatie heeft die in de mens niet aanwezig zijn. Hier werd echter geen bewijs voor gevonden. Langdurig vasten alleen heeft in dit muismodel nog niet tot een lage bloedsuiker geleid.

Een ander mogelijk model voor het bestuderen van MCAD deficiëntie is het computermodel van de vetzuuroxidatie dat recentelijk beschreven is. Dit computermodel is een model voor de vetzuuroxidatie in de lever van de rat en kan nauwkeurig voorspellen welk effect bijvoorbeeld MCAD deficiëntie kan hebben op de stapeling van tussenproducten en op andere processen in de vetzuuroxidatie. De vetzuuroxidatie is door zijn structuur (het is een cyclus) gevoelig voor overbelasting bij een groot aanbod van langketen vetzuren. Het computermodel met MCAD deficiëntie bleek zelfs gevoeliger voor overbelasting bij een groot aanbod van langketen vetzuren dan het computermodel voor de normale situatie. Bij een voldoende groot aanbod van langketen vetzuren "zakte het systeem in elkaar" en kon het niet meer functioneren. Dit model dient weliswaar nog aangepast te worden voor de vetzuuroxidatie in de muizenlever, maar toch bieden deze resultaten nieuwe inzichten voor vervolgstudies naar de oorzaak van het acuut ontstaan van een mogelijk levensbedreigend klinisch beeld bij MCAD deficiëntie.

De resultaten uit het computermodel komen overeen met wat we beschrijven in **Hoofdstuk 7**. In dit overzichtsartikel beschrijven we hoe de mitochondriële functie gereguleerd wordt door complexe feed-forward and feedback mechanismen en de mogelijke rol van secundaire processen die het gebruik van voedingsstoffen monitoren (nutrient-sensing processen genoemd) in het ontstaan van klinische symptomen bij defecten in de vetzuuroxidatie of in de oxidatieve fosforylering (een ander systeem dat een belangrijke rol speelt in de energieproductie in mitochondriën). In een zebravismodel voor een defect in de vetzuuroxidatie (multiple acyl-CoA dehydrogenase deficiëntie), bleken deze processen verstoord door het onderliggende gendefect. Ook in muismodellen en patienten met defecten in de oxidatieve fosforylering werden aanwijzingen voor verstoringen in deze processen gevonden. In patienten met MCAD deficientie zijn de nutrient-sensing processen nooit bestudeerd.

Nutrient-sensing processen zijn uitgebreide complexe netwerken. Persoonsgebonden verschillen in het functioneren van deze netwerken kunnen mogelijk bijdragen aan het risico dat een patiënt loopt op een metabole ontsporing. Mogelijk spelen deze processen een rol bij het ontstaan van symptomen in het geval van MCAD deficiëntie. Studies die het metabolisme op verschillende niveaus bestuderen zijn nodig om hier meer inzicht in te krijgen.

HOOFDPUNTEN UIT DIT PROEFSCHRIFT

Samenvattend werden de volgende hoofdpunten beschreven in dit proefschrift:

- Het maken van een individuele risico-inschatting na een positieve hielprik is mogelijk door te kijken naar de MCAD restactiviteit (**Hoofdstuk 2**).
- Kinderen met een MCAD restactiviteit van meer dan 10% van normaal hebben een normale vastentolerantie en *in vivo* MCAD restactiviteit, zoals werd vastgesteld met een fenylpropionzuur belastingstest. Echter, het effect van ziekte/koorts op de MCAD restactiviteit dient uitgezocht te worden voordat het behandelbeleid voor deze kinderen versoepeld kan worden (**Hoofdstuk 3**).
- De energiebalans in spier is tijdens inspanning verstoord in patiënten met MCAD deficiëntie. Mogelijk wordt dit veroorzaakt door een verschuiving van vetverbandende spiervezels (type I oxidatieve spiervezels) naar suikerafbrekende spiervezels (type II glycolytische spiervezels) (**Hoofdstuk 4**).
- In MCAD deficiënte patiënten werden tekenen van oxidatieve stress en een verstoorde antioxidatieve afweer gevonden. Vrije carnitine medicatie leek de oxidatieve stress te verergeren, terwijl riboflavine medicatie een beschermend effect leek te hebben (**Hoodstuk 5**).
- Het muismodel voor MCAD deficiëntie kan bijdragen aan het ontrafelen van de mechanismen achter de klinische symptomen bij MCAD deficiëntie, ook al zijn de MCAD restactiviteiten aanzienlijk hoger in de muis dan in klinisch presenterende patiënten. Simulaties in een computermodel voor vetzuuroxidatie in rattenlever voorspelden een toegenomen gevoeligheid voor overbelasting met tussenproducten in het geval van MCAD deficiëntie ten opzichte van de normale situatie (**Hoofdstuk 6**).
- Metabole ziekten kunnen nutrient-sensing processen verstoren en daardoor de mitochondriële stofwisseling beïnvloeden. Mogelijk draagt deze verstoring bij aan de klinische symptomen die gezien worden bij defecten in de vetzuuroxidatie, zoals MCAD deficiëntie (**Hoofdstuk 7**).

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CURRICULUM VITAE

Catharina Maria Louise (roepnaam Nienke) Touw werd op 9 april 1985 geboren in Nieuwegein. De eerste 11 jaar van haar leven groeide zij op in IJsselstein, waarna zij naar Rotterdam verhuisde. Daar rondde zij in 2002 het Marnix Gymnasium af. Aansluitend verhuisde zij naar Utrecht om te studeren aan University College Utrecht. Na afronding van de Bachelor of Science (cum laude) en een semester aan de University of California, Los Angeles, startte zij in 2006 met de 4-jarige geneeskunde opleiding aan de School of Utrecht Medical Masters (SUMMA). Tijdens deze studie kreeg zij interesse in de Metabole Ziekten en genetica, wat leidde tot de succesvolle afronding van verschillende wetenschappelijke stages naar het syndroom van Hurler, onder leiding van dr. T.J. de Koning.

In 2010 werd de opleiding afgerond en verhuisde Nienke naar Groningen, waar zij 15 maart 2010 startte met het promotieonderzoek naar MCAD deficiëntie onder begeleiding van Prof. dr. G.P.A. Smit, Prof. dr. D.J. Reijngoud en Dr. T.G.J. Derks. Dit onderzoek heeft geleid tot de resultaten die beschreven zijn in dit proefschrift.

Sinds 1 februari 2014 is Nienke werkzaam als arts-assistent bij de afdeling Klinische Genetica in het UMC Utrecht.

Nienke is getrouwd met Mathieu Volker en samen wonen zij in Utrecht.

LIST OF PUBLICATIONS

From genome to phenome – Simple inborn errors of metabolism as complex traits. CML Touw, TGJ Derks, BM Bakker, AK Groen, GPA Smit, DJ Reijngoud. *Accepted for publication in Biochim Biophys Acta.* 2014 Jun 3. PMID: 24905735

Experimental evidence for protein oxidative damage and altered antioxidative defence in patients with medium-chain acyl-CoA dehydrogenase deficiency. CML Touw[#], TGJ Derks[#], GS Ribas, GB Biancini, CS Vanzin, G Negretto, CP Mescka, DJ Reijngoud, GPA Smit, M Wajner, CR Vargas. *Accepted for publication in J Inherit Metab Dis.* 2014. March 13. PMID: 24623196

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