The role of peroxisomes in malnutrition and metabolic disease

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Chapter 6

iPSC-derived liver organoids as a tool to study Medium Chain Acyl-CoA Dehydrogenase deficiency

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Submitted
ABSTRACT

Background

Medium chain acyl-CoA dehydrogenase deficiency (MCADD) is an inherited metabolic disease, characterized by a mutation in the ACADM gene. Interestingly, even with the same mutation, patients often present with very heterogeneous symptoms, ranging from fully asymptomatic to life-threatening hypoketotic hypoglycemia. The mechanisms underlying this heterogeneity remain unclear. Therefore, there is a need for in vitro models of MCADD that recapitulate the clinical phenotype as a tool to study the pathophysiology of the disease.

Methods

Fibroblasts of control and symptomatic MCADD patients with the c.985A>G (p.K329E) were reprogrammed into induced pluripotent stem cells (iPSCs). iPSC were then differentiated into hepatic expandable organoids (EHOs), further matured to Mat-EHOs, and functionally characterized.

Results

EHOs and Mat-EHOs performed typical hepatic metabolic functions, such as albumin and urea production. The organoids metabolized fatty acids, as confirmed by acyl-carnitine profiling and high-resolution respirometry. MCAD protein was fully ablated in MCADD organoids, in agreement with the instability of the mutated MCAD protein. MCADD organoids accumulated medium-chain acyl-carnitines, with a strongly elevated C8/C10 ratio, characteristic clinical phenotype of the disease. Notably, C2 and C14 acyl-carnitines were found decreased in MCADD Mat-EHOs. Finally, MCADD organoids exhibited differential expression of genes involved in mitochondrial β-oxidation and peroxisomal coenzyme A metabolism, particularly upregulation of NUDT7.

Discussion

iPSC-derived organoids of MCADD patients recapitulated the major clinical phenotype of the disease. Mat-EHOs expressed relevant pathways involved in putative compensatory mechanisms, notably CoA metabolism. The upregulation of NUDT7 expression may play a role in preventing excessive accumulation of dicarboxylic acids in MCADD. This patient-specific-hepatic organoid system is a promising platform to study the phenotypic heterogeneity between MCADD patients.
INTRODUCTION

Medium-chain acyl-CoA dehydrogenase (MCAD) is one of the flavoenzymes that catalyze the first step of mitochondrial β-oxidation of fatty acids. It oxidizes medium-chain acyl-CoA into 2-enoyl-CoA. MCAD deficiency (MCADD) is an autosomal recessive disease and the most common fatty acid oxidation disorder (FAOD) with a prevalence of 1/8,300 in the Netherlands\(^1\). Interestingly, people homozygous for an \textit{ACADM} mutation can present various clinical phenotypes. While some patients remain asymptomatic throughout life, some experience life-threatening hypoketotic hypoglycemia when exposed to catabolic stress (e.g. fasting and intercurrent illness)\(^2\)-\(^4\). After diagnosis, the most common treatment relies on avoidance of fasting and an emergency regimen\(^5\)-\(^6\). While the mutation c.985A>G (p.K329E) is estimated to account for more than 90% of pathogenic alleles, the implementation of MCADD in newborn screening has revealed several \textit{ACADM} variants of unknown significance\(^7\). The clinical risk associated with individual variants and the mechanisms underlying metabolic decompensation remain unclear\(^7\),\(^8\). Patients with residual MCAD activity, equal or above 10%, have been referred to as mild and often remain asymptomatic\(^1\). However, within the group that is homozygous for the classical c.985A>G (p.K329E) mutation, patients may present with a wide range of symptoms and disease severity, suggesting a limited genotype-phenotype correlation. Therefore, it is likely that genetic variation beyond the \textit{ACADM} gene, the environment, and epigenetics play a major role in the development of a metabolic crisis\(^9\).

Preclinical, mechanistic studies of the pathophysiology underlying MCADD have traditionally relied on the use of animal models\(^10\)-\(^12\). However, rodents are equipped with an extra dehydrogenase, long-chain acyl-CoA dehydrogenase (LCAD), which shows overlapping substrate specificity with MCAD\(^13\) and may therefore mask the phenotype. Additionally, a full \textit{ACADM} deletion cannot elucidate the effect of individual point mutations in the \textit{ACADM} gene. This makes human and patient-derived in vitro models an attractive alternative for the study of MCADD. Organoids are 3D multicellular structures that proliferate in vitro, while recapitulating several functions of the organ of origin. They can be obtained from primary tissue as well as from pluripotent and adult stem cells\(^14\). Liver organoids recapitulate several hepatic functions such as albumin production, bile acid production, and CYP3A4 activity\(^15\). Since they were first described\(^16\), organoids have been used for an increasing number of applications including the study of differentiation, organ development\(^17\), and (metabolic) disease\(^18\), as well as drug screening\(^19\). For primary tissue, highly invasive liver
biopsies are required. In contrast, hepatobiliary organoids can also be derived from induced pluripotent stem cells (iPSCs)\textsuperscript{20–23}. The latter can be obtained from fibroblasts, lymphoblasts, or even urine cells, providing an expandable source of hepatic cells through a minimally invasive procedure\textsuperscript{24}. Several protocols have been developed for differentiating iPSCs into hepatobiliary organoids. Although these organoids remain closer to fetal than to adult tissue\textsuperscript{25–27}, they represent an important organ-specific system to understand not only the disease mechanism but also patient-specific phenotypes and symptomatology.

