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ARTICLE

Altered mitochondrial metabolism in peripheral blood cells from patients with inborn errors of β-oxidation

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Abstract

Inborn errors of mitochondrial fatty acid oxidation (FAO), such as medium-chain acyl-CoA dehydrogenase deficiency (MCAD) and very long-chain acyl-CoA dehydrogenase deficiency (VLCAD) affects cellular function and whole-body metabolism. Carnitine uptake deficiency (CUD) disturbs the transportation of fatty acids into the mitochondria, but when treated is a mild disease without significant effects on FAO. For improved clinical care of VLCAD in particular, estimation of FAO severity could be important. We have investigated whether the oxygen consumption rate (OCR) of peripheral blood mononuclear cells (PBMCs) obtained from patients with MCAD, VLCAD, and CUD can be used to study cellular metabolism in patients with FAO defects and to determine the severity of FAO impairment. PBMCs were isolated from patients with VLCAD ($n = 9$), MCAD ($n = 5$–7), and CUD ($n = 5$). OCR was measured within 6-hours of venous puncture using the Seahorse XFe96. The PBMCs were exposed to glucose alone or with caprylic acid (C8:0) or palmitic acid (C16:0). OCR was significantly lower in cells from patients with β-oxidation deficiencies (MCAD and VLCAD) compared to CUD at basal conditions. When exposed to C16:0, OCR in VLCAD cells was unchanged, whereas OCR in MCAD cells increased but not to the levels observed in CUD. However, C8:0 did not increase OCR, as would be expected, in VLCAD cells. There was no clear relationship between clinical severity level and OCR. In patients with β-oxidation deficiencies, changes of mitochondrial respiration in PBMCs are detectable, which indicate that PBMCs have translational potential for studies of β-oxidation defects. However, further studies are warranted.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Inborn errors of mitochondrial fatty acid β-oxidation (FAODs) affect cellular function and whole-body metabolism. The clinical severity of certain FAODs at the time of diagnosis may be challenging. Peripheral blood mononuclear cells (PBMCs) are a heterogeneous group of cells often used to study the biology of

disease. However, the translational potential of PBMCs in FAODs has not been determined before and little is known regarding the importance of FAODs in this cell group.

WHAT QUESTION DID THIS STUDY ADDRESS?

In order to investigate the potential of using PBMCs from patients with FAODs, we investigated cellular metabolism in PBMCs derived from patients with medium-chain acyl-CoA dehydrogenase deficiency (MCAD), very long-chain acyl-CoA dehydrogenase deficiency (VLCAD), and carnitine uptake deficiency (CUD).

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

FAOD is used in human PBMCs for energy production. Mutations in the VLCAD or the MCAD enzymes seem to affect the overall mitochondrial function, whereas CUD seems to have a normal FAOD rate as compared to results in the literature. Normal FAOD is essential for overall mitochondrial function in human PBMCs.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Studying PBMCs shows an interesting translational potential and for further understanding the pathophysiology of FAODs. If this method is developed further, it may be used as a simple and easily accessible tool to study mitochondrial function in a number of inborn errors of metabolism.

INTRODUCTION

Inborn errors of mitochondrial fatty acid oxidation (FAODs) are autosomal recessive disorders that can be detrimental to cellular function and whole body metabolism.[1] FAODs are caused by disruption of either mitochondrial β-oxidation, or of the carnitine shuttle-dependent transport of fatty acids across mitochondrial membranes.[2] Medium-chain acyl-CoA dehydrogenase deficiency (MCAD; OMIM #201450) and very long-chain acyl-CoA dehydrogenase deficiency (VLCAD; OMIM #201475) are two of the most common β-oxidation defects.[3] The symptoms range from asymptomatic to life-threatening.[1] Global prevalence of FAODs is estimated to 6.5 per 100,000 live births.[4]

MCAD is caused by mutations in the *ACADM* gene and is considered to be a relatively mild disease, but with an increased risk of energy deficiency, hypoglycemia, and metabolic acidosis in catabolic situations and when the patient is exposed to prolonged fasting.[5] VLCAD, caused by mutations in the *ACADVL* gene, results in increased risk of energy deficiency with a broad phenotypic variation from mild to severe forms.[1] Symptoms range from exercise- or fasting-induced muscle symptoms to rhabdomyolysis, hepatic involvement, and, in some cases, severe cardiac symptoms. Sudden death may occur already in the neonatal period.[6] The main treatment for MCAD is avoidance of prolonged fasting, carnitine is supplemented when necessary.[7] VLCAD treatment consists of fasting avoidance, ensuring adequate energy intake, restricted intake of long chain fatty acids, and supplementation with medium-chain triglycerides; additional treatment compounds, including triheptanoin, are currently under investigation.[8]

Carnitine uptake deficiency (CUD; OMIM #212140) is caused by mutations in the *SLC22A5* gene affecting the production of the carnitine transporter protein, OCTN2, which is necessary for the transportation of carnitine over the cell membrane.[9] Defects in OCTN2 lead to excess loss of carnitine in the urine, with subsequent low plasma and decreased intracellular levels.[10] This leads to secondary effects on β-oxidation. Untreated, CUD may cause hypoglycemia, hyperammonemia, cardiomyopathy, and hypotonia.[9] CUD is mainly treated with supplementation of L-carnitine, which leaves the patient asymptomatic with plasma levels of carnitine and acyl-carnitines within normal range, indicating intact mitochondrial β-oxidation.[9] Even though mice with severe untreated CUD show abnormal mitochondria, the respiratory chain remains intact.[11]

