Exploring the mechanisms underlying the phenotype of MCAD deficiency with Systems Medicine

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Chapter 6

Towards identifying *in vitro* metabolic adaptations that explain symptomatology in MCADD individuals
A short report

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Abstract

In this study we investigated cultured skin fibroblasts of young symptomatic (n=4) and asymptomatic MCAD-deficient (MCADD) individuals (n=6), including an older MCADD individual (30 years) who remained asymptomatic. The control skin fibroblast (n=6) were obtained from individuals apparently free of a metabolic disease. Under basal culturing conditions, C6-C10 acyl-carnitine secretion and intracellular C6- and C8-acyl-carnitine concentrations were higher in MCADD fibroblasts than in control fibroblasts. In addition, the fibroblasts of the eldest asymptomatic MCADD individual showed higher acyl-carnitine secretion than those of the other MCADD individuals. Quantitative, targeted proteomics showed an upregulation of SCAD in the same asymptomatic MCADD individual. Otherwise no adaptations in the proteome were found in the MCADD fibroblasts. The results illustrate the potential and the challenges of a personalized medicine strategy. We discuss how the culture conditions may be optimized to reveal metabolic adaptations, for instance by imposing low glucose concentrations.

Introduction

MCAD deficiency is one of the most prevalent inherited metabolic diseases and the most common inborn error of the mitochondrial fatty acid oxidation (mFAO) pathway [1–6]. The MCADD prevalence at birth was 1/8300 in the Netherlands between 2007 and 2015 [7]. mFAO plays an important role in energy homeostasis during fasting and conditions of high-energy demand. MCAD-deficient (MCADD) individuals run a risk of metabolic crisis under such a condition, especially if prolonged fasting occurs together with intercurrent illness, such as airway or gastrointestinal infections [8–11]. Acute symptoms include life-threatening hypoketotic hypoglycemia, coma and seizures. Interestingly, even within the subpopulation of individuals with a homozygous loss-of-function c.985A>G mutation in the MCAD-encoding *ACADM* gene, symptoms can range from fatal hypoketotic hypoglycemia early in life to a complete lack of symptoms throughout life [10,12–20]. Genetic and environmental modifiers could play a role in the heterogeneity in vulnerability to or robustness against metabolic stress. How this vulnerability or robustness arises is as yet unclear.

As already indicated in the General Introduction in Chapter 1 of this thesis, in the past decades reliable untargeted and targeted multi-omics methods have been developed. This offers the opportunity to study inborn errors of metabolism on levels not previously possible [21–23]. The application of these multi-omics technologies on cultured skin fibroblasts originating from symptomatic and asymptomatic MCADD individuals can be a first step to gain insight into regulatory mechanisms underlying symptomatology in MCAD deficiency. In addition, it might even allow us to identify meaningful biomarkers that predict whether a patient could develop symptoms and which adaptations in energy metabolism could underlie their robustness or vulnerability. Previously, we observed adaptations in the proteome of mitochondrial energy metabolism in cultured skin fibroblasts of MCADD individuals compared to controls. The levels of several mFAO proteins, including VLCAD, MCKAT, MTP subunit A and ETF subunit B, were higher in mitochondria isolated from skin fibroblasts of three MCADD patients with the homozygous c.985A>G *ACADM* mutation than in mitochondria from control fibroblasts [24]. The latter study focused on the development of the targeted proteomics methodology. The patient fibroblasts illustrated the potential of the technique. Due to the very low number of MCADD individuals, however, no conclusions of any clinical relevance could be drawn. In order to study the effect of modifiers on the symptomatology in MCADD, a larger number of both symptomatic and asymptomatic MCADD individuals is required.

In the study presented here, we explored whether symptomatic and asymptomatic MCADD fibroblasts and control fibroblasts show differences in their mitochondrial energy metabolism under baseline conditions (standard culture medium with 6.1 mM glucose). We measured the proteome of mitochondrial energy metabolism [24] and the intracellular and extracellular acyl-carnitine profiles of fibroblasts of 4 symptomatic, 6 asymptomatic and 6 individuals apparently free of a metabolic disease. In the first part of this study, one biological replicate for each skin fibroblast cell line was generated, as we searched for differences between the groups. The results of the analysis suggested major adaptations in only one individual: both the proteome and the acyl-carnitine profile of the eldest asymptomatic MCADD individual differed substantially from that of all other MCADD individuals. These potentially functional adaptations could underlie the lack of symptoms in this individual. Therefore, we extended the study and repeated the analysis of the proteome and acyl-

carnitine profile in a total of 3-4 biological replicates fibroblast cultures of each individual, to validate the initial experimental findings. The main results are presented in this chapter.

Results

Cohort and their characteristics

We used primary human skin fibroblasts from MCADD individuals homozygous for the loss-of-function c.985A>G ACADM mutation (Table 1).

Table 1: Summary of patient characteristics

Patient ID	Gender	Gene mutation	Manifes-tation	Age of onset/ Diagnosis (years)	Clinical manifestation
1	M	c.985A>G	Sympt	2	Found sub-comatose in bed, with hypoketotic hypoglycemia, after an episode of diarrhea.
2	F	c.985A>G	Asympt	1	Older sibling presented at age 1 with MCADD-related metabolic crisis. Thereafter, Pt2 was treated with frequent feedings and a fat-restricted diet. Admitted several times as a precaution during intercurrent illness, but without documented hypoglycemia or other severe symptoms.
3	F	c.985A>G	Asympt	1.5	Diagnosed after twin sibling died at 1.5 y during a flu episode. Treated with frequent feedings and L-carn.
4	F	c.985A>G	Asympt	11	Older sibling of Pt3. No documented derangements.
5	M	c.985A>G	Sympt	2	Presented with post-operative hypoglycemic coma, leading to reanimation and severe disability.
6	F	c.985A>G	Asympt	0	Younger sibling of Pt5. Treated from near birth with frequent feedings. No documented derangements.
7	F	c.985A>G	Sympt	2	Found lethargic and hypotonic in bed, accompanied by seizures and hypoglycemia.
8	M	c.985A>G	Sympt	1	Comatose and hypoglycemic after two days of vomiting and high fever.
9	M	c.985A>G	Asympt	0	Younger sibling of Pt8. Treated from near birth with frequent feedings, L-carn and vitamin B2. No documented derangements.
10	F	c.985A>G	Asympt	30	Discovered by metabolic profile after positive NBS of daughter. No documented derangements as child.