Patient-specific iPSC-derived MCADD hepatobiliary organoids could serve (i) to study the effect of different mutations in the \textit{ACADM} gene itself and (ii) to study compensatory mechanisms that may depend on genetic variation outside the \textit{ACADM} gene. To realize this goal, it is important that organoids recapitulate the major disease phenotype. Moreover, pathways that have been hypothesized to play a compensatory role in asymptomatic patients, should be active in the organoids. Putative compensatory enzymes and pathways include short-chain acyl-CoA dehydrogenase\textsuperscript{28}, peroxisomal β-oxidation\textsuperscript{29} and coenzyme A metabolism\textsuperscript{28}. Whereas the MCAD enzyme is localized in the mitochondria, peroxisomes are single membrane organelles equipped with their own β-oxidation pathway. They are involved in many metabolic processes and highly abundant in liver cells. They are renowned for their ability to oxidize branched-chain and very-long-chain fatty acids\textsuperscript{30}, yet they are also capable of oxidizing medium-chain fatty acids (MCFA)\textsuperscript{29,31} and fatty dicarboxylic acids\textsuperscript{32}. To the best of our knowledge, there are currently no experimental studies reporting on the role of peroxisomes in any of the deficiencies of mitochondrial dehydrogenases (SCADD, MCADD and VLCADD).

The goal of this study was to establish and characterize an iPSC-derived hepatobiliary organoid system for the study of MCADD. iPSCs derived from fibroblasts of symptomatic MCADD patients with the classical c.985A>G (p.K329E) mutation were differentiated into hepatic organoids and compared to organoids from healthy controls. Here we demonstrate that MCADD organoids recapitulate typical diagnostic MCADD markers. Moreover, mature organoids upregulate peroxisomal markers, making them a suitable system to study patient-specific differences in peroxisomal metabolism. Finally, we report a minor regulation of peroxisomal CoA metabolism, already in these organoids of symptomatic MCADD patients.
RESULTS

Generating induced pluripotent stem cells from control and MCADD patient fibroblasts

Control and MCADD fibroblasts were reprogrammed into iPSCs following a previously published protocol\textsuperscript{33}. Immunofluorescence confirmed the expression of NANOG, OCT4, SOX2, SSEA-4, Tra-1-60 and Tra-1-81 indicating the pluripotency of the iPSCs (Supplementary figure 1).

Generating expandable hepatic organoids (EHOs) from control and MCADD iPSCs

In order to differentiate iPSCs into hepatic organoids, a previously published protocol\textsuperscript{34} was slightly adapted (Figure 1a): to supplement Wnt3a and hRspon1, conditioned medium was used instead of recombinant proteins. At day 8 the cells were transferred from 2D culture and embedded into BME domes and cultured in expansion medium (EM) for at least 10 days. Morphological changes in the cells were observed at the different stages of the differentiation (Figure 1b). While most of the organoids were cystic and contained clear lumina, more complex structures could also be observed. Some organoids formed “lobule-like” structures emerging from a central structure (Figure 1b). At this stage, expandable hepatic organoids (EHO) could be cryopreserved and - after thawing - expanded in culture for several passages without any clear morphological changes.

In order to assess cell identity at the different stages of differentiation, the gene expression profile of the organoids was characterized (Figure 1c-e). We defined different developmental stages including iPSCs, definite endoderm (DE), hepatoblasts (HB), and EHOs (Figure 1a and b), and compared them to human liver biopsies. Endoderm-specific markers \textit{SOX17}, \textit{FOXA2}, \textit{GATA4}, and \textit{GATA6} were all induced at DE stage. \textit{FOXA2}, \textit{GATA4}, and \textit{GATA6} stayed higher than in the iPSCs at all stages, while \textit{SOX17} declined in EHOs, resembling the liver reference (Figure 1c). Early hepatic specification markers \textit{TBX3} and \textit{HNF4-\alpha} were already observed in the DE stage but peaked in the EHOs. For alpha-fetoprotein (AFP), a fetal hepatocyte marker, we observed a peak in expression at the stage of EHOs and barely any presence in the earlier stages, nor in liver tissue. The hepatic marker albumin was not observed until later stages of the differentiation (HB) and showed its maximal expression in the EHOs. Interestingly, the albumin expression was very high in the EHOs and almost comparable to that in human liver (Figure 1d). Finally, the expression of early biliary markers \textit{CK19} and \textit{SOX19} (Figure 1e) illustrates the presence of more than one cell type in the organoids. Together, these results
highlight that the differentiation protocol from iPSCs into EHOs recapitulates the early stages of hepatic development.