The FAODs are typically identified via newborn screening (NBS) based on acylcarnitine profiles.[12] The MCAD diagnosis is confirmed by genetic analysis of the *ACADM* gene, and CUD by analysis of the *SLC22A5* gene.[5,9] The diagnosis of VLCAD is based on plasma levels or ratios of acylcarnitines,[13] and confirmed by genetic analysis of the *ACADVL* gene.[12] In addition, the activity of the VLCAD enzyme can be measured in fibroblasts or lymphocytes to attempt to assess the severity of the disorder.[2] However, the diagnostic methods used today cannot reliably predict the clinical phenotype and need

for treatment, which are currently assessed by clinical observation.[12] Hence, we speculate whether evaluation of mitochondrial oxygen consumption can add information regarding the clinical phenotype. Single studies of mitochondrial respiration have been performed in fibroblast from patients with VLCAD.[14] However, harvesting of fibroblast leaves the patient scarred and is time-consuming.[15]

The mitochondrial FAO is complex. It has recently been proposed to consist of an integrated molecular architecture to enable the transfer of reducing equivalents between the FAO and the electron transport chain (ETC).[16] However, FAODs affect not only FAO, but also have secondary effects on, for example, systemic composition of complex lipids.[17] Studying mitochondrial metabolism easily and accessibly directly from patients with FAODs, would hence be of great value to increase our knowledge of the ramifications of these disorders on the mitochondrial metabolism.

Peripheral blood mononuclear cells (PBMCs) include T lymphocytes, B lymphocytes, NK cells, hematopoietic stem cells/hematopoietic progenitor cells, and dendritic cells.[18] This heterogeneous group of cells is often used to study the biology of health and disease.[19,20] The metabolism of PBMCs has been studied recently,[21,22] however, the importance of fatty acid oxidation in this cell group is not completely characterized. In specific white blood cell populations, studies have shown that lymphocytes and monocytes have all the biochemical prerequisites to utilize glucose, lipids, and amino acids—particularly glutamine.[23] The nutritional demands of PBMCs are complex, however, glucose is a major source for oxidative phosphorylation in some PBMCs (e.g., T-cells).[24] In addition, long-chain fatty acid β-oxidation has been shown to contribute with sizeable energy production in naïve T-cells, indicating that T-cells are flexible in their nutrient use.[25] Animal studies indicate that the metabolism of PBMCs may be similar to that of other organs.[26,27]

In this study, we investigated the feasibility of using human PBMCs to determine mitochondrial respiration shortly after venous puncture in patients with VLCAD, MCAD, and CUD, which in the future may reduce the need of fibroblasts to diagnose and study these disorders, as well as allowing for structural and dynamic studies of mitochondrial function instead of whole cell metabolism.

In addition, we explored the effects of the different fatty acid oxidation defects on mitochondrial respiration in resting human PBMCs using different substrates, glucose alone or with addition of caprylic (C8:0) or palmitic (C16:0) acid. Finally, we investigated whether the results could be translated to clinical severity in patients with VLCAD.

METHODS

Study population

Patients with MCAD (n = 5–7), VLCAD (n = 9), or CUD (n = 5) were included in this study. All individuals were recruited between February and May of 2019 at the Karolinska University Hospital, Stockholm, Sweden. The mean ages of the patients with MCAD and VLCAD were 7.6 ± 3.5 and 5.7 ± 2.2 years, respectively. Patients with CUD were matched for age, body mass index (BMI), and BMI-standard deviation score (SDS), which is BMI adjusted for age.[28] The mean age of patients with CUD was 6.0 ± 2.9 years. The inclusion criterion was genetically confirmed diagnosis of MCAD, VLCAD, or CUD. Exclusion criteria were age over 18 years and, for patients with CUD, clinical symptoms and altered levels of acyl-carnitine and/or carnitine in plasma. All parents gave their written informed consent regarding the participation of their children in the study. The study was approved by the Institutional Review Board of Uppsala University, Sweden, decision number 2006/005, 2009-09-30.

The study was carried out in accordance with the Declaration of Helsinki.

Blood sampling

Blood samples were obtained in the morning after breakfast, in line with the patients' respective dietary regimen. Venous blood (2 ml) was drawn in an EDTA-vacutainer (Becton Dickinson, Franklin Lakes, NJ) using a patent venous catheter, after local anesthesia by a mixture of Lidocaine and Prilocaine (EMLA, AstraZeneca, Cambridge, UK). Complete blood counts were determined at the Laboratory of Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden, using the SYSMEX XN instrument (Sysmex, Kobe, Japan).

Isolation of PBMCs

PBMCs were isolated using a density gradient Lymphoprep (Axis-Shield, Oslo, Norway) and all samples were analyzed within 6 h after venous puncture, as previously recommended.[29] After isolation via Lymphoprep, the PBMCs are considered to be comprised of ~ 85% lymphocytes and 15% monocytes.[30] PBMCs were isolated at room temperature in the presence of anticoagulants to prevent activation of the cells.[31,32]

Fatty acid preparation

Fatty acids were prepared as previously described.[33,34] In short, 100 mM C16:0 or C8:0 acid solution (both Sigma

Aldrich, St. Louis, MO, USA) were dissolved in 50% ethanol at 60°C. Assay medium (Agilent, Santa Clara, CA) composed of 143 mM NaCl, 5.4 mM KCl, 0.91 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 2 mM Glutamax, 3 mg/l Phenol Red, with the addition of 0.5% fatty acid free bovine serum albumin (BSA; Boehringer Mannheim GmbH, Mannheim, Germany) was used for diluting the fatty acid solutions to a final concentration of 0.5 mM. Both fatty acid solutions were then kept at 37°C for at least 30 min and pH was adjusted to 7.40. No carnitine was added.