Sympt: symptomatic, Asympt: Asymptomatic. Categorization of symptomatology according to [10]. M: male, F: female. L-carn: L-carnitine. Pt: Patient.

The c.985A>G *ACADM* mutation causes impairment of the MCAD protein folding [25] and protein instability [26,27] resulting in degradation of the mutated protein and lack of activity [28]. We specifically studied fibroblasts that originated from MCADD patients (4 Symptomatic and 6 asymptomatic individuals from 7 families) who were clinically ascertained before the

introduction of the newborn screening (NBS) program and of whom the symptomatology had been previously described [10]. It should be noted that some of the individuals that were categorized as asymptomatic, received treatment before the average age of disease presentation (Table 1). These children were diagnosed after a sibling presented with symptoms. In fact, the median (range) age of onset in symptomatic group was 2 (1-2) years, while the median age of diagnosis in the asymptomatic group was 1.25 (0-30) years. As treatment can interfere with the natural course of disease development, it is not certain whether some of the MCADD individuals categorized as asymptomatic are truly asymptomatic. Therefore, interpretation of results obtained in fibroblasts of asymptomatic MCADD individuals should be considered with caution. Importantly, patient 10 (Pt10) was the eldest MCADD individual in the studied cohort. Pt10 has remained asymptomatic throughout life without treatment. She was identified at age 30 because of a positive result in the newborn screening of her newborn child. Pt10 can thus be considered the most ascertained asymptomatic MCADD individual. For this reason, the results of Pt10 will be presented and discussed separately. Characteristics of the control cohort are given in Supplementary Table 1.

Acyl-carnitines

First, we performed an explorative study on a single sample of each of the cultured skin fibroblasts of all individuals. The motivation for this approach was that we expected differences between the groups rather than between the individuals. We measured acyl-carnitine profiles and the targeted proteome of mitochondrial energy metabolism. The concentrations of C6-, C8- and C10:1-acyl-carnitine, but not of C10-acyl-carnitine, were higher in the culture medium of MCADD fibroblasts than in that of control fibroblasts (Supplementary Figure S1). As the fresh medium already contained acyl-carnitines, we also calculated the change in acyl-carnitine concentrations in the medium during cultivation and normalized the result to cellular protein content, to account for differences in confluence of the cells. The MCADD fibroblasts secreted more C6- and C8-acyl-carnitines compared the control fibroblasts. In addition, the fibroblasts from Pt10 secreted more C6-C10-acyl-carnitine than the other MCADD fibroblasts (Figure 1A-D). We also measured intracellular acyl-carnitine levels. This showed that symptomatic and asymptomatic MCADD fibroblasts had significantly higher intracellular C6-, C8- but similar C10:1- and C10-acyl-carnitine concentrations compared to control fibroblasts (Figure 1E-H). Furthermore, we did not observe differences between the intracellular concentrations of acyl-carnitines in the skin fibroblast of Pt10 and those of the symptomatic and other asymptomatic fibroblasts (Figure 1E-H). Other acyl-carnitines were not different between any of the groups (data not shown).
Since Pt10 differed from all other individuals, we set out to validate the observations with multiple replicate fibroblast cultures per individual. The concentrations of C6-, C8-, and C10:1-acyl-carnitine in the medium of all but one patient cell line (Pt7) were higher than those of the control fibroblasts (Supplementary Figure S2A-C). Several patient fibroblast cultures had also higher C10-acyl-carnitine levels in the medium (Supplementary Figure S2D).

