Disrupted fat distribution and composition due to medium-chain triglycerides in mice with a β-oxidation defect1–4

Sara Tucci, Ulrich Flögel, Marga Sturm, Elena Borsch, and Ute Spiekerkoetter

ABSTRACT

Background: Because of the enhanced recognition of inherited long-chain fatty acid oxidation disorders by worldwide newborn screening programs, an increasing number of asymptomatic patients receive medium-chain triglyceride (MCT) supplements to prevent the development of cardiomyopathy and myopathy.

Objective: MCT supplementation has been recognized as a safe dietary intervention, but long-term observations into later adulthood are still not available. We investigated the consequences of a prolonged MCT diet on abdominal fat distribution and composition on liver fat.

Design: Mice with very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCADD) were supplemented for 1 y with a diet in which MCTs replaced long-chain triglycerides without increasing the total fat content. The dietary effects on abdominal fat accumulation and composition were analyzed by in vivo 1H- and 13C-magnetic resonance spectroscopy (9.4 Tesla).

Results: After 1 y of MCT supplementation, VLCADD mice accumulated massive visceral fat and had a dramatic increase in the concentration of serum free fatty acids. Furthermore, we observed a profound shift in body triglyceride composition, ie, concentrations of physiologically important polyunsaturated fatty acids dramatically decreased. 1H-Magnetic resonance spectroscopy analysis and histologic evaluation of the liver also showed pronounced fat accumulation and marked oxidative stress.

Conclusion: Although the MCT-supplemented diet has been reported to prevent the development of cardiomyopathy and skeletal myopathy in fatty acid oxidation disorders, our data show that long-term MCT supplementation results in a severe clinical phenotype similar to that of nonalcoholic steatohepatitis and the metabolic syndrome. Am J Clin Nutr 2011;94:439–49.

INTRODUCTION

The introduction of newborn screening programs for fatty acid oxidation disorders (FAOD) in many countries worldwide led to the identification of a constantly increasing number of FAOD patients who remain asymptomatic until severe catastrophic situations occur (1). Typical symptoms develop during periods of prolonged fasting or because of infectious illnesses and comprise hypoketotic hypoglycemia, hepatic encephalopathy, cardiomyopathy, and skeletal myopathy (2). The most common inherited disorder of the mitochondrial β-oxidation of long-chain fatty acids (LCFAs) is considered to be very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCADD), which has a regional incidence of 1:30,000 (3–5). The clinical phenotype is very heterogeneous and involves organs and tissues that mostly rely on fatty acid β-oxidation, such as skeletal muscle and heart (4, 6, 7), but the liver may also be affected.

Therapeutic approaches to prevent metabolic derangement during situations of increased energy demand include avoidance of fasting and a fat-restricted and fat-modified diet, in which long-chain triglycerides (LCTs) are fully or in part replaced by medium-chain triglycerides (MCTs) (3, 8, 9). MCT preparations are in use for many clinical conditions and are also used for intravenous lipid solutions. The ingredients consist mainly of saturated fatty acids (SFAs; C-8 and C-10). In fact, medium-chain fatty acids (MCFAs) are able to bypass the first step of β-oxidation catalyzed by VLCAD and are supposed to supply tissues and organs with the required energy. Several reports highlight the clinical efficacy of MCTs in the prevention and treatment of cardiomyopathy and skeletal muscle symptoms (10, 11). Nevertheless, MCT supplementation as a mainstay of treatment in LCFA oxidation defects is widely debated, especially with respect to asymptomatic patients identified by newborn screening. Although an MCT diet has been considered a safe dietary intervention and largely applied in LCFA oxidation defects, recent studies in a mouse model of VLCADD showed detrimental effects on lipid homeostasis and clearance during short-term MCT use (12, 13).

Because FAOD patients routinely undergo MCT therapy in the clinical setting over a prolonged period of time, in the current study we investigated the consequences of long-term MCT supplementation in mice with VLCADD (VLCAD2/2). These mutants have been shown to be suitable as a model for VLCADD, because symptoms and phenotypes arise by triggers very similar to those for humans, such as fasting, cold exposure, and physical exercise (8, 9, 14–16). Here, in vivo studies based on 1H- and 13C-magnetic resonance (MR) techniques were applied to analyze noninvasively the effects of MCTs on abdominal fat distribution

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2 ST and UF contributed equally to this work.

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and composition and on liver fat. These data were subsequently correlated with clinical standard liver and serum variables and with liver histology.