Different functional assays as well as immunostainings were performed in the EHOs to determine their “liver-like” state. Immunostainings of the EHOs in culture revealed the expression of more mature hepatic markers ALB and HNF4-α (Figure 1f). Finally, the functionality of EHOs was confirmed by the secretion of albumin and urea into the supernatant (Figure 1g).
Figure 1. Protocol for the generation of human expandable hepatic organoids (EHOs) from iPSCs. (A) Schematic depiction of the differentiation protocol from iPSCs to hepatobiliary organoids. (B) Representative images depicting morphological changes at different stages of the differentiation. From left to right: iPSCs, DE, HB, cystic EHOs and “lobule-like” EHOs. Scale bars, 200 µM for B1-3 and 500 µM for B4-5. (C) Relative gene expression at different stages of the differentiation of endoderm markers, (D) early hepatocyte markers and (E) early biliary specification. Data represents 3 biological replicates from independent control donors. For C-E, human liver samples were included in the analysis. (F) Immunofluorescent staining of EHOs using albumin (green) on top and HNF4 (green) bottom, with nuclei in blue and cell membrane in red. Scale bar = 100 µm. (G) Upper graph: Urea released in supernatant by organoids (blue) and human precision-cut liver slices (PCLS) (yellow) in 24 hours. Lower graph: Albumin released in supernatant by organoids (blue) in 24 hours. Data represents 10 biological replicates for the organoids and 3 biological replicates for the PCLS from independent experiments. All graphs: error bars indicate SEM.
Next, we assessed the carnitine profile of the organoids to confirm the accumulation of medium-chain acyl-carnitines, characteristic of MCADD patients. Control and MCADD organoids were incubated with palmitate and L-carnitine for 24 hours and the acyl-carnitine profile was assessed both intracellularly and in the supernatant. While no changes were observed in short- and long-chain acyl-carnitines (Suppl Figure 2), MCADD organoids accumulated medium-chain acyl-carnitines with 6 to 10 carbon atoms (C6, C8, and C10 in Figure 2cd). Moreover, intracellularly the C8/C10 ratio, an important diagnostic biomarker of MCADD in clinical practice, was strongly upregulated in MCADD organoids (from 11 to 20) (Figure 2d). The same pattern was observed in the supernatant where the C8/10 ratio was upregulated from 6 to 8 (Figure 2d). The values are in the same range as those observed in the plasma of severe MCADD patients (median 13.1), whereas mild MCADD patients (≥ 10% residual MCAD activity) had a median C8/10 ratio of 3.10 and healthy controls 0.81.
**Figure 2.** MCADD EHO organoids display an MCADD-characteristic phenotype in culture. (A) Representative immunoblot images illustrating the lack of MCAD protein in MCADD organoids; data represents 2 MCADD lines and 2 control lines. (B) Intracellular triglyceride levels in control and MCADD organoids. On the left organoids were stimulated with bovine serum albumin (BSA). The graph on the right depicts the values after treating the organoids with 0.5mM of BSA-Palmitate; data represents the mean of 6 biological replicates ± SEM (Unpaired two-tailed t-test) (C) Oxygen consumption rate in organoids measured at state 3 (stimulated with ADP, octanoyl carnitine and malate) and uncoupled (state U); Data represents the mean of 5 biological replicates ± SEM. (D) Upper panel: medium chain acyl-carnitines measured in the organoids incubated with palmitate and L-carnitine for 24 hours (C6, C8 and C10) and C8/C10 ratio; data represents 6 biological replicates from independent cultures ± SEM. Lower panel: Medium-chain acylcarnitine measured in supernatant (C6, C8 and C10) and C8/ C10 ratio. Data represents 6 biological replicates from independent cultures ± SEM. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 one-tailed unpaired t test).

**Maturation of EHOs into Mat-EHOs increases peroxisome abundance**

Although peroxisomes are specialized in their ability to oxidize very-long-chain, branched and dicarboxylic fatty acids, they are also capable of oxidizing
medium-chain acyl-CoA. Given the indications of higher number of peroxisomes in hepatic cells, we decided to first further differentiate the EHOs into mature EHOs (Mat-EHOs) in order to stimulate peroxisome proliferation.

EHOs were kept in expansion medium (EM) for 4-5 days after passage and then transferred to maturation medium (MM) (Figure 3a). Maturation medium contains different cytokines that allow the EHOs to mature into a more hepatocyte-like state. EHOs in MM underwent morphological changes, shrinking in size and developing thicker outer edges. (Figure 3b). Maturation was confirmed by upregulation of mature hepatocyte markers HNF4-α, AFP, Albumin and CYP2C9. Matured organoids also showed lower expression of cholangiocyte markers TBX3 and CK19. Moreover, genes coding for enzymes involved in peroxisomal fatty-acid oxidation (ABCD1, ACOX1, and CROT) were significantly upregulated (Figure 3c). Finally, Mat-EHOs secreted higher levels of albumin into the supernatant than EHOs, in line with a more hepatocyte-like phenotype (Figure 3d).
Figure 3. Maturation of EHOs into Mat-EHOs. (A) Schematic depiction of the maturation protocol (B) Representative brightfield images of EHOs and Mat-EHOs. Scale bar = 500 µm. (C) Relative gene expression of mature hepatic markers and peroxisomal markers in EHOs and Mat-EHOs. Data represents 8 biological replicates from independent experiments ± SEM. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 two-tailed unpaired t test). (D) Albumin secreted into the supernatant after 24 hours in EHOs and Mat-EHOs. Data represents 4 biological replicates ± SEM. (*P<0.05, **P<0.01, two-tailed unpaired t test).

Peroxisomal adaptation in MCADD

Subsequently, we investigated if mitochondrial and peroxisomal fatty-acid oxidation pathways (Figure 4a) were regulated in response to loss of MCAD protein. In order to mimic the fasting conditions, which often trigger symptoms in patients, we exposed the Mat-EHO organoids to glucose-free medium in the presence of BSA-palmitate and carnitine. Again similar to the clinical phenotype, medium-chain acyl-carnitines accumulated in MCADD and the ratio C8/C10 was strongly elevated (Figure 4b-c). Interestingly, and unlike in
the EHOs, in Mat-EHOs short-chain acyl-carnitine (C2) and long-chain myristoyl-carnitine (C14) were downregulated (Figure 4b).