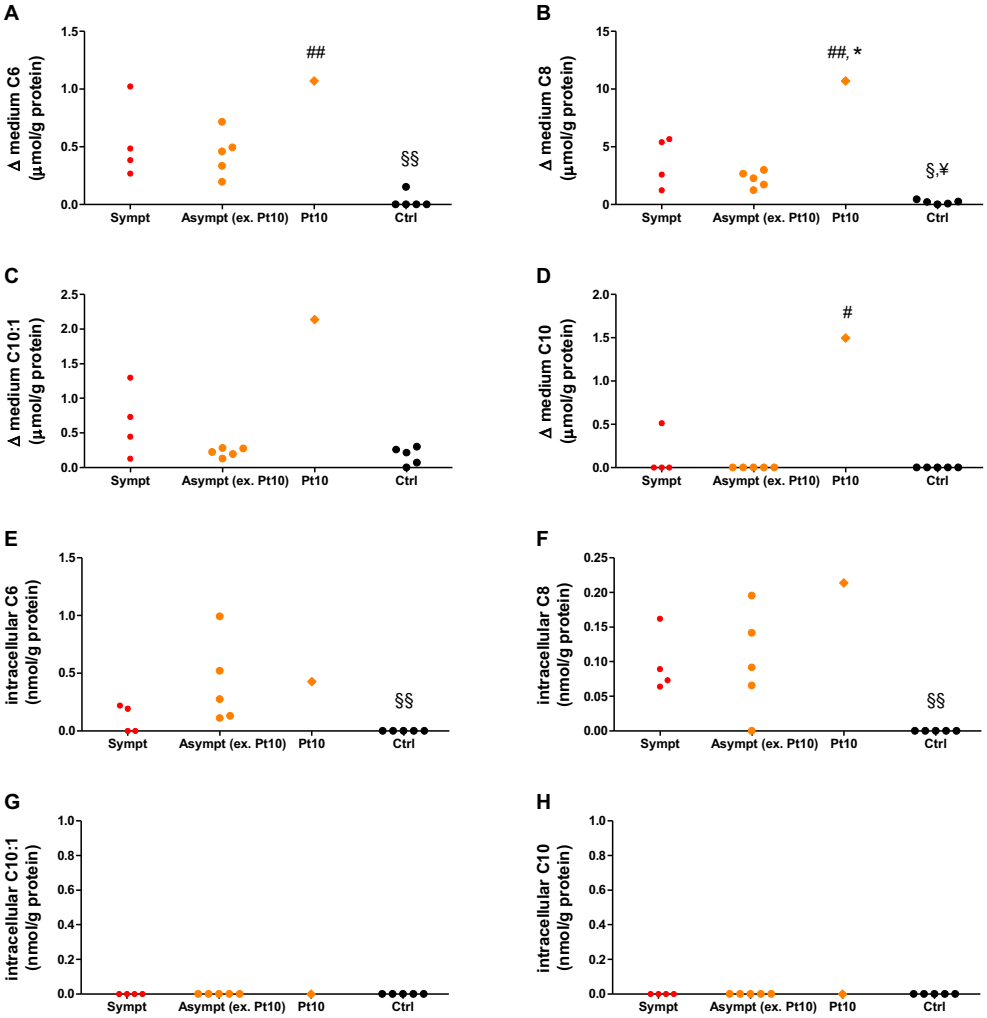

Figure 1. C6-C10-acyl-carnitine secretion into in the medium (A-D) and intracellular C6- and C8-acyl-carnitine concentrations (E-F). Secretion of acyl-carnitines is shown as the difference in concentration of acylcarnitines in the medium before and after culture of fibroblasts, normalized for the cellular protein concentration. The result pertain to the explorative part of the study. n=4, 5, and 4 different individuals, for symptomatic, asymptomatic and control fibroblasts, respectively. The control fibroblasts were C1, C3, C4, C5 and C6.K (Supplementary Table ST1). §: significant difference compared to MCADD fibroblasts (including Pt10), #: significant difference compared to MCADD fibroblasts (excluding Pt10), ¥: significant difference compared to Pt10, ‡: significant difference compared to symptomatic MCADD fibroblasts, †: significant difference compared to asymptomatic MCADD fibroblasts, &: Significant compared to the other fibroblasts. *: significant difference compared to control fibroblasts. One symbol denotes $p<0.05$, two symbols denotes $p<0.01$.

Consequently, both symptomatic and asymptomatic MCADD groups had significantly higher C6-C10-acyl-carnitine concentrations in the medium than the control group (Supplementary Figure S2E-H). In addition, the control fibroblasts consumed C6-C10-acyl-carnitines, while MCADD fibroblasts did not (Supplementary figure S3 and Figure 2A-D).

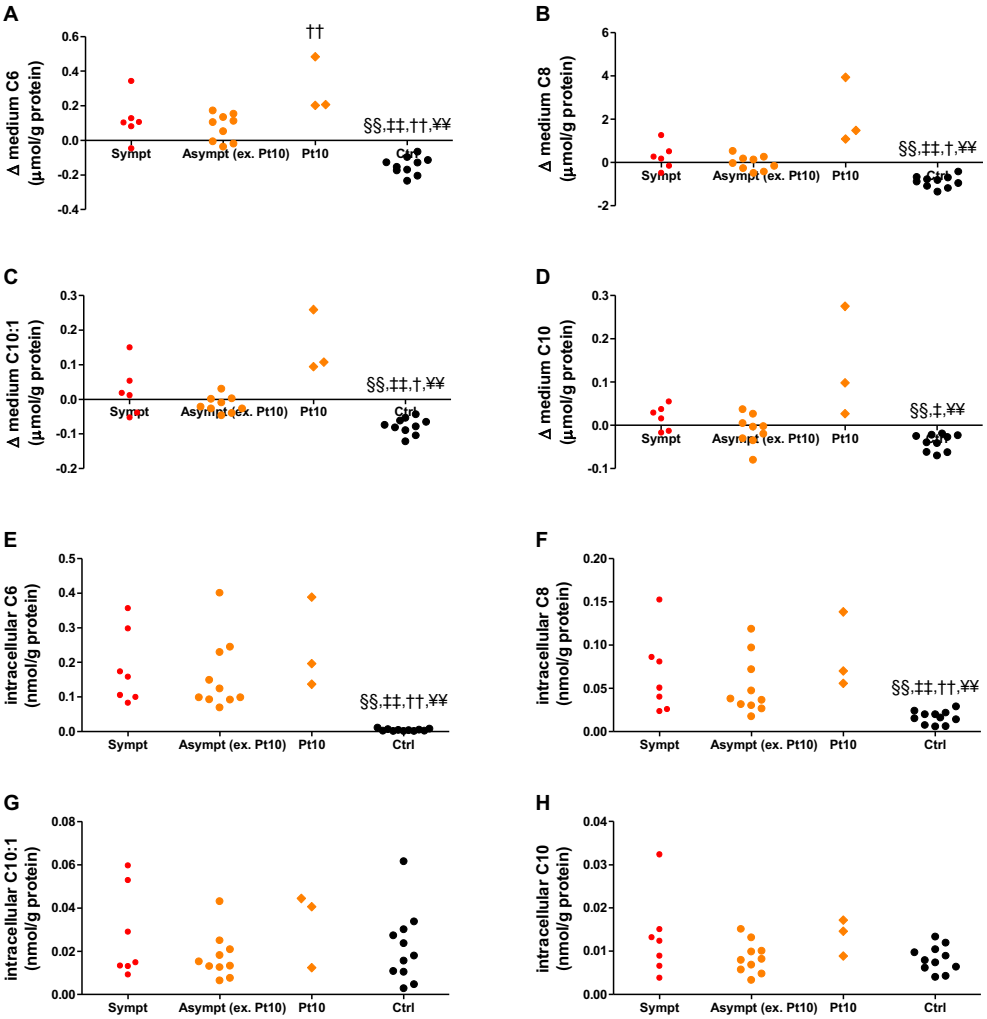

Figure 2. C6-C10-acyl-carnitine secretion into in the medium (A-D) and intracellular C6-C10-acyl-carnitine concentrations (E-H). Secretion of acyl-carnitines is shown as the difference in concentration of acyl-carnitines in the medium before and after culture of fibroblasts, normalized for the cellular protein concentration. The results pertain to the validation phase of the study. n=1 for Patient ID # 7 and control fibroblast #C5, n=2 for Patient ID #1-6, 8-9 and control fibroblasts #C3,4,6K and n=3 for Patient ID #10 and control fibroblast #C6.W. Significance visualization is according to Figure 1.