Oxygen consumption and extracellular acidification rates

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were analyzed using the XFe96 flux analyzer (Agilent). Following isolation, 500,000 PBMCs, counted by BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) according to the MIFlowCyt standard,[35] were placed into a well of an XFe96 cell culture microplate pre-coated with 3% ECM gel (Sigma-Aldrich, St. Louis, MO) and 5 μg/ml fibronectin (Sigma-Aldrich). The cells were then incubated for 1 h at 37°C in assay medium (Agilent) with 5 mM glucose and 5 mg/ml fatty acid-free BSA (basal condition) with or without the supplementation of 0.5 mM caprylic acid (C8:0) or 0.5 mM palmitic acid (C16:0). For all conditions, pH was adjusted to 7.40. For each subject, 6–8 replicates of each condition were analyzed. Basal OCR and ECAR were measured in parallel during 20 min. Subsequently, 50 μM of the carnitine palmitoyl transferase 1 (CPT-1) blocker etomoxir (Sigma-Aldrich) was added and the effect was measured during 25 min. Next, 25 μM of the ATP-synthase inhibitor oligomycin (Sigma-Aldrich) was added in order to determine ATP-coupled respiration. After 65 min 37.5 μM carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (Sigma-Aldrich), which collapses the mitochondrial proton gradient and allows uninhibited electron flow through the ETC and oxygen to be maximally consumed by complex IV, was added in order to determine maximal respiration. A mixture of 2.5 μM Rotenone (Sigma-Aldrich) and 25 μM Antimycin A (Sigma-Aldrich), which effectively shut down the mitochondria by inhibiting complex I and complex III, respectively, was added at 85 min to determine non-mitochondrial respiration.

The extracellular acidification rate (ECAR), reflecting glycolysis, was determined in parallel by measuring the changes in pH every minute during the first 20 min of the experiment. The average changes in pH before the addition of etomoxir (i.e., the ECAR in the basal state), are presented.

In order to eliminate the contribution of background OCR, the decrease in OCR after the addition of etomoxir in the condition without fatty acids was subtracted from the decrease in OCR in the conditions with fatty acids, according to the formula: average effect of etomoxir on OCR (in condition with fatty acids) – average effect of etomoxir on OCR (in condition without fatty acid). The average decrease in OCR for both fatty acid conditions was then determined as described above.

Residual enzymatic activity

VLCAD enzymatic activity, determined with the electron transfer flavoprotein fluorescence reduction assay, was performed at the Metabolic laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden, as previously described.[36] In short, lymphocytes were isolated using Ficoll-Paque. Subsequently, the lymphocytes were centrifuged in a preparation buffer. A reagent mixture was then added with the substrate palmitoyl-CoA. Then, 5 μL catalase and 5 μL glucose oxidase were added and the analysis was then performed at 378 nm exciting wavelength and 488 nm emitting wavelength. Total protein content was measured using the Lowry method. Fibroblasts were used as controls for these measurements.

Clinical parameters

Acyl-carnitines were measured by either a Xevo TQ or Xevo TQ-S (Waters, Millford, MA) at the Center for Inherited Metabolic Diseases, Karolinska University Hospital, Solna, Sweden. Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), creatine kinase (CK), and glucose were measured at the Karolinska University Hospital Clinical Chemistry Department using the COBAS 8000 (Roche Diagnostics, Indianapolis, IN).

Statistical analysis

All data are presented as mean ± SD unless otherwise specified. Differences between groups were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Pearson's regression analysis was used to determine correlations. The p values less

than 0.05 were considered statistically significant. All statistical analyses were performed in GraphPad Prism version 8.4 (GraphPad Software, La Jolla, CA). Results in figures are illustrated in boxplots, showing minimum to maximum with all points included, if not stated otherwise.

RESULTS

Clinical characteristics

There were no major differences in age, BMI, BMI-SDS, or sex distribution among the CUD, MCAD, and VLCAD groups. However, there was a large variation in BMI within the MCAD group, driven by one outlier. Notably, the CK was higher ($p = 0.0071$) in the VLCAD group, as compared to the MCAD group (Table 1). No differences were seen in ASAT, ALAT, or fasting glucose among the three groups. Acyl-carnitine profiles matched the patient groups, with elevated C14:1-carnitine and C16:0-carnitine for VLCAD and C8:0-carnitine for MCAD (Table 1), respectively. The platelet counts were higher in the VLCAD group ($361 \pm 44 \times 10^9$ cells/L) than in both the CUD ($326 \pm 18 \times 10^9$ cells/L, $p < 0.0001$) and MCAD ($308 \pm 51 \times 10^9$ cells/L, $p < 0.0001$) groups. Complete blood count did not differ in other aspects (data not shown). All patients with CUD had normal acyl-carnitine and carnitine levels in plasma (Table 1 and data not shown). Individual data for patients with VLCAD and patients with MCAD are shown in Tables 2 and 3, respectively.