Interestingly, we observed that several genes encoding mitochondrial β-oxidation enzymes were downregulated in the MCADD Mat-EHOs relative to controls, including medium-chain ketoacyl-CoA thiolase (MCKAT) (encoded by \textit{ACAA2}), hydroxyacyl-coenzyme A dehydrogenase (M/SCHAD) (encoded by \textit{HADH}) and carnitine/acylcarnitine carrier protein (CACT) (encoded by \textit{SLC25A20}) (Figure 4d). Given the ability of peroxisomes to oxidize MCFA, it has been hypothesized that in the absence of MCAD, excess medium-chain acyl-CoAs can also be channeled to peroxisomes, where they undergo further β-oxidation until C6-acyl-CoA, which may be shuttled back into the mitochondria to be oxidized by SCAD\textsuperscript{29} (Figure 4a). For the peroxisomal β-oxidation, the genes encoding for the transporters \textit{ABCD1} and \textit{ABCD3} (ATP binding cassette transporter subfamily D; \textit{ABCD1} and \textit{ABCD3}), the oxidase \textit{ACOX1} (acyl-CoA oxidase 1; \textit{ACOX1}), DBP and LBP (D-bifunctional protein and L-bifunctional protein; \textit{HSD17B4} and \textit{EHHDAH}, respectively) and the thiolases \textit{ACAA1} and \textit{SCPx} (\textit{ACAA1} and \textit{SCP2}, respectively) were evaluated (Figure 4e). Apart from a non-significant decline in \textit{ACOX1} expression, other measured genes were not regulated in MCADD organoids.

In conclusion, all the observed adaptations showed downregulation, rather than upregulation of putative compensatory pathways.
iPSC-derived liver organoids as a tool to study Medium Chain Acyl-CoA Dehydrogenase deficiency

Figure 4. Adaptations of mitochondrial and peroxisomal β-oxidation in MCADD in Mat-EHOs. (A) Schematic depiction of the hypothesized interplay between mitochondria and peroxisomes in the β-oxidation of medium chain fatty acids. In green, the targets analyzed in D-E. (B) Acyl-carnitines measured in supernatant collected after 24 hours in glucose-free medium supplemented with BSA-palmitate and L-carnitine. (C) C8/C10 ratio; For B-C, data represents 6 biological replicates for control and 4 for MCADD ± SEM (*P<0.05, **P<0.01 two-tailed unpaired t-test). (D) Relative gene expression of genes involved in the mitochondrial β-oxidation. (E) Relative gene expression of genes involved in the peroxisomal β-oxidation. For E and D, organoids were grown in glucose-free medium supplemented with BSA and L-carnitine. Data represents 6-7 biological replicates for control and 5-6 for MCADD ± SEM. (*P<0.05, **P<0.01, two-tailed unpaired t test). Control (grey) and MCADD (red).

In agreement with the changes observed in the acyl-carnitine profile (Figure 4b), a recent in silico study, based on a detailed kinetic model of human fatty-acid oxidation, predicted decreased levels of short-chain and C12-14 acyl-CoA and acyl-carnitines in MCADD28. Such decrease has been suggested to be associated with a concomitant depletion in the free coenzyme A (CoA) levels and, consequently, reduced entry of long-chain fatty acids into the pathway28,37. CoA is an essential intracellular cofactor involved in several metabolic pathways.
and its level is dynamically regulated to adjust to the metabolic state through biosynthesis and turnover (Figure 5a)\textsuperscript{38}.

Next, we measured the relative gene expression of different genes involved in coenzyme A biosynthesis and total CoA levels (Figure 5b-c). This process occurs partly in the cytosol and partly in the mitochondria (Figure 5a). PANK2, encoding the mitochondrial pantothenate kinase, was slightly, but significantly downregulated in the MCADD organoids (Figure 5c). In addition to CoA biosynthesis, carnitine acyl-transferases and acyl-thioesterases (ACOT) also modulate the size of the CoA and acyl-CoA pools in different intracellular compartments. Peroxisomes also contain several enzymes involved in CoA recycling (Figure 5a), among them two carnitine acyl-transferases: carnitine octanoyl-transferase (CROT) and carnitine acetyl-transferase (CRAT), in charge of converting Acyl-CoAs into their respective acyl-carnitine form, with the concomitant release of free CoA. MCADD organoids showed substantially lower CROT and CRAT expression than healthy controls (Figure 5d). NUDT7, a gene that encodes a peroxisomal Nudix hydrolase, which hydrolyzes CoA and acyl-CoA species into 4’pantotheine and acyl-4’-pantotheine\textsuperscript{39,40} and also plays a role in the metabolism of dicarboxylic acids\textsuperscript{41,42} was clearly upregulated in MCADD organoids (Figure 5d). The total CoA pool (free CoA plus acyl-CoA) did not differ between MCADD and control Mat-EHOs (Figure 5b). Taken together, we observe a regulation of some genes involved in CoA metabolism, but an obvious compensatory response could not be identified.
iPSC-derived liver organoids as a tool to study Medium Chain Acyl-CoA Dehydrogenase deficiency

Figure 5. CoA biosynthesis and metabolism in Mat-EHOs. (A) Schematic depiction of the hypothesized effect of MCADD on CoA pools and compensatory mechanism from peroxisomes. In green, the targets analyzed in C-D. (B) Total coenzyme A pools in control (grey) and MCADD (red) organoids. Organoids were grown in glucose-free medium supplemented with BSA-palmitate and L-carnitine. Data represents 5 biological replicates ± SEM. (C) Relative gene expression of genes involved in CoA biosynthesis. Data represents 7 biological replicates for the control group and 5 for MCADD ± SEM. (*P<0.05, **P<0.01, two-tailed unpaired t test). (D) Relative gene expression of genes involved in CoA metabolism. For C and D, organoids were grown in glucose-free medium supplemented with BSA and L-carnitine. Data represents 7 biological replicates for the control group and 5 for MCADD ± SEM. (*P<0.05, **P<0.01, two-tailed unpaired t test).
Chapter 6