In line with the explorative study, the fibroblasts of Pt10 secreted significantly more C6-C10-acyl-carnitine than the fibroblasts of all other MCADD individuals combined (Supplementary figure S3 and Figure 2A-D). Finally, the fibroblasts of symptomatic and asymptomatic MCADD individuals had significantly higher intracellular C6- and C8-, but similar C10:1- and C10-acyl-carnitine concentrations compared to control fibroblasts (Supplementary figure S4 and Figure 2E-H). There was no difference between the intracellular acyl-carnitine concentrations of the fibroblasts of Pt10 and that of other symptomatic or asymptomatic MCADD individuals

(Supplementary figure S4 and Figure 2E-H). Overall, these results suggest that cells of Pt10, the eldest asymptomatic MCADD individual, secrete more C6-C10-acyl-carnitines.

Targeted proteomics

The measured mitochondrial proteins comprise enzymes involved in mFAO and TCA cycle, core subunits of each of the respiratory-chain complexes, and transporters required to transport metabolites into and out of the mitochondria [24]. In all control samples MCAD was detected, while it was low in the patient samples (Figure 3A). Otherwise there were no significant differences between MCADD and control fibroblasts in the explorative phase of the study.

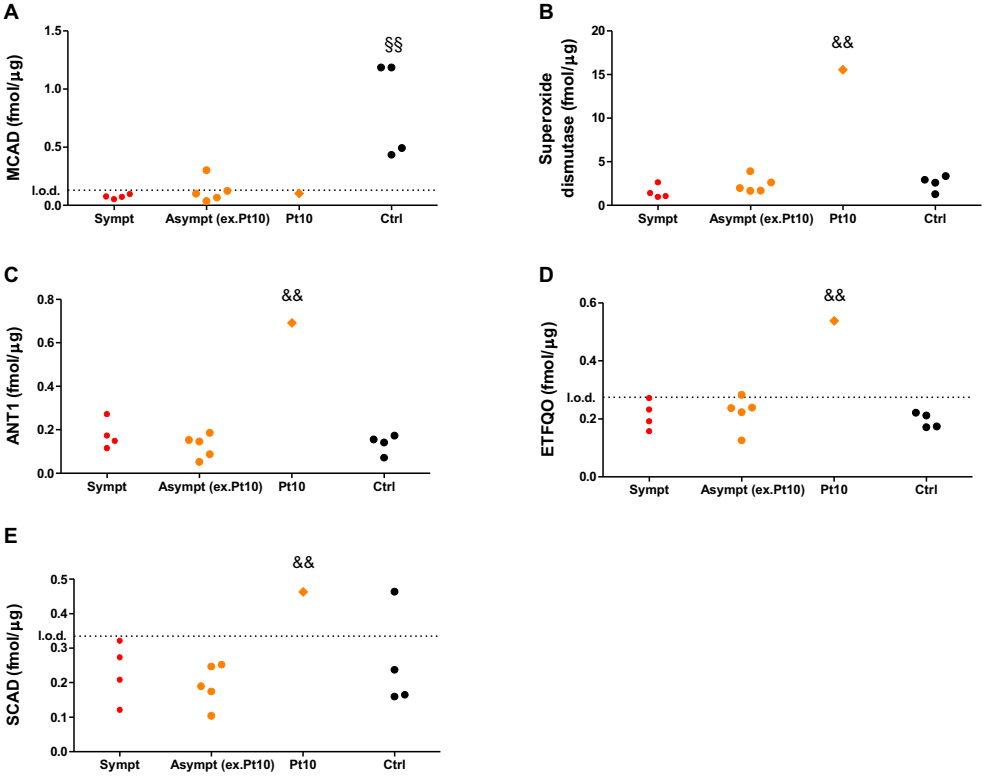

Figure 3. Selection of proteins of the exploratory phase of the study. Depicted are the proteins where Pt10 fibroblasts showed the largest difference in concentration compared to the fibroblasts of the other MCADD and control individuals and SCAD. Significance visualization is according to Figure 1.

In the exploratory phase of the study, it appeared that the proteome of the fibroblasts of Pt10 was quite different, showing higher levels of several proteins than the other fibroblasts (top 3 and SCAD shown in Figure 3B-E). The protein concentrations that were most elevated in Pt10 cells compared to the other fibroblasts, were those of mitochondrial superoxide dismutase 2 (coded by SOD2), ADP/ATP translocase 1 (ANT1, coded by SLC25A4) and ETF dehydrogenase (ETF-ubiquinone oxidoreductase, ETFQO, coded by ETFDH) (Figure 3B-D). In addition, Pt10 showed higher levels of SCAD compared to the other fibroblasts (Figure 3E).

In the validation phase of the study we confirmed the low MCADD concentrations in MCADD compared to control fibroblasts (Figure 4A and Supplementary Figure S5A). The fibroblasts of the MCADD individuals showed slight but significantly higher levels of the antioxidant protein Peroxiredoxin-6 (coded by Prdx6) (Figure 4B and Supplementary Figure S5B). This is in line with protein oxidative damage and altered antioxidant defense observed in MCADD individuals [29]. In addition, the fibroblasts Pt10 did not show significant differences in proteome compared to any of the groups, except for significantly higher concentrations of SCAD in fibroblast of Pt10 compared to the other asymptomatic fibroblasts (Figure 4C and Supplementary Figure S5C).