MATERIALS AND METHODS

Animals

VLCAD<sup>−/−</sup> mice were kindly provided by AW Strauss (currently Cincinnati Children’s Hospital, Cincinnati, OH) and have been generated as described in detail previously (17). Experiments were performed on sixth- to seventh-generation intercrosses of C57BL6+/129sv VLCAD genotypes. Littermates served as controls, and the mice were genotyped as described previously (17). Groups consisting of 5 to 7 mice (1-yr-old) were investigated under well-fed, nonfasting conditions. All animal studies were performed with the approval of the Heinrich-Heine-University Institutional Animal Care and Use Committee and in accordance with the Committees’ (LANUV) guidelines.

Diet composition and supplementation

After weaning, at ≈5–7 wk of age, mice of each genotype were divided in 2 groups and were fed with different diets for 1 y. The first group received a normal purified mouse diet containing 5% crude fat in the form of LCTs, corresponding to 12% of metabolizable energy as calculated with Atwater factors (ssniff EF R/M Control, ssniff; Spezialdiäten GmbH, Soest, Germany). The second group was fed with a diet corresponding as well to 12% of total metabolizable energy but in which 4.4% from a total of 5% fat was MCT (CeresMCT-oil, basis GmbH, Oberpfaffenhofen, Germany), whereas the remaining 0.6% was derived from soy bean oil to provide the required essential LCFAs. Both diets based on purified feed ingredients contained the same nutrient concentration as follows: 94.8% dry matter, 17.8% crude protein (N × 6.25), 5% crude fat, 5% crude fiber, 5.3% crude ash, 61.9% nitrogen free extract, 36.8% starch, 12% of metabolizable energy, and 19% of metabolizable energy, respectively. All mice groups received water ad libitum.

Magnetic resonance imaging and spectroscopy analysis

General

Data were recorded on a Bruker DRX 9.4 Tesla Wide Bore (89 mm) nuclear magnetic resonance spectrometer operating at frequencies of 400.13 MHz for <sup>1</sup>H and 100.62 MHz for <sup>13</sup>C measurements. Experiments were carried out by using a Bruker microimaging unit (Micro 2.5) equipped with an actively shielded 40-mm gradient set (capable of 1 T/m maximum gradient strength and 150 µs rise time at 100% gradient switching) and Paravision 4 as operating software. <sup>1</sup>H-MR images and spectra were taken from a 30-mm saw resonator, and proton-decoupled <sup>13</sup>C-MR spectra were acquired with a 12 × 8 mm transmit/receive <sup>13</sup>C surface coil (Bruker) inserted into the resonator and fixed over the mice’s abdomen.

The mice were anesthetized with 1.5% isoflurane in a water-saturated gas mixture of 20% oxygen in nitrogen applied at a rate of 75 mL/min by manually restraining the animal and placing its head in an in-house-built nose cone. Respiration was monitored with a pneumatic pillow positioned at the animal’s back. Vital function was acquired by using an M1025 system (SA Instruments, Stony Brook, NY) to synchronize data acquisition with respiratory motion. Throughout the experiments mice were breathing spontaneously at a rate of ≈100 min<sup>−1</sup> and were kept at 37°C. Animals were placed within the resonator so that in z-direction (30 mm) the field of view (FOV) covered the abdomen from just below the diaphragm down to the pelvis. The entire protocol—including animal preparation, fat MRI and <sup>1</sup>H, and <sup>13</sup>C-MRS—lasted ≈1 h and was well tolerated by all mice, which recovered from anesthesia within 1–2 min after removal of the nose cone.

Abdominal fat content and distribution by <sup>1</sup>H-MRI

Abdominal fat content was determined by acquisition of images with a 2D <sup>1</sup>H multislice turbo spin echo sequences with and without fat suppression. Data were taken from a FOV of 30 × 30 mm<sup>2</sup> with a matrix of 256 × 192, which resulted in a spatial resolution of 117 × 117 µm<sup>2</sup> after zero filling (RARE factor, 8; TE, 10.8 ms; TR, 3.5 s; slices, 30; slice thickness, 1 mm; averages, 2; acquisition time, ≈3 min). With the use of the same receiver gain, 2 image sets were recorded with and without chemical shift selective fat suppression. To produce essentially fat-only images, fat-suppressed data were subtracted in absolute intensity mode from nonsuppressed data sets by using an in-house-developed software module based on the LabVIEW package (National Instruments, Austin, TX). For further analysis, all data sets (suppressed, nonsuppressed, and fat only) were imported into the 3D visualization software Amira (Mercury Computer Systems, Mérignac, France). With reference to the corresponding fat-suppressed anatomic images, signals in fat-only images were associated with visceral or subcutaneous (subdivided into deep and superficial) areas by using the Segmentation Editor of Amira. For quantification of the fat content, the integral was calculated over the segmented areas and was related to the total volume analyzed as determined from the anatomic reference images.