DISCUSSION

In this paper we have established a patient-specific iPSC-derived hepatic organoid system for the study of MCADD. These organoids recapitulated the metabolic profile with high levels of medium-chain acyl-carnitines and an elevated C8/C10 ratio that is typical of severe MCADD patients. Furthermore, the Mat-EHOs expressed pathways that are relevant for future studies of the pathophysiology of the disease. It may not be surprising that we observed only minor adaptations in putative compensatory pathways, since the MCADD organoids were derived from symptomatic patients. Compensatory pathways would rather be expected to be more pronounced in asymptomatic patients. Nevertheless, we observed some conspicuous differences between MCADD organoids and controls in respect of CoA metabolism, evidenced by reduced levels of C14-acyl-carnitine and upregulation of NUDT7 in MCADD.

Studying metabolic functions in iPSC-derived hepatobiliary MCADD organoids

Discovery of organoids in 2009\(^1\), revolutionized the field of biomedical research, providing an alternative to traditional primary hepatocytes. Recent work has focused on the development of organoids from iPSCs. iPSC-derived hepatobiliary organoids have often been described as closer to fetal tissue\(^2\), and advancing their maturity levels to that of liver tissue remains a challenge. Here we compared different maturation stages of the organoids for the study of MCADD. While the original protocol uses a 3-step procedure which relies on spontaneous aggregation of hepatic single cells\(^3\), we found that this step was hard to reproduce and did not yield enough biomass for metabolic readouts. Therefore, for the purpose of our study, EHOs were matured to Mat-EHOs (equivalent of “pre-maturation” stage in the reference paper\(^4\)). At this stage, Mat-EHOs were found to already exhibit liver-like phenotypes such as typical hepatocyte mRNA markers, albumin and urea secretion, and the presence of functional mitochondria and peroxisomes.

Importantly, both MCADD EHOs and Mat-EHOs showed accumulation of medium-chain acyl-carnitines (C6-C10), recapitulating a major clinical phenotype. Interestingly, only MCADD Mat-EHO organoids presented reduced levels of short- and long-chain acyl-carnitine relative to their control counterparts. These changes are in alignment with a recent in silico study from our group, based on a detailed computational model of the fatty-acid oxidation, which predicted reduced levels of short- and long-chain acyl-CoAs and their corresponding acyl-carnitines in MCADD\(^5\). Decreased short-chain
acyl-CoA or acyl-carnitine levels are not surprising, since they are downstream of the deficient MCAD enzyme. C14, however, is upstream of MCAD, and at first sight it might therefore be expected to be elevated or unchanged. In the computational model the reduced C14-acyl-CoA could be attributed to a limitation of free CoA availability. Free CoA was sequestered into medium-chain acyl-CoAs, which strongly accumulated in MCADD. The decrease of free CoA, in turn limited the entry of new long-chain fatty acids into the mFAO pathway and thereby led to a reduced C14-acyl-CoA. Thus, our data provides experimental validation of this non-intuitive computational prediction.

Together these data suggest that the maturation step into Mat-EHO organoids, accompanied by peroxisome and mitochondrial enrichment and a representative acylcarnitine profile, is a relevant step to study the pathophysiological mechanisms of MCADD.

Adaptations of mitochondrial and peroxisomal β-oxidation and CoA metabolism in MCADD

While peroxisomes have been reported to oxidize MCFAs, we did not observe any regulation of peroxisomal enzymes involved in the import and oxidation of fatty acids in MCADD organoids, which suggests this pathway was not further activated.

In addition to the oxidation of fatty acids, peroxisomes play a central role in the oxidation of dicarboxylic fatty acids (DCA). Under high fatty acid (FA) supply, such as fasting and mFAO disorders, excess FAs are channeled to DCA via ω-oxidation. DCA accumulation in the liver can be toxic, causing inflammation, fibrosis and death. In MCADD, patients in metabolic crisis have been reported to exhibit accumulation of medium-chain dicarboxylic fatty acids (MC-DCA) and high excretion in urine. Excess DCAs can be metabolized by the peroxisomal β-oxidation or via the activity of nudix hydrolase 7 (NUDT7). The expression of peroxisomal ABCD3 transporter and LBP, both playing a major roles in DCA oxidation, was not regulated in the MCADD organoids. Recent animal studies suggest that Nudt7 contributes to the regulation of dicarboxylic fatty acid metabolism in the liver. Male mice lacking Nudt7 (Nudt7 -/-) exposed to high fat diet showed accumulation of MC-DCA. The authors proposed a major role of NUDT7 in the regulation of the levels of MC-DCA. The upregulation NUDT7 expression in the MCADD organoids may therefore play a role in preventing excessive accumulation of MC-DCA that otherwise could be toxic.
Conclusion and Outlook

In conclusion, the Mat-EHO organoids derived from iPSCs of severe MCADD patients show a phenotype that is characteristic for these patients and provide a good basis for future studies into patient-to-patient variability. For instance, they can be used to assess the phenotype of genetic variants of the ACADM gene that give rise to different residual activities. More interestingly, they can be used to study putative compensatory pathways in asymptomatic patients with a classical c.985A>G and zero residual MCAD activity. Furthermore, nutritional interventions and different stressors can be modulated in vitro, to study liver-specific aspects of MCADD pathophysiology and potential therapeutic interventions.