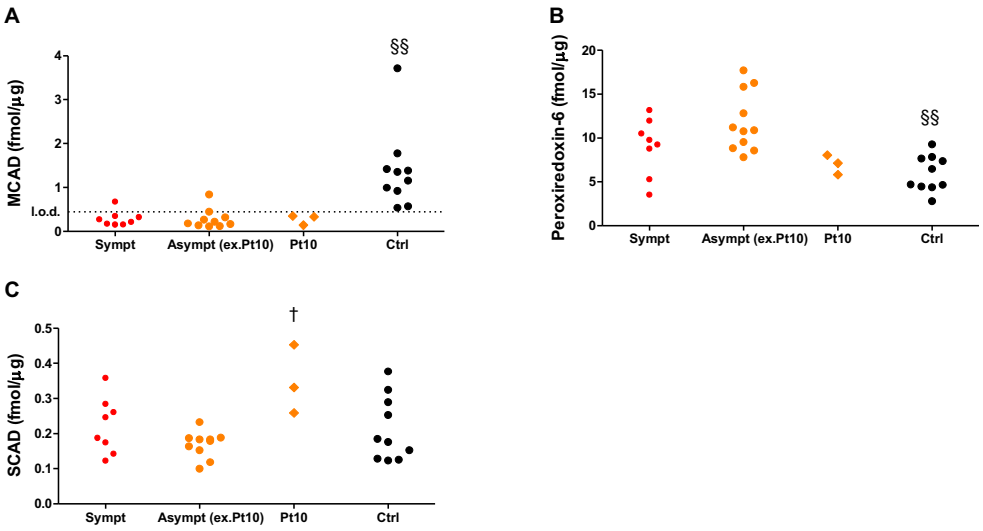

Figure 4. Selection of proteins of the validation part of the study. n=2 for Patient ID #1-9 and control fibroblasts #C3,C4,C6.K and C6.W and n=3 for Patient ID #10 Significance visualization is according to Figure 1.

Discussion

In this study we investigated whether there are metabolic adaptations in cultured skin fibroblasts of MCADD individuals that separate symptomatic from asymptomatic MCADD individuals. We did not observe consistent differences in acyl-carnitine concentrations between fibroblast of symptomatic and asymptomatic MCADD individuals, except for Pt10, the eldest MCADD individual with a lifelong absence of symptoms. It appears, from both phases of the study that fibroblast of Pt10 secrete intracellular accumulated acyl-carnitines more efficiently. Nevertheless, the intracellular concentrations of acyl-carnitines remained high compared to control fibroblasts. The question arises, what causes the higher rate of acyl-carnitine secretion. It could be a result of higher rates of mFAO with concomitant acyl-carnitine production in fibroblasts of Pt10 compared to the fibroblasts of the other MCADD individuals. Alternatively, the proteins involved in the secretion of acyl-carnitines could be upregulated in Pt10. This was not tested, since the targeted method did not include any transporters outside the mitochondria. Proteomics showed hardly any adaptations in MCADD cells compared to controls. This is consistent with earlier findings in MCAD-KO mouse liver (**Chapter 3** and [24]). We observed adaptations in the proteome of Pt10, but they were less

pronounced in the validation phase than in the exploratory phase of the study. Therefore, we could not unambiguously establish if they contribute to the lack of symptoms in Pt10. Upregulation of SCAD, as consistently observed in the exploratory and the validation phase, could be a functional adaptation. This enzyme catalyzes the same reaction as MCAD, with an overlapping set of substrates, and can therefore partly compensate for the loss of MCAD.
Under the culture conditions tested in this study, with 6.1 mM of glucose, the cells may not depend strongly on fatty-acid oxidation. To elicit larger and consistent adaptations, more stringent conditions may be required, such as lower glucose levels and higher fatty-acid concentrations.

Materials and Methods

Patients and controls

Primary human skin fibroblasts originated from patients homozygous for the c.985A>G *ACADM* mutation, who were clinically ascertained before 2007 were selected. Symptomatology was categorized according to [10]. In short, the symptomatic phenotype was defined by the occurrence of at least one acute metabolic crisis with documented hypoglycemia/coma/encephalopathy or seizures. Control fibroblasts were from patients without a documented metabolic disease were used (Supplementary table ST1). The fibroblasts were obtained from the Department of Clinical Genetics of the University Medical Center Groningen. The Medical Ethical Committee of the University Medical Center Groningen confirmed that the Medical Research Involving Human Subjects Act was not applicable and that official study approval by the Medical Ethical Committee was not required (METc 2016/590). Under these circumstances and when data are used anonymously, individual subject's informed consent is not required, as per institutional and national rules. (METc 2016/590).