Liver fat by <sup>1</sup>H-MRS

For the analysis of liver fat, localized respiratory-triggered <sup>1</sup>H-MR spectra were acquired from a 3 × 3 × 3 mm<sup>3</sup> voxel placed in the middle of the right liver lobe. After manual shimming, the spectra were recorded by using a PRESS sequence with outer volume suppression (TE, 20 ms; TR, 600 ms; spectral width, 6 kHz; data size, 2 k; average, 16; acquisition time, ≈10 s). Exponential weighting resulting in a 10-Hz line broadening was applied before Fourier transformation. Chemical shifts were referenced to the water signal at 4.8 ppm. After the manual phase and baseline correction, signals arising from water and lipid protons were integrated for quantification of the liver fat content. The results are given as a percentage of the total <sup>1</sup>H signal.

Abdominal fat composition by <sup>13</sup>C-MRS

After global shimming of the whole FOV, nonvolume selective proton-decoupled <sup>13</sup>C-MR spectra were recorded over the entire abdominal region for determination of fat composition (rectangular pulse, ≈60° at coil center; TR, 2 s; spectral width, 2 kHz; data size, 4 k; composite pulse decoupling with WALTZ-16;
average, 560; acquisition time, ~18 min). Exponential weighting resulting in a 10-Hz line broadening was applied before Fourier transformation, and chemical shifts were referenced to the methylene resonance of fatty acids at 29 ppm. After the manual phase and baseline correction, individual contributions of the signals from carboxylic, olephinic, methylene, and methyl moieties were quantified by integration. Signal assignments and calculation of the amount of SFAs, MUFA, and polyunsaturated fatty acids (PUFA) and the average chain length was carried out according to data in the literature (18, 19).

Twenty-four hours after nuclear magnetic resonance spectroscopy, the mice were starved for 60 min and then killed by carbon dioxide asphyxiation. Blood samples were collected by heart puncture, and the serum was obtained by centrifugation at 16,000 g for 10 min and was stored at −80°C for further analysis. The liver was rapidly removed and either immediately frozen in liquid nitrogen or transferred in 10% formaldehyde for histology.

Histologic evaluation

Liver tissue was excised from the eviscerated animals and fixed in 10% formin. For light microscopy examination, the tissues were embedded in paraffin and sectioned at 5 μm. Liver slices were stained with hematoxylin and eosin (H&E) for assessment of steatosis, inflammation, and necrosis or with Sirius red for assessment of fibrosis. To determine lipid content, 10-μm thick cryostat sections were collected on Superfrost slides and stained with Sudan III. Steatosis was rated by a blinded investigator according to the percentage of hepatocytes containing macrovesicular fat: 0 is none, 1 is up to 33%, 2 is 33-66%, and 3 is >66% (20). The degree of inflammation was graded to none, mild, moderate, and severe. The histopathologic scoring of fibrosis stage was as follows (20):

1) Stage 1: zone 3 perisinusoidal/pericellular fibrosis, focally or extensively present
2) Stage 2: zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis
3) Stage 3: zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis
4) Stage 4: cirrhosis

Liver homogenates, enzyme activity, and lipid content

Liver was homogenized in ice-cold phosphate-buffered saline (pH 7.3) and centrifuged at 4°C and 16,000 × g for 15 min to pelletize any cell debris. The clear supernatant fluid was immediately used for the enzyme assays or stored at −80°C. Protein concentrations in tissue homogenates were measured by using the bovine serum albumin method as described previously (21).

Reduced glutathione (GSH) was measured in liver homogenates by using an enzymatic kit (Glutathione Assay Kit; Bio Trend, Cologne, Germany). Glutathione peroxidase (GPX) activity was determined by calculating the oxidation rate of NADPH to NADP* spectrophotometrically at 340 nm for 4 min as previously described (22, 23). The concentration of thiorbitaric acid–reactive substances (TBARS) resulting from the decomposition of lipid peroxide products was determined fluorimetrically as previously described (24). Triglyceride concentrations were measured in liver as duplicates by using enzymatic kits (EnzyChrom Triglyceride Assay Kit; Bio Trend, Cologne, Germany) following the manufacturer’s instructions.

Analysis of serum lipids and transaminases

Free fatty acid (FFA) and lipoprotein concentrations were measured as duplicates in serum samples as described previously (13). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured at 37°C accordingly to the International Federation of Clinical Chemistry and Laboratory Medicine procedures (25, 26).