Cellular oxygen consumption rate

The cells responded to the added substrates in accordance with expectations for the three different conditions (Figure 1a–c). Basal OCR was lower in cells from patients with MCAD and VLCAD compared to CUD cells (Figure 2a). Cells from patients with MCAD had a higher basal OCR than those from patients with VLCAD ($p = 0.034$) in the presence of 0.5 mM C16:0. However, no differences were seen between these two groups when cells were exposed to C8:0. The ATP-coupled contribution to OCR was lower in both β-oxidation deficiency groups, regardless of treatment, compared to the CUD group (Figure 2b). Maximal OCR was lower in both the VLCAD and MCAD groups compared to the CUD group regardless of substrate (Figure 2c) but no differences were seen between the MCAD and VLCAD cells. For the non-mitochondrial OCR (Figure 2d), cells from patients with VLCAD had lower non-mitochondrial OCR than cells from patients with CUD in the presence of C8:0 and C16:0, respectively. However, cells from patients with MCAD had higher non-mitochondrial OCR than those from patients with VLCAD in the C16:0 ($p = 0.0024$) but not in the C8:0 condition.

The percentage of increase of basal OCR when comparing the conditions without fatty acids to the condition with

TABLE 1 Clinical characteristics of the study group

	CUD ($n = 5$)	MCAD ($n = 7$)	VLCAD ($n = 9$)
Age (years)	6.0 ± 2.9	7.6 ± 3.5	5.7 ± 2.2
Weight (kg)	22.1 ± 7.7	36.1 ± 31.6	22.5 ± 8.0
Height (cm)	110.0 ± 21.4	126.7 ± 20.6	113.3 ± 14.3
BMI	16.0 ± 0.8	21.1 ± 8.6	17.1 ± 2.6
BMI-SDS	0.2 ± 0.3	1.27 ± 1.6	0.6 ± 1.2
Sex (female/male)	2/3	3/4	2/7
ALAT (μkat/L)	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.7
ASAT (μkat/L)	0.7 ± 0.1	0.5 ± 0.2	1.4 ± 2.2
CK (μkat/L)	5.92 ± 5.87	1.79 ± 0.7	21.09 ± 55.85[a]
C8:0-Carnitine (μmol/L)	<LOD	3.2 ± 4.0	<LOD
C14:1-Carnitine (μmol/L)	<LOD	<LOD	2.1 ± 2.2
C16:0-Carnitine (μmol/L)	<LOD	<LOD	0.62 ± 0.05
Fasting glucose (mmol/L)	4.6 ± 0.2	4.7 ± 0.5	4.6 ± 0.7

Abbreviations: BMI, body mass index; BMI-SDS, body mass index-sodium dodecyl sulfate; CK, creatinine kinase; CUD, carnitine uptake deficiency; LOD, limit of detection; MCAD, medium-chain acyl-CoA dehydrogenase deficiency; VLCAD, very long-chain acyl-CoA dehydrogenase deficiency.

Presented as mean ± SD.

[a]$p < 0.05$ vs. MCAD.

TABLE 2 Disease specific clinical characteristics, including genetic mutations and adverse events, in the VLCAD group

ID	Gestational age (weeks)	Screening detected	Age at genetic diagnosis (months)	Genetic mutation	Enzyme activity	Dietary treatment	Emergency regimen	Clinical phenotype	Clinical events
001	38	Yes	1	c.848T>C c.1816T>C	30	Yes	Yes	Intermediary	Neonatal lethargy and weight loss, mild CK-elevations during infections
002	38	Yes	1	c.848T>C c.1837C>T	11	Yes	Yes	Intermediary	Neonatal lethargy, gastrostomy due to feeding difficulties. No decompensations.
003	40	No	2	c.343DelG c.343DelG	13	Yes	Yes	Severe	Diagnosed after neonatal hypoglycemia. Recurrent severe rhabdomyolysis events.
004	40	Yes	2	c.1591C>T c.1678+1 G>C	20	No	Yes	Intermediary	No neonatal events. Clinically stable, one episode with mild CK-elevation.
005	38	Yes	2	c.848T>C c.1387C>T	12	Yes	Yes	Intermediary	Mild CK-elevation during gastroenteritis, no symptoms from 2 years of age.
006	38	Yes	2	c.708-709DelCT –	54	No	Yes	Asymptomatic	Repeated pathologic acyl carnitine profile, no clinical symptoms.
007	38	Yes	2	c.1238T>C c.1837C>T	12	Yes	Yes	Intermediary	Moderate dietary treatment, no decompensations.
008	38	Yes	2	c.848T>C c.848T>C	Not taken	Yes	Yes	Intermediary	One episode of CK-elevation.
009	39	Yes	2	c.848T>C c.1838G>C	9	Yes	Yes	Severe	Repeated hospitalizations with CK-elevations, Port-a-Cath.

Abbreviations: CK, creatinine kinase; VLCAD, very long-chain acyl-CoA dehydrogenase deficiency.

TABLE 3 Disease-specific clinical characteristics, including genetic mutations and adverse events, in the MCAD group

ID	Gestational age (weeks)	Screening detected	Age at genetic diagnosis (months)	Genetic mutation	Enzyme activity	Carnitine treatment	Emergency regimen	Hypoglycemia	Clinical events
1	40	Yes	1	c.985A>G c.985A>G	1.1	Yes	Yes	No	Several hospitalizations due to infections or vomiting during first years of life. Obesity.
2	39	No	112	c.985A>G c.985A>G	0.4	Yes	Yes	No	Diagnosed after NBS of sibling. No clinical symptoms. Obesity.
3	38	Yes	1	c.985A>G c.985A>G	2.8	No	Yes	No	One hospitalization due to pneumonia.
4	39	Yes	1	c.985A>G c.1073A>T	–	No	Yes	No	Several hospitalizations due to infections or vomiting during first years of life.
5	40	No	40	c.985A>G c.199T>C	1.6	Yes	Yes	No	Diagnosed after NBS of sibling. No clinical symptoms.
6	40	Yes	2	c.985A>G c.199T>C	1.9	Yes	Yes	No	No clinical symptoms.
7	38	Yes	1	c.985A>G c.985A>G	0	Yes	Yes	No	Several hospitalizations due to infections or vomiting during first years of life.