Cell culture and harvest

The cells were cultured at 37°C in 5% CO_2 in Ham's F-10 Nutrient Mix (Thermo Fisher Scientific), supplemented with 10% fetal calf serum (FCS, Gibco) and 1% penicillin/streptomycin (PenStrep, Gibco). For the experiments, the culture medium was supplemented with 400 µM L-carnitine (experimental medium). For targeted proteomics, cells were first seeded at ~75% confluency in T75 culture flasks (Corning life sciences). After 24 hours, the cells were incubated in experimental medium. After 72 hours, the cells were washed with PBS (DPBS, Gibco), trypsinized with 0.25% trypsin EDTA (Gibco) and resuspended in fresh medium. The cells were spun down (5' at 1,000 rpm) and washed with 10 ml PBS three times. Finally, the cells were aliquoted ($2{\cdot}10^5$ - $1{\cdot}10^6$ cells per aliquot) in 1.5 mL Protein LoBind tubes (Eppendorf), pelleted (5' at 1,500 rpm) and stored at -80°C until further processing and analysis. Cell pellets were lysed in 25 µL ice-cold NP-40 lysis buffer (0.1% NP-40, 0.4 M NaCl, 10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0) for 20 minutes, vortexed shortly, spun on a rotator at 40 rpm and 4°C for 15 minutes and spun for 10 minutes at 12,000 g and 4°C. 20 µL of the supernatant was transferred to a second LoBind tube and 5 µL was used for protein determination (Pierce© BCA Protein Assay kit, Thermo Scientific). Per time point 3 wells of a 6-well plate (Greiner Bio-One, 05T160) were seeded at ±75% confluency. 24 hours after seeding, cells were incubated with 1 ml of experimental medium. For the metabolomics, spent medium was collected and cells were harvested on ice. Cells from two 6-cm wells were washed twice with ice-cold PBS, scraped with 500 µL methanol (-20°C), aliquoted (1000 µl per aliquot) and stored at -80°C until further analysis. For protein determination, cells in one well were scraped with 200 µL ice-cold PBS, aliquoted and stored at -80°C.

Targeted quantitative proteomics of mitochondrial proteins

A set of >50 mitochondrial proteins involved in substrate transport, oxidative phosphorylation (OXPHOS), mitochondrial fatty-acid β-oxidation, tricarboxylic acid cycle, and antioxidant activity were

quantified in the preprocessed cell samples using isotopically labelled standards (^{13}C-labeled lysines and arginines) derived from synthetic protein concatemers (QconCAT) (PolyQuant GmbH, Bad Abbach, Germany) according to Wolters *et al.* [24].

Acyl-carnitine levels

Acyl-carnitine levels were measured according to Derks *et al.* [12].

Statistical analysis

The significance of differences, were assessed using parametric (one-way ANOVA and subsequent LSD or Dunnet's post hoc test) or nonparametric tests (Kruskal-Wallis ANOVA Dunn's post hoc test) depending on their distribution. At a p value of <0.05 the null hypothesis was considered to be refuted. `Statistical analyses were performed using SPSS (v22) and the data was visualized using GraphPad Prism software (GraphPad Software Inc., version 5.00, 2007).

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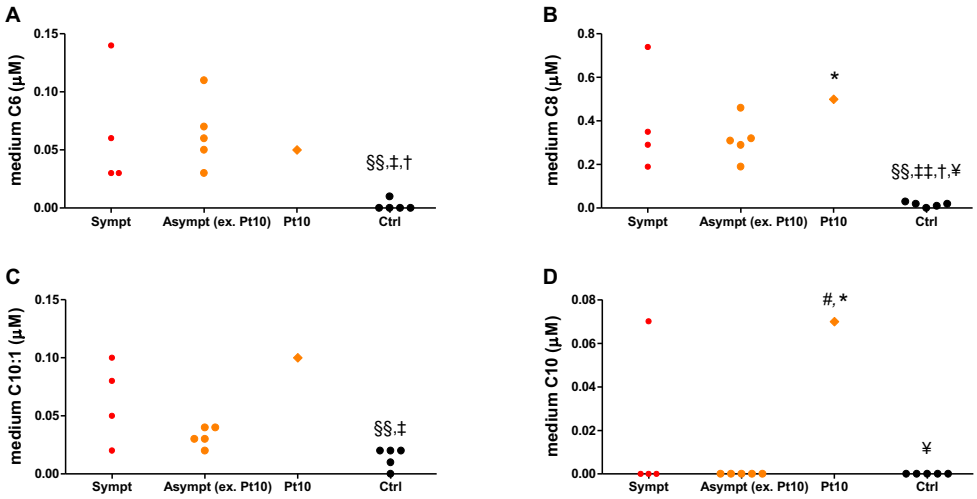
Supplementary Figures and tables

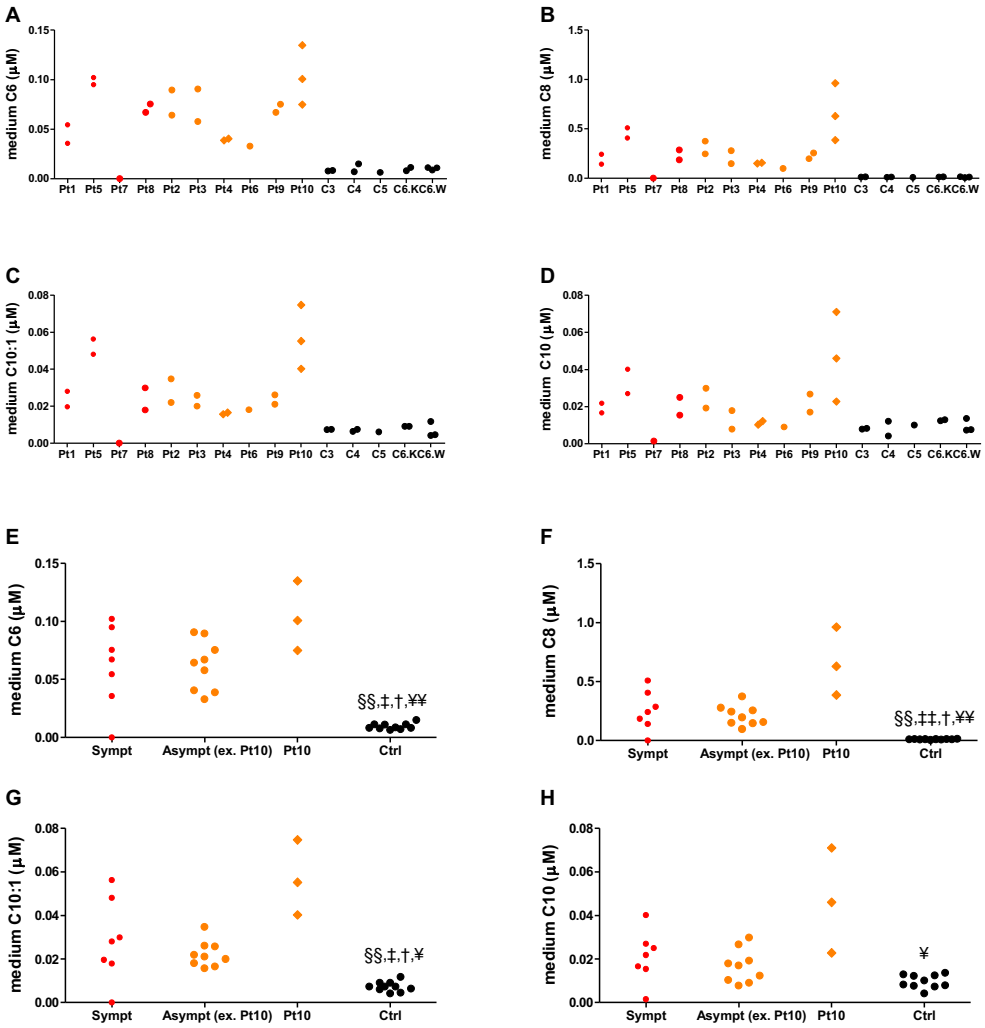
Supplementary table ST1: Characteristics of the individuals from which the control fibroblasts originated.