METHODS

Human fibroblasts

Fibroblasts from patients without documented heritable metabolic diseases (n=2) and also from symptomatic patients with MCADD carrying the classical c.985A>G missense mutation (n=2) were obtained from the Department of Genetics of the University Medical Center Groningen. Patients 5 and 8, previously described in the literature28, were chosen for this study because of their classical mutation, symptomatology and normal growth of fibroblasts. Control fibroblasts from C104 and C105 were also described in the literature 28. All patients were born prior to the implementation of the neonatal MCADD screening in the Netherlands (2007). Both MCADD patients were symptomatic, suffered at least one recorded metabolic crisis, resulting in hospitalization with hypoglycaemia (<2.6 mmol/L), coma, and/or seizures.

Fibroblasts were cultured in Ham’s F-10 Nutrient Mix (Thermo Fisher Scientific 11550043), supplemented with 10% FCS (Gibco) and 1% penicillin/streptomycin (Gibco).

Generation and culture of iPSCs from patient fibroblasts

Human fibroblasts were reprogrammed into induced pluripotent stem cells following the previously published protocol33. iPSCs were cultured in Matrigel (Corning)-coated plates in mTeSR™ Plus medium (STEMCELL Technologies). Cells were passaged every 4-6 days and medium was changed every other day.
Generation of EHOs from iPSCs

iPSCs were differentiated into expandable hepatic organoids (EHOs) following the previously published protocols for hESCs\textsuperscript{51,52} with some adaptions. iPSC cells were cultured in Matrigel-coated plates and placed in RPMI1640 medium supplemented with 1X B27-Supplement (Invitrogen), 100ng/ml Activin A (Peprotech), and 30% Wnt3a-conditioned medium (kindly provided by Hans Clevers). Wnt3aCM was removed after 24 hours and cells were kept in the same medium supplemented with 100ng/ml Activin A and 2X B27-supplement for 48 hours. The cells were then transferred to Hepatocyte Culture Medium (HCM) (Lonza, CC-3198) without EGF and supplemented with 20ng/ml BMP4 and 10ng/ml FGF2 (both Peprotech) for the next 5 days. After 5 days, the cells were dissociated using 0.25% Trypsin-EDTA and embedded in BME domes (5000-10000 cells per 50µL dome) in Corning 24-well plates. The cells were cultured in an expansion medium (expansion medium) containing Ad. DMEM/F12 (Gibco) supplemented with 1X Glutamax, 1X HEPES, P/S (all Gibco), 1X B-27 Supplement, 1X N-2 Supplement (all Invitrogen), 10mM Nicotinamide (Sigma Aldrich), 1.25mM N-acetylcysteine (Sigma Aldrich), 50ng/ml EGF (Peprotech), 10μM Forskolin (TOCRIS), 10nM Iso-leu gastrin (Sigma Aldrich), 30% Rspon1-CM (Kindly provided by Calvin J. Kuo) and 10% Wnt-3a CM.

Medium was changed every 2-3 days. Organoids were passaged every 7-9 days by manually disrupting the BME domes and split in a ratio 1:8-1:10 depending on the line and supplemented with 10 μM Y 27632 dihydrochloride (Axon Medchem) for 2 days after passaging.

Maturation of EHOs into Mat-EHOs

Expandable hepatic organoids were matured into hepatocyte-like EHOs (Mat-EHOs) following the previously published protocol\textsuperscript{51}. Organoids were kept in expansion medium for 4-6 days after passage, and then placed in maturation medium (MM) containing Ad. DMEM/F12 supplemented with 1X Glutamax, 1X HEPES, P/S, 1X B-27 Supplement, 1X N-2 Supplement, 10mM Nicotinamide, 1.25mM N-acetylcysteine, 10nM Iso-leu gastrin, 50 ng/ml HGF, 25 ng/ml BMP7 and 25 ng/ml FGF4 (all growth factors from Peprotech). Organoids were kept in maturation medium for 4 days prior to collection for downstream analysis.
Organoid imaging and immunofluorescence

For brightfield imaging, organoids were imaged using an AxioObserver Z1 compound microscope (Carl Zeiss), 2.5× and 5× objectives and an AxioCam MRm3 CCD camera (Carl Zeiss).

For immunofluorescence images, organoids were processed following the previously published protocol [53]. After collection with ice-cold Ad. DMEM/F12, the organoids were kept on ice for 10 minutes to ensure BME degradation. After centrifugation at 80G at 4°C, the organoids were reconstituted in 4% PFA and kept at 4°C for 45 minutes. After fixation, organoids were permeabilized using PBS containing 0.1% Tween 20 and kept in that buffer for 2 days. Primary and secondary antibody incubations were performed in PBS containing 0.1% Tween 20 and 0.2% BSA. Primary and secondary antibodies are listed on supplementary table 2. Organoids were imaged using an ImageXpress Micro Confocal High-Content Imaging System (Molecular devices) with a 20X water-immersion objective (molecular devices). 50 slices with 2μm step size were acquired for each wavelength. Image processing was performed using Fiji v1.8.0.