Real-time polymerase chain reaction analysis

Total liver RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany). Forward and reverse primers for β-actin (BC138614), fatty acid synthase (FASN; NM_007988.3), sterol regulatory element binding transcription factor 1 (SREBP-1c; BC056922.1), and stearoyl-coenzyme A desaturase (SCD1; NM_009127.4), annotated in Table 1, were designed with the FastPCR program (R Kalendar; Institute of Biotechnology, Helsinki). Real-time polymerase chain reaction (PCR) was performed in a single-step procedure with the QuantiTect SYBR Green RT-PCR (Qiagen) on an Applied Biosystems 7500 Sequence Detection System in Micro Amp 96-well optical reaction plates capped with MicroAmp optical caps (Applied Biosystems, Foster City, CA) as previously described (27). The values in all samples were normalized to the expression level of the internal standard.

Statistical analysis

MR data are presented as means ± SDs. All other reported data are presented as means ± SEMs. The n value denotes the number of animals tested. The significance of differences was analyzed by using Student’s t tests for paired and unpaired data. The effect of the 2 variables, diet and genotype, was analyzed by using 2-factor analysis of variance with a Bonferroni post hoc test. (GraphPad Prism 5; GraphPad Software, San Diego CA). Differences were considered significant if P < 0.05.

### TABLE 1

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<th>Reverse 5’ → 3’</th>
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<td>TTGATAGAAGACCGGTAGCG</td>
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<td>FASN</td>
<td>TCTGGATCCCGACCGGGCTACCC</td>
<td>TCCCGGGTGTGCCCTGTCAAAGG</td>
</tr>
<tr>
<td>SCD1</td>
<td>AGATCTCAGGCCTTCACAGCACC</td>
<td>GACGGATGCTCTTCACAGGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>TAGGCACCAGGGTGATGGA</td>
<td>CTCCATGTCTGCCCAGTTGG</td>
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1 SREBP-1c, sterol regulatory element binding protein; FASN, fatty acid synthase; SCD1, stearoyl-coenzyme A desaturase.
RESULTS

Animal weights and dietary intake

To study the long-term effect of the MCT diet, the mice were fed for 1 y with either an LCT diet containing 5% fat or with a diet in which 4.4% fat was replaced with MCT, whereas the remaining 0.6% contained the essential LCT in the form of soybean oil. The daily dietary intake did not differ between the groups, independent of the diet received. Over a period of 3 wk, the WT and VLCAD$^{-/-}$ mice consumed 3.36 $\pm$ 0.13 and 3.38 $\pm$ 0.38 g of the LCT diet, whereas the intake from the MCT diet was 3.14 $\pm$ 0.36 compared with 3.18 $\pm$ 0.26 g, respectively. With the normal LCT diet, no significant differences were observed in mean body weights between the WT and VLCAD$^{-/-}$ groups, although the VLCAD$^{-/-}$ mice had higher weights (32.17 $\pm$ 2.61 g compared with 28.94 $\pm$ 2.25 g). After 1 y of the MCT diet, the VLCAD$^{-/-}$ mice had a slight but significantly higher mean body weight (32.49 $\pm$ 0.75 g) than did the WT mice (28.73 $\pm$ 1.23 g) with the same dietary regimen ($P < 0.05$).

MCT diet alters abdominal fat distribution and composition

To gain more detailed insight into the effect of a prolonged MCT diet on lipid homeostasis, we analyzed the abdominal fat distribution using $^1$H-MR imaging (MRI). The analysis of fat-only images showed that the VLCAD$^{-/-}$ mice fed the MCT diet had a significantly higher overall fat content per measured body volume than did the WT mice fed the same dietary regimen (Figure 1A, left; $P < 0.05$). The classification of abdominal fat distribution by $^1$H-magnetic resonance ($^1$H-MR). Signals in fat-only images were associated with visceral or subcutaneous (subc.) areas, subdivided into deep and superficial, with reference to corresponding fat-suppressed anatomic images. A: Quantitative analysis showed an $\sim$2-fold increase in abdominal fat in mice with very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCAD$^{-/-}$) after long-term supplementation with medium-chain triglycerides (MCT), which was predominantly caused by an increase in visceral fat. Values are expressed as means $\pm$ SDs ($n$ = 5–7). B: Representative $^1$H-MR fat-only images of wild-type (WT) and VLCAD$^{-/-}$ mice after 1 y of long-chain triglyceride (LCT) and MCT supplementation. *Significantly different from WT mice within the same diet group, $P < 0.05$ (2-factor ANOVA and Student’s $t$ test).
in visceral and deep/superficial subcutaneous fat showed that this was predominantly caused by an increase in visceral fat (Figure 1A, right) and only to a minor extent by alterations in subcutaneous fat, as also evident in Figure 1B.