ID	Age (years)	Indication	Outcome
C1	42	Sudden death	Acute cardiac death
C2	72	Sudden death	Cardiac arrest, neurodegenerative disease
C3	56	migraine	No genetic origin of disease
C4	1	Sudden death	Septic shock
C5	0	Sudden death	Rhesus antagonism
C6.K	Presumably 20's	Scientific Research	-
C6.W	0	Not available	Not available

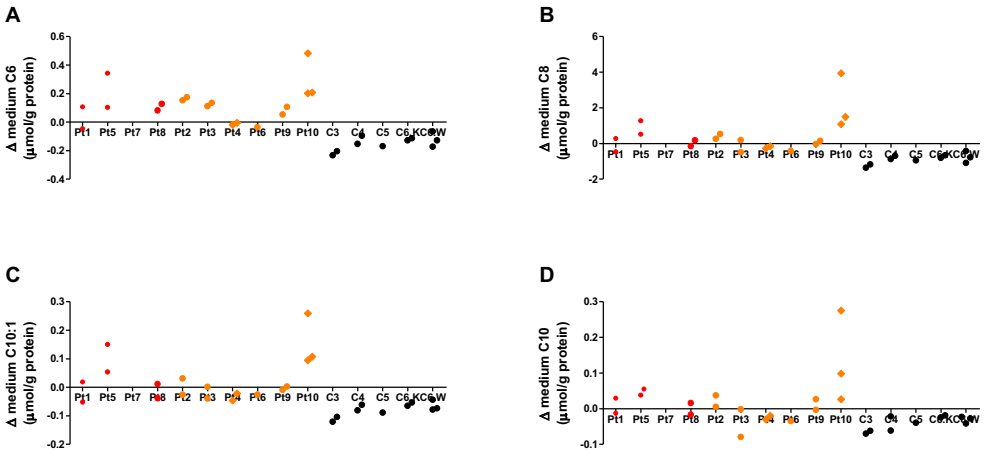
'Age' refers to the age at which fibroblasts were taken. 'Indication' describes the reason for biopsy. 'Outcome' describes disease outcome. No metabolic origin of disease was identified.



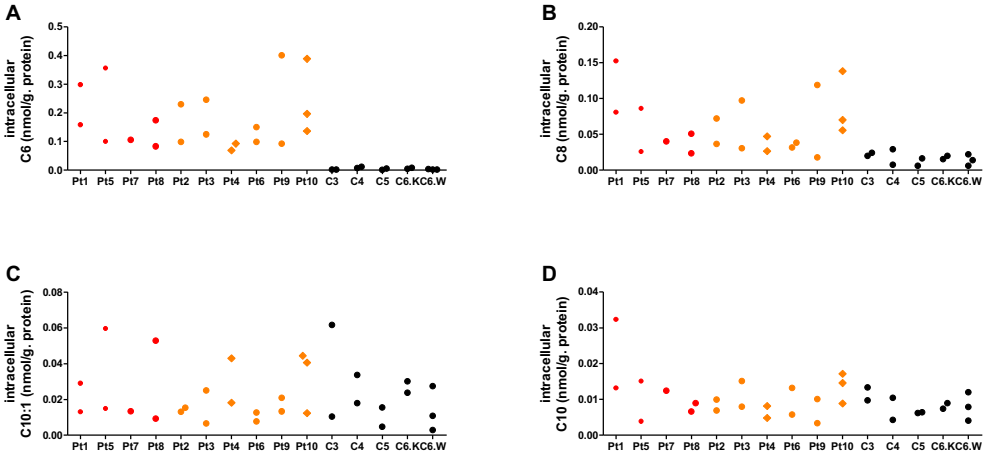
Supplementary figure S1. Concentration of acyl-carnitines in the medium. The results pertain to the exploratory phase of the study. Details as in Figure 1. Significance visualization is according to Figure 1.