RNA isolation, reverse transcription and quantitative real-time qPCR

RNA was isolated from the organoids using RNase easy Kit (Qiagen) as described by the manufacturer. NanoDrop (NanoDrop Technologies) was used to assess the quality and yield of total RNA. M-MLV Reverse Transcriptase (200U/μl) (Invitrogen) was used to perform reverse transcription as established by the manufacturer. qPCR was performed in 384 well format in duplicates (5 -10 ng per well) using FastStart Universal SYBR Green Master (Rox) (Sigmal Aldrich) using QuantStudio 7 Flex (Thermo Fischer Scientific). All primer (Integrated DNA technologies Inc) sequences are listed in supplementary table 1. β-actin served as endogenous control, and was used for normalization.

Hepatocyte functional assays

For albumin measurements, the supernatant was collected after 24 hours. Levels of albumin in the supernatant were measured with the human albumin ELISA kit (Abcam) as described by the manufacturer using the Synergy H4 Hybrid Microplate Reader (BioTek Instruments Inc).

Urea secretion was assessed in the organoids supernatant using the QuantiChrom™ Urea kit as described by the manufacturer.
All results were normalized to protein content.

**Immunoblotting**

The protocol followed for immunoblotting was slightly modified from a previously published paper\(^{54}\). Organoids were collected using ice-cold Ad. DMEM/F12 and kept on ice for 10 minutes to degrade the BME. Organoids were then centrifuged at 290\(g\) for 5 minutes at 4\(^{\circ}\)C and washed with cold PBS prior to another centrifugation step. After the second centrifugation, the organoids were reconstituted in radio immunoprecipitation assay (RIPA) buffer containing 1\% IGEPAL CA-630, 0.1 \% SDS, and 0.5 \% sodium deoxycholate in PBS. RIPA buffer was supplemented with Phosphatase Inhibitor Cocktail 2 (Cat. No. P5726) and Cocktail 3 (Cat. No. P0044) and Complete Protease Inhibitor Cocktail (Cat. No. 1186145001) (All Sigma Aldrich). Sonics Vibra cell VCX130 (Sonics & Materials Inc.) was used to sonicate organoid lysates using 4 pulses of 10 second on, 30 seconds off at an amplitude of 30\%. Lysates were then centrifuged at 12000 rcf for 10 minutes at 4\(^{\circ}\)C to ensure the precipitation of cell debris. Protein content was determined using Pierce BCA Protein Assay Kit (ThermoScientific) and all samples were adjusted to the lowest concentration value. Lysates were adjusted with Laemmli loading buffer (5X: 60 mM Tris-Cl pH 6.8, 10% glycerol, 1% SDS, 0.05% Bromophenol Blue, 1% beta-mercaptoethanol). Protein separation was done in SDS-PAGE 10-14\% using a Mini PROTEAN Tetra Vertical Electrophoresis Cell system (Bio-Rad, 1658029FC). For western blot, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon®-P, Millipore).

**Fat isolation and triglyceride quantification**

Organoids were collected in 1x TBS (137 mM NaCl, 2.7 mM KCl, 66 mM Tris, pH 7.4) in MiliQ water. Fat was extracted in chloroform: methanol in a ratio 2:1. The levels of hepatic triglycerides were quantitatively determined using the DiaSys Triglyceride FS kit (Holzheim). Results were normalized to protein content.

**High resolution respirometry**

Organoids were collected at day 8-10 using ice-cold Ad. DMEM/12 and kept on ice for 10 minutes. Organoids were then centrifuged at 290 \(g\) at 4\(^{\circ}\)C for 5 minutes and washed with 2\(\mu\)L of MiR05 buffer, followed by another spin and finally reconstituted in 600\(\mu\)L of MiR05 buffer, containing 110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 20 mM HEPES, 0.5 mM EGTA, 10 mM KH2PO4, 3 mM MgCl2, and 1 mg/ml bovine serum albumin, at pH 7.1.
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Oxygen consumption rate was measured in the organoids using a two-channel high-resolution Oroboros Oxygraph-2 k (Oroboros). Organoids were first permeabilized by addition of digitonin (0.02 mg/ml). The maximal coupled respiration was measured in the presence of 1mM ADP, 25 μM octanoylcarnitine and 2mM malate (state 3). Basal respiration was determined by the subsequent addition of 0.002 mg/ml of oligomycin to block ATP synthase (state 4). Finally, uncoupled respiration (state U) was measured after subsequent administration of 1.5 μM carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP). Oxygen consumption rates were normalized to protein concentration.

**Acylcarnitines measurements**

EHO organoids were incubated with 0.5mM BSA-conjugated palmitate (Sigma-Aldrich, P9767) and 2 mM l-carnitine (Sigma Aldrich) for 24 h prior to collection in PBS. Acylcarnitines were measured both in supernatant and intracellularly following the published protocol55.

**Total CoA measurements using HILIC-MS/MS analysis**

Mat-EHO organoids were incubated in glucose-free maturation medium supplemented with 0.5mM BSA-conjugated palmitate and 2 mM l-carnitine for 24 h. Organoids were collected in ice-cold medium and washed 2X in ice-cold PBS. Samples were prepared as described elsewhere56. Briefly, the pellet was reconstituted in 600 μL MilliQ H₂O and the lysate was sonicated using a Sonics Vibra cell VCX130 (25 seconds, 50% amplitude, 2 times). Lysates were centrifuged at 14000 rpm for 15 minutes at 4°C. In a new tube, 80 μL Tris (2-carboxyethyl)phosphine hydrochloride (10 mM) was added to 400 μL supernatant and, incubated for 15 minutes at room temperature. Next, samples were spun down (14000 rpm, 15 minutes, 4°C). In a new tube, 40 μL ammonia solution was added to 400 μL supernatant (1.25% v/v) and, incubated shaking at 500 rpm at 60°C for 60 minutes. Lastly, samples were dried using a SpeedVac (Eppendorf) and reconstituted in 100 μL ice-cold 80% methanol.