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase deficiency; NBS, Newborn screening.

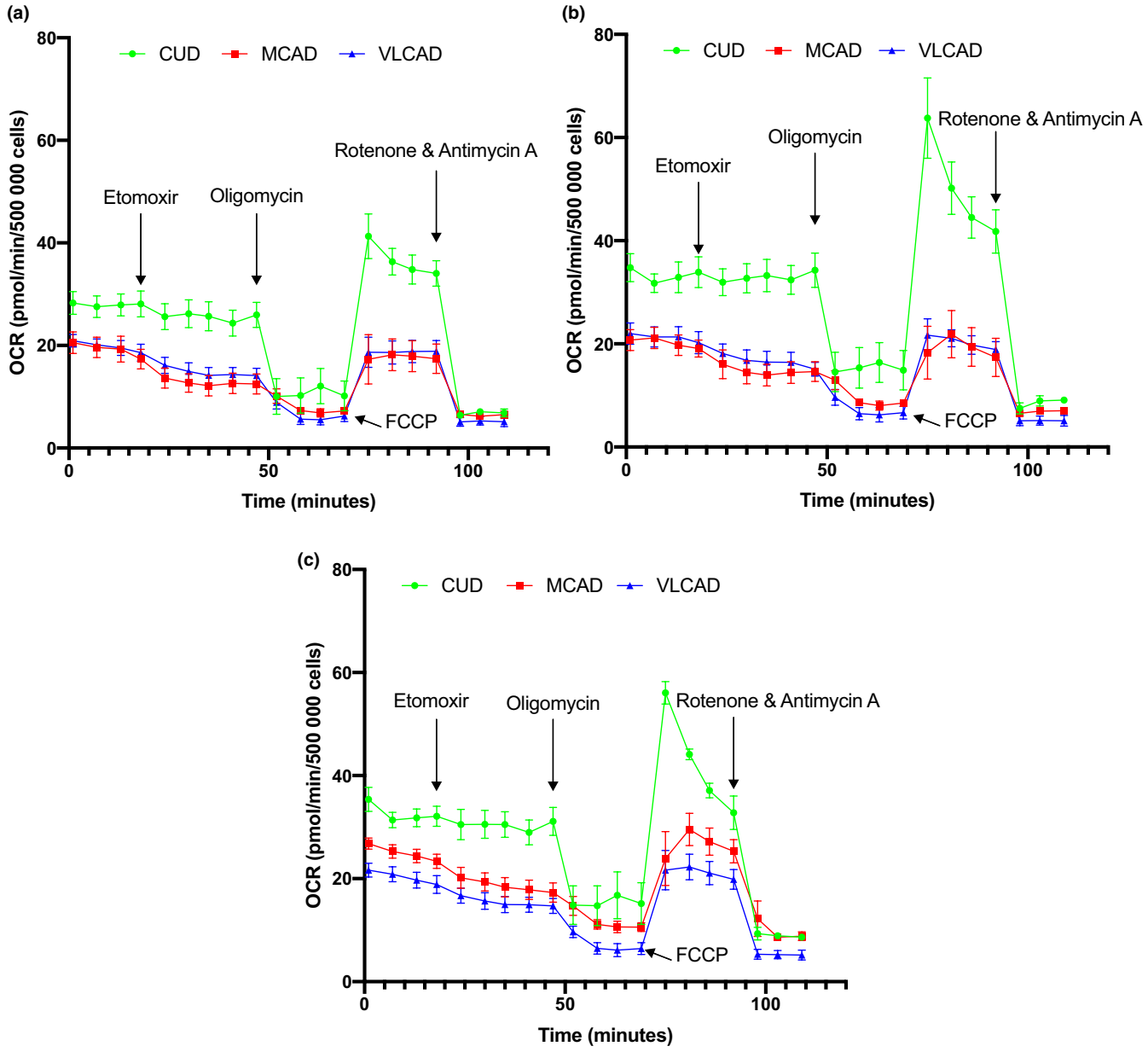

FIGURE 1 Cellular oxygen consumption rate (OCR). Flux analysis, presented as mean ± standard error of the mean, of medium chain acyl-CoA dehydrogenase deficiency (MCAD; $n = 6$), very long chain acyl-CoA dehydrogenase deficiency (VLCAD; $n = 9$), and carnitine uptake deficiency (CUD; $n = 5$) peripheral mononuclear blood cells (PBMCs) in the condition of (a) 0.5% fatty acid free BSA and 5 mM glucose, (b) 0.5% fatty acid free BSA, 5 mM glucose and 0.5 mM caprylic acid (C8:0), (c) 0.5% fatty acid free BSA, 5 mM glucose and 0.5 mM palmitic acid (C16:0). BSA, bovine serum albumin

fatty acids was higher ($p = 0.0085$) for MCAD cells compared to VLCAD cells when they were exposed to C16:0 (Figure 2e). The cells from patients with CUD had an attenuated OCR after the addition of etomoxir in the presence of C16:0 compared to C8:0 ($p < 0.05$). However, no decrease was seen for cells from patients with VLCAD or MCAD (Figure 2f).