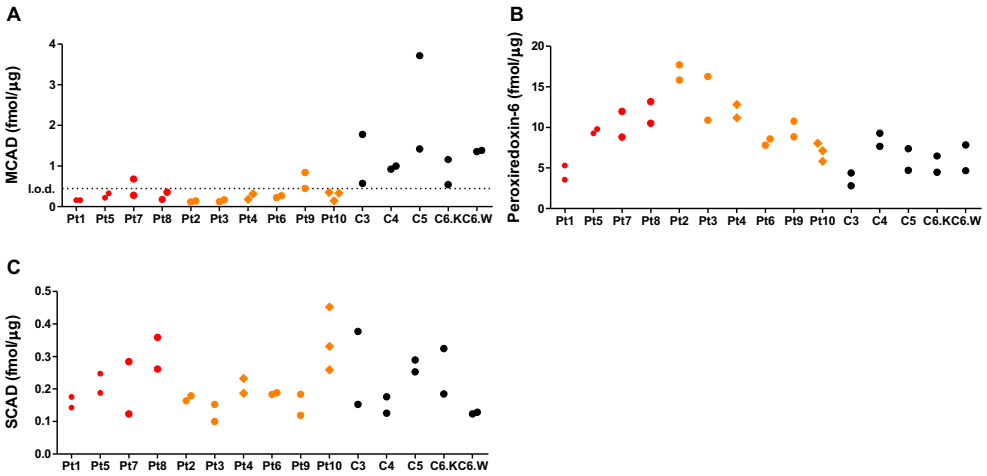
Supplementary figure S2. Concentration of acyl-carnitines in the medium, depicted for the fibroblasts separately (A-D) and depicted as symptomatic, asymptomatic and control fibroblast groups (E-H). The results pertain to the validation phase of the study. Details as in Figure 2. Significance visualization is according to Figure 1.



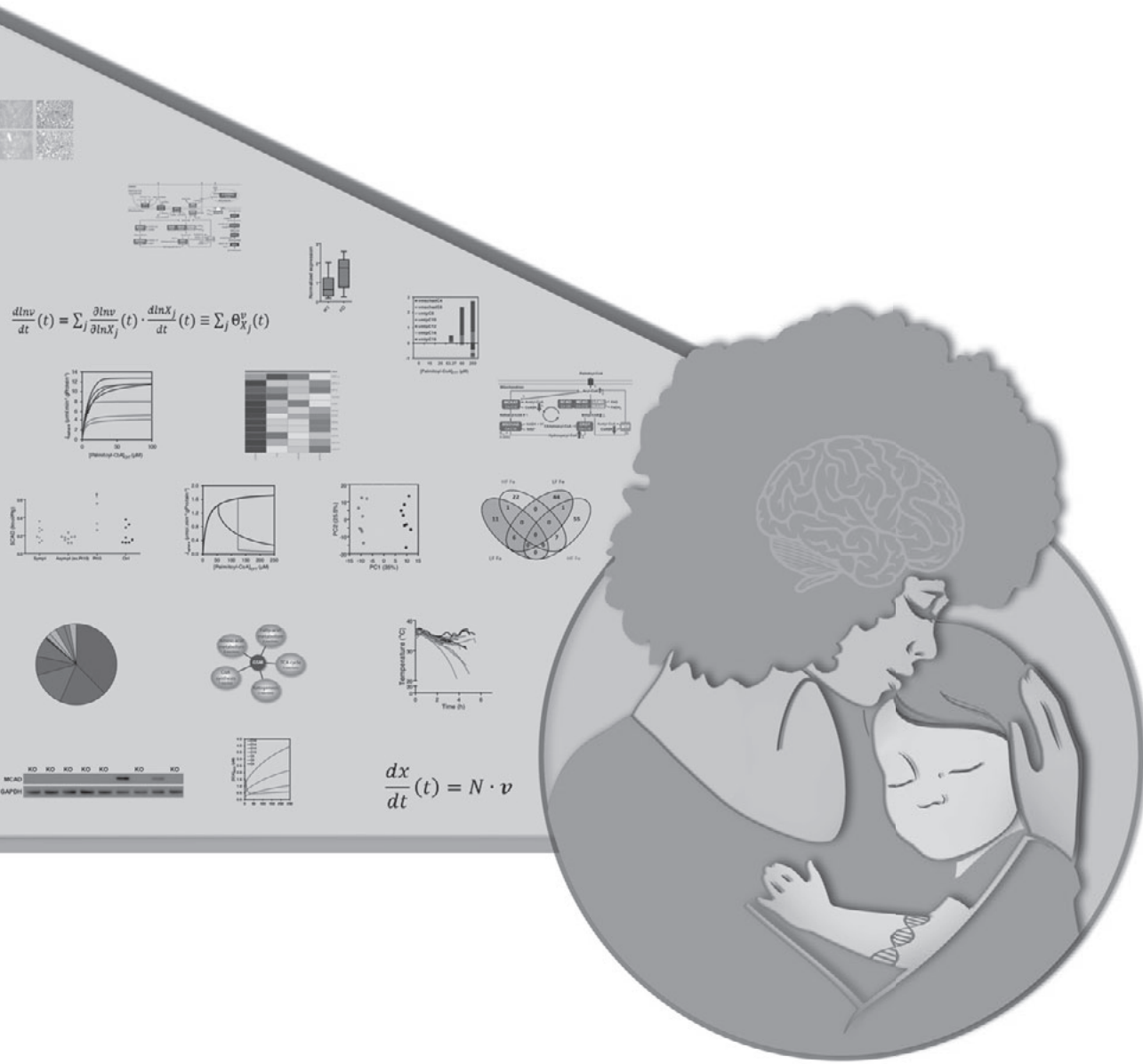
Supplementary figure S3. Acyl-carnitine secretion into in the medium, depicted per fibroblast cell line. The results pertain the validation phase of the study. Details as in Figure 2.



Supplementary figure S4. Intracellular acyl-carnitine concentrations, depicted per fibroblast cell line. The results pertain to the validation phase of the study. Details as in Figure S2.



Supplementary figure S5. Selection of proteomic results of the validation phase of the study, depicted per fibroblast cell line. Details as in Figure 4. Significance visualization is according to Figure 1.


$\frac{dlnv}{dt}(t) = \sum_j \frac{\partial lnv}{\partial lnX_j}(t) \cdot \frac{dlnX_j}{dt}(t) \equiv \sum_j \theta^v_{X_j}(t)$
KO KO KO KO KO KO KO
MCAD
GAPDH
$\frac{dx}{dt}(t) = N \cdot v$