Coenzyme A was extracted by a two-step protocol using chloroform/methanol/ water based on the Bligh and Dyer approach57. The detailed sample preparation and HILIC-MS/MS protocol are described in detailed in the literature58.
Human Precision-Cut Liver Slices (hPCLS)

PCLS with approximately 250–300 μM thickness were prepared using a Krumdieck Tissue Slicer (Alabama Research and Development), as described elsewhere. Individual slices were kept in culture for 24h in Williams E medium (WE), containing 25 mM glucose, 0.5% BSA, 1 mM l-carnitine, and gentamycin (Invitrogen). The plates were kept under a continuous supply of 80% O₂/5% CO₂, shaking at 70 rpm.

Human liver tissue was collected from a transplantation donor at the University Medical Center Groningen (UMCG, Netherlands). The liver was used for research purposes after being rejected for transplantation with the approval of the Research Ethics Committee of UMCG.

Statistical Analyses

All results are expressed as mean ± standard error of the mean (SEM). Analyses were performed using GraphPad Prism Software Version 9.02 (Graphpad Software). Statistical significance was determined as *P value < 0.5, **P value < 0.01 and ***P value < 0.001; no indication means no significant changes (ns). For iPSC work, biological replicates are considered individual donors. For organoid work, due to low number of patients, biological replicates are considered organoids coming from the two same patients or healthy controls that were at least one passage apart.

Informed consent

The use of historical patient fibroblasts was approved by the Medical Ethical Committee of the University Medical Center Groningen and confirmed according to the Dutch law.

FUNDING

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REFERENCES


SUPPLEMENTARY FILES

Supplementary Figure 1. Expression of iPSC markers in iPSC control line.

Immunofluorescent staining of DAPI (blue) and (A) SOX2 (green), (B) SSEA4 (red), (C) Tra1-60 (green) and NANOG (red) and (D) Tra1-81 (green) and OCT4 (red).
Control organoids in grey and MCADD organoids in red. Data represents 6 biological replicates ± SEM. (*P<0.05 one-tailed unpaired t-test).
### Supplementary Table 1. List of primer sequences used in RT-qPCR

<table>
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<tr>
<th>Gene Name</th>
<th>Forward and reverse primer sequence (5’- 3’)</th>
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</table>
| SOX17     | Fwd: CGCACGGAAATTTGAACAGTA  
                        Rev: GGATCAGGGACCTGGTCACAC |
| FOXA2     | Fwd: ACTACCCCGGCTACGGTTTC  
                        Rev: AGGCCCGTTTTGTCCTGGA |
| GATA4     | Fwd: CTCCTTTCTCAGCAGAGCTGTGA  
                        Rev: CTCTGCTACAGCCAGTAGGATT |
| GATA6     | Fwd: CCCACAACACAACCACAGC  
                        Rev: GCGAGACTGACGCCTATGTA |
| TBX3      | Fwd: TTTCAAAATTCTCGGAGATGGGTGTC  
                        Rev: ACGACCTTTGACATGCAGTCTCC |
| HNF4a     | Fwd: ACTACATCAACGACCGCAGT  
                        Rev: AGTTCCCGGGCATAAAAAAGTAAG |
| Albumin   | Fwd: GAGACCAGAGTTGATGATG  
                        Rev: AGTTCCCGGGCATAAAAAAGTAAG |
| AFP       | Fwd: CTTTGCGCTCGCTGCTATGA  
                        Rev: GCATGTGATTTAACAAGCTGCT |
| CK19      | Fwd: ACGACCATTAGGACCTCGGG  
                        Rev: TCCCACCTGGCCCCCTACGTA |
| SOX9      | Fwd: ACGACGAGACCGGAG  
                        Rev: CTGGTACTTGTAATCCGGGTG |
| ABCD1     | Fwd: CTTCTGGAGACGCGCTGTGAT  
                        Rev: TTCGATATTGCAATCCGGGTG |
| ABCD3     | Fwd: GTCCTTTAGCACAACCGCAATGG  
                        Rev: CTCTTCCGCAGCCATTTGGAC |
| NUDT7     | Fwd: CTCCTGGCTTGTGGCTCTGATG  
                        Rev: TGCTCTGGTACCTCAGCTACC |
| NUDT19    | Fwd: GCACCACTCGCAGCTTTTGACA  
                        Rev: GTTGCCCCAGGAGAGGACCC |
| SLC27A2   | Fwd: GTGGAGAAAGATGAAACCTGTCCG  
                        Rev: CTGAGCCTTTTCTGCCAGCATAG |
| ACBD2     | Fwd: GCTGCGCAAGGATGAGACTCAA  
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| ACOT4     | Fwd: CTTGCGACGTGGCTACTACGT  
                        Rev: CCTAGAAATCGCCCGAACAGCC |
| ACOT8     | Fwd: GCTGACCACTGAGTGCTCTAG  
                        Rev: AGTGACAGCAGTACCCCTCATG |
| ACOX1     | Fwd: GGGCGCATACGAGAAGAGGACT  
                        Rev: AGTGAAAGCTTCAGTGCCAGC |
| EHHADH    | Fwd: CGGAGCATCGTGAAAACACAGCA  
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| SCP2      | Fwd: GACAAGGTGCAACGCTGGTTGA  
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Fwd, forward primer; Rev, reverse primer.
**Supplementary Table 2. List of primary and secondary antibodies used in immunoblotting.**

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