Due to a problem with one of the injection ports, some analyses for the MCAD group were lost. There was no problem in the basal state. All data that were obtained are included in this study. Hence the number for MCAD varies between five and seven.

Extracellular acidification rate

ECAR was higher for cells from patients with MCAD than VLCAD, regardless of exposure to glucose, C8:0, or C16:0 (Figure 2g). In addition, in the C8:0 condition, ECAR was higher in the MCAD group than in the

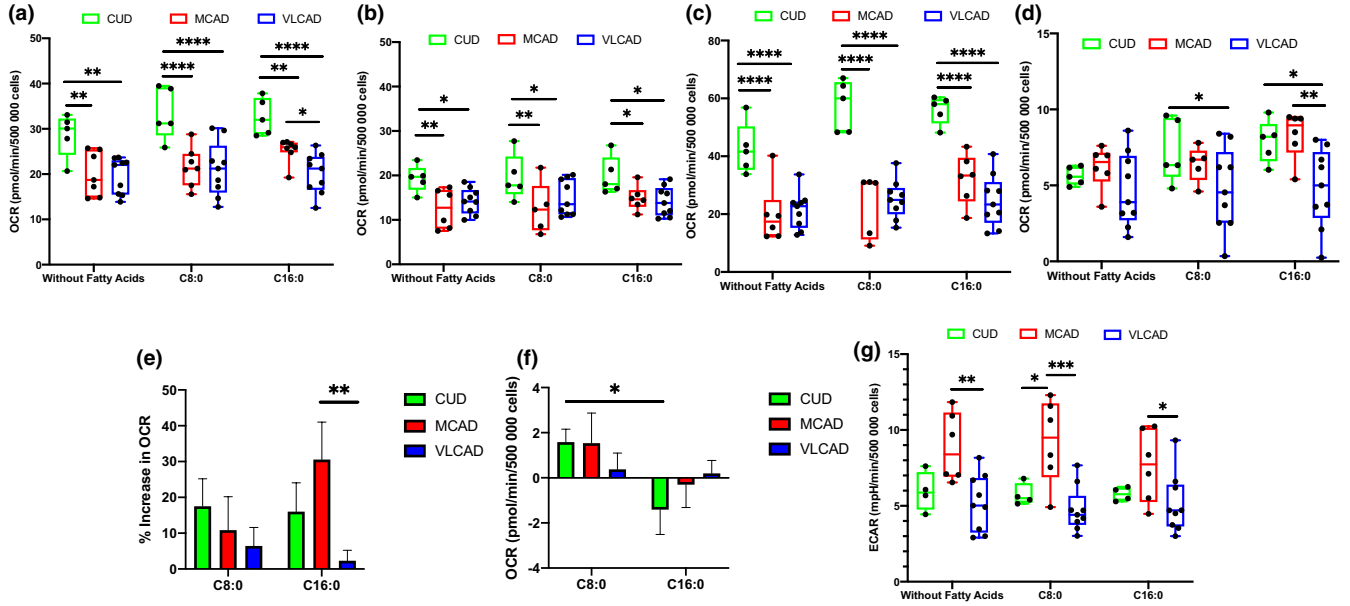

FIGURE 2 Cellular oxygen consumption rate (OCR). Aspects of cellular metabolism analyzed in peripheral mononuclear blood cells (PBMCs) from patients with carnitine uptake deficiency (CUD; $n = 5$), medium chain acyl-CoA dehydrogenase deficiency (MCAD; $n = 6$), or very long chain acyl-CoA dehydrogenase deficiency (VLCAD; $n = 9$). OCR showed as pmol/min. ECAR, extracellular acidification rate, showed as change in mpH/min. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (a) Basal OCR (MCAD $n = 7$). (b) ATP-coupled OCR (in C8:0 condition MCAD $n = 5$), (c) Maximal OCR (in C8:0 condition MCAD $n = 5$), (d) Non-mitochondrial OCR (in C8:0 condition MCAD $n = 5$), (e) Bar chart with standard error of the mean (SEM) of change in OCR after addition of C16:0, (f) Bar chart with SEM of effect of etomoxir, (g) ECAR (CUD $n = 4$)

CUD group ($p = 0.0188$), but not in the basal or C16:0 condition.

Individual OCR

Because VLCAD is a disease with a broad phenotype, where severity is difficult to determine with diagnostic tools, all patients with VLCAD were categorized into mild, medium, or severe disease in accordance with their clinical events (Table 2). The patients with VLCAD were then color-coded according to their clinical categorization, and their individual OCRs in the C16:0 condition are shown in Figure 3a. No clear pattern or association was seen between clinical categorization and OCR. In addition, every individual was then coded as CUD, MCAD, or VLCAD and plotted together in the C16:0 condition, where the highest MCAD or VLCAD OCR was 29.6 pmol/min and the lowest CUD OCR was 29.75 pmol/min (Figure 3b).

Correlations between OCR and enzymatic activity

In order to investigate how well the OCR results overlapped with enzymatic residual activity, we correlated the enzymatic activity and different aspects OCR using linear regression models. Enzymatic activity is assessed in the clinic only for patients with VLCAD, hence only VLCAD was included in these analyses. In the C16:0 condition, basal OCR had an R^2 of 0.24 ($p = 0.22$), ATP-coupled OCR had an R^2 of 0.52 ($p < 0.05$), no other association were seen (Table 4).

DISCUSSION

This study, using resting PBMCs from patients with CUD, MCAD, or VLCAD, shows that fatty acid oxidation is affected in these cells, and that the FAODs translate into altered mitochondrial respiration in PBMCs. Not only was the fatty acid oxidation rate diminished in PBMCs from patients with β-oxidation defects, as expected, but total energy production was also reduced. Importantly, we show that it is possible to determine mitochondrial bioenergetics shortly after venous puncture in these easily accessible cells, which presents interesting translational potential for the investigated disorders.

The PBMCs from the patients showed OCR in the same range as previous studies on PBMCs using similar numbers of cells.[32,37] Patients with CUD had similar OCR to that reported for healthy controls in previous studies.[32] PBMCs from patients with MCAD or VLCAD had reduced overall cellular energy metabolism for almost every analysis

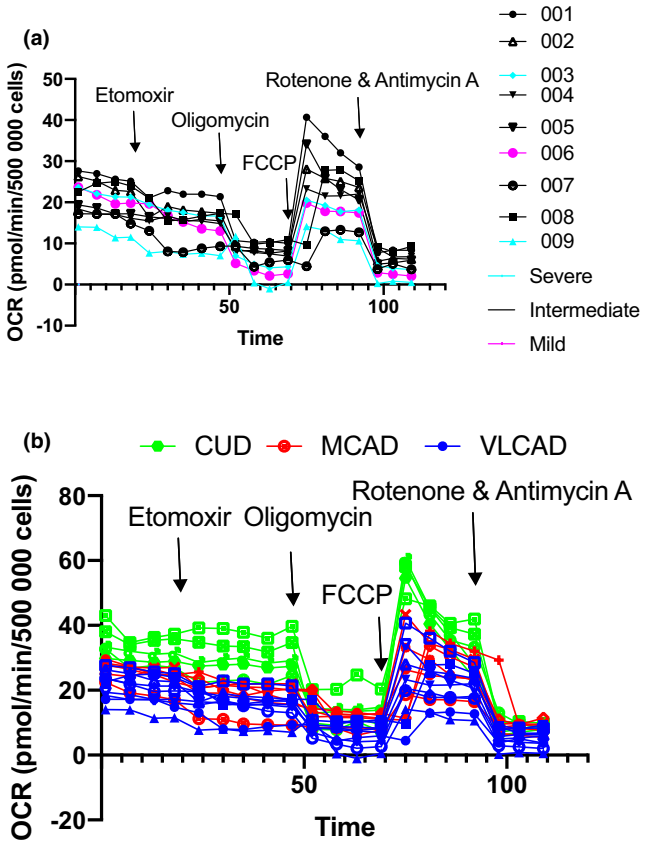

FIGURE 3 Individual oxygen consumption rate (OCR). Individual readings from the XFe96 flux analyzer. (a) Shows the C16:0 condition for very long chain acyl-CoA dehydrogenase deficiency (VLCAD; $n = 9$). Every reading from each individual included in the study is shown in b in the C16:0 condition. CUD, carnitine uptake deficiency ($n = 5$). MCAD, medium chain acyl-CoA dehydrogenase deficiency ($n = 6$)

TABLE 4 Associations between enzymatic activity (nkat/g) of VLCAD and different aspects of OCR in the VLCAD group ($n = 9$)

Analysis	Beta coefficient	R^2	*p* value
Basal OCR	1.826	0.24	0.22
ATP-coupled OCR	4.034	0.52	0.044
Non-mitochondrial OCR	−0.18	0.0009	0.94

Abbreviations: OCR, oxygen consumption rate; VLCAD, very long-chain acyl-CoA dehydrogenase deficiency.

conducted. Cells from patients with MCAD and VLCAD had reduced capacity to oxidize nutrients compared to cells from patients with CUD, not only for the fatty acids C8:0 or 16:0, but also for glucose, which is in line with current knowledge from fibroblasts.[14] Interestingly, cells from patients with MCAD utilized glycolysis to a greater extent than cells from patients with VLCAD regardless of substrate and CUD when exposed to C8:0.

The PBMCs from patients with VLCAD showed impaired oxidation capacity, with lower basal OCR and ATP production in all three nutrient conditions, including—unexpectedly—when exposed to C8:0, which theoretically should not be affected. It has previously been proposed that patients with VLCAD have upregulated MCAD enzyme activity to compensate for their deficient VLCAD enzyme.[12] We did not see an increase in OCR in the VLCAD cells when supplementing with C8:0. Indeed, it has been shown that when exposed to an abundance of long-chain fatty acids (LCFAs), CD8 + T-cells accumulate LCFAs rather than oxidizing them, impairing mitochondrial function and triggering transcriptional reprogramming of the lipid metabolism, leading to a reduction of overall fatty acid metabolism.[38] Hence, we speculate that VLCAD leads to secondary effects on the mitochondria, which manifests as a reduction of the capacity to oxidize not only C16:0 but also C8:0. This poses questions regarding the efficacy of the use of medium chain triacylglycerides in the treatment of these patients. The PBMCs from the patients with MCAD responded differently. Exposed to C16:0, they showed increased basal and non-mitochondrial OCR compared to VLCAD cells. However, the basal OCR was still lower than that of the CUD cells. This indicates that PBMC mitochondrial metabolism was not affected in cells from patients with carnitine-treated CUD. Our findings are in line with a previous study on the bioenergetics of fibroblasts from patients with VLCAD, where the authors found reduced basal and ATP-coupled OCR in patients compared to controls.[14] Bioenergetics have also been shown to be reduced in the mitochondria of cardiac muscle cells in VLCAD rats.[39] In addition, oxidative phosphorylation in the mitochondrial ETC has been reported to be altered in MCAD mice and human fibroblasts from patients with MCAD.[40] It has been speculated that the energy defect in VLCAD is more complex than being a disorder of the fatty acid oxidation, which points to a pathophysiology beyond fatty acid oxidation only.[14,41] In fact, the maximal respiratory capacity of the PBMCs from the patients with β-oxidation deficiencies was significantly lower than that of cells from patients with CUD. The maximal respiration was close to the basal respiration in the patients with MCAD and VLCAD in every environment. This indicates that their metabolism is close to full capacity already in the basal state, regardless of the nutrient. However, this was not true for PBMCs from patients with CUD, which increased their maximal respiration. In compliance with this finding, a study on naïve T-cells from CPT-1 knockout mice (TCpt1a) showed that TCpt1a cells failed to increase their maximal mitochondrial respiration when C16 was used as the main substrate.[42] This could possibly explain the complex clinical symptoms of particularly VLCAD, such

as fatigue, lethargy, and exercise intolerance in many patients, even under optimal treatment conditions.[43]

To investigate how well the OCR overlapped with enzymatic activity in fibroblasts or lymphocytes, we correlated aspects of cellular OCR to enzymatic activity in the patients with VLCAD. We found a correlation only with ATP-coupled OCR, but not with non-mitochondrial OCR or basal OCR. Indicating that the method is useful to stratify different aspects of mitochondrial metabolism as opposed to whole cell metabolism, which opens possibilities to investigate the pathophysiology of VLCAD further.

Higher concentrations of reactive oxygen species (ROS) in mitochondria are found in several disorders of the ETC, although not yet evaluated in FAODs.[14] It is known that an increased concentration of superoxide can lead to molecular and DNA damage and cellular dysfunction.[44,45] Seminotti et al.[14] have shown that by blocking ROS in models of VLCAD, cellular bioenergetics improve significantly. Hence, increased ROS from the impaired fatty acid oxidation in cells from patients with MCAD or VLCAD could explain our finding of overall reduction in capacity to oxidize nutrients.

From the basal state when 5 mM glucose was present, only MCAD cells showed a significant increase in OCR compared to the VLCAD group when exposed to C16:0. This is consistent with previous knowledge from T-cells, which mainly utilize glucose and glutamine as nutrient sources in the resting state,[46] where glucose is particularly necessary for cell survival.[47] However, the differences between OCR of PBMCs from patients with CUD and β-oxidation deficiencies were increased when the cells were exposed to an abundance of fatty acids. This indicates that in resting PBMCs, fatty acid oxidation does occur.

Unexpectedly, ECAR from the MCAD cells was increased compared to cells from VLCAD or CUD. ECAR is generally considered to indicate glycolysis.[48] When activated, lymphocytes upregulate aerobic glycolysis in order to meet their increased energy demands.[46] Our results cannot be explained by different activation, oxygen supply, nutrients, or exposure to stressors. In the MCAD cells, an inability to oxidize medium chain—but not long chain—fatty acids, seemed to shift cellular energy utilization from oxidative phosphorylation toward glycolysis in PBMCs. This finding warrants further studies.

With globally extended NBS, milder forms of FAODs are found, which raises problems regarding choice of treatment strategies. In the clinical situation, it is often difficult to assess the severity of the disorder and the need for treatment, and a way to rapidly and reliably analyze cellular metabolism and energy regulation could be of great importance. In this study, we show that it is possible to estimate cellular energy metabolism in PBMCs from patients with MCAD and VLCAD within 6-hours of venous puncture in a clinical setting. Indeed, PBMCs from patients with β-oxidation defects (i.e., MCAD or VLCAD), showed lower OCR than PBMCs from patients with CUD in every individual tested. Even though there was no clear distinction between clinically estimated level of severity and OCR outcome, the method shows translational potential for mitochondrial disease and may obviate the necessity to obtain and culture fibroblasts or myocytes in order to study the mitochondrial energy metabolism. This provides an opportunity to investigate cellular energy metabolism directly from a routine blood sample.

Measurements of mitochondrial function in PBMCs are a weighted average of the cellular metabolism from a heterogeneous population of cells.[49] Hence, it cannot be considered an optimal sensor of metabolic function. However, PBMCs are easily harvested in the clinic and the availability of the cells must be taken into consideration when discussing their translational potential.

This study has a few limitations. First, the low number of patients makes it difficult to find correlations. The disorder with the most limited number of patients are the patients with VLCAD and we included almost all patients with VLCAD who are followed by our department. Second, the analysis of the PBMCs had to be made within 6 h of venous puncture as the cells needed to be fresh, which somewhat reduce clinical accessibility. Third, the enzymatic activity was measured in lymphocytes, as is clinical practice in Sweden, and therefore this study does not compare OCR with enzymatic activity in patients' fibroblasts. However, fibroblasts are used as controls for the enzymatic measurements.

CONCLUSIONS

Mutations in the VLCAD or the MCAD enzymes affect mitochondrial respiration, as recorded from isolated PBMCs from the patients. Using PBMCs from patients with β-oxidation deficiency show possible translational potential in studies and clinical assessment of VLCAD and MCAD deficiency. However, further studies are warranted, such as a stable isotope substrate oxidation study.

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

R.S., M.H., D.O., A.N., and P.B. wrote the manuscript. R.S., J.C., M.H., A.N., and P.B. designed the research. R.S., J.C., and H.M. performed the research. R.S., M.H., A.N., P.B., J.C., H.M., A.C., C.H., and D.O. analyzed the data. P.B. contributed new reagents/analytical tools.

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