In the same experimental setting, natural abundance $^{13}$C-MR spectra were acquired for the parallel analysis of abdominal fat composition. Characteristic $^{13}$C-MR spectra for the VLCAD$^{-/-}$ mice after 1 y of the LCT and MCT diets are shown in Figure 2A. The most striking difference was the dramatic drop in signal intensity for polyunsaturated carbons $[\Delta_2, (\Delta-1)_2]$. Quantification of the spectra showed a PUFA content of only 13 ± 5% with the MCT diet and 49 ± 6% with the control diet ($P < 0.05$). Concomitantly, we found a massive increase in MUFAs ($P < 0.05$) and a moderate up-regulation of SFA levels (Figure 2B; $P < 0.05$). However, these effects were not specific for VLCAD$^{-/-}$ mice, but were similarly found in WT mice (Figure 2B). Surprisingly, in both groups, MCT supplementation had only a minor effect on the average FA chain length in abdominal fat (Figure 2C) as calculated from the ratio of signal intensities for terminal and nonterminal carbons (18). Although the MCT diet comprised mainly C-8 and C-10 triglycerides, the average FA chain length in triglycerides incorporated into abdominal tissue was still found to be in the range of 16 to 17 carbons—even after an intake of this diet for 1 y. Of note, the average FA length was slightly but significantly shorter in MCT-fed VLCAD$^{-/-}$ mice than in all others mouse groups, as shown in Figure 2C ($P < 0.05$).

**Increased liver fat in VLCAD-deficient mice after MCT therapy**

To determine intrahepatic lipid accumulation, in a last step of the MR session localized in vivo, $^1$H-MRS were acquired from the middle of the right liver lobe. Under control conditions (LCT

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**FIGURE 2.** Analysis of triglyceride composition in abdominal fat. A: In vivo $^{13}$C-magnetic resonance (MR) spectra acquired over the abdomen of mice with very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCAD$^{-/-}$) after long-term supplementation with medium-chain triglycerides (MCT) and long-chain triglycerides (LCT). Arrows indicate the dramatic decrease in signals for polyunsaturated fatty acids (PUFAs) $[\Delta_2, (\Delta-1)_2]$ on MCT therapy. For signal assignments, refer to the structure and nomenclature of the triglycerides given at the top. Relative contribution of saturated fatty acids, monounsaturated fatty acids (MUFAs), and PUFAs to the overall triglyceride content (B) and average fatty acid chain length (C) in wild-type (WT) and VLCAD$^{-/-}$ mice after 1 y of LCT and MCT supplementation. The data were calculated from $^{13}$C-MR spectra after signal integration by using established relations (18, 19) and are expressed as means ± SDs ($n = 5–7$). *Significantly different from WT mice within the same diet group, $P < 0.05$ (2-factor ANOVA and Student’s $t$ test). #Significant differences between mice within the same genotype under different dietary conditions, $P < 0.05$ (2-factor ANOVA and Student’s $t$ test). Carbox., carboxylic.
diet), the WT and VLCAD<sup>−/−</sup> mice did not show substantial differences in intrahepatic lipid concentrations (Figure 3A). However, as can be seen from representative spectra shown in Figure 3B, <sup>1</sup>H-MRS showed pronounced alterations in both liver fat content and composition in VLCAD<sup>−/−</sup> mice fed the MCT diet. The ratio of water to fat signals indicated a massive increase in liver fat, whereas the loss of the signal for the double allylic protons [A − 1], reflected the dramatic decrease in PUFA content, which was also observed at the entire abdominal level (see above). Quantification of the spectra showed almost twice as much liver fat in the VLCAD<sup>−/−</sup> mice fed the MCT diet than in those fed the LCT diet (P < 0.05); however, only a tendency toward an increase in liver fat was observed in the WT mice fed the MCT diet, which was not significant (Figure 3A).

Severe structural and functional impairment in liver of VLCAD<sup>−/−</sup> mice fed the MCT diet

To substantiate the in vivo MR findings, mice livers were processed for histologic evaluation and enzymatic analysis. WT and VLCAD<sup>−/−</sup> mice had higher mean liver weights with the MCT diet than with the LCT diet, but significant differences were detected only in WT mice (Table 2; P < 0.05). In contrast, histologic analysis showed that an MCT diet resulted in cloudy swelling of hepatocytes with or without microvesicular, and rarely macrovesicular vacuolization, in ≈50% of the WT mice (Figure 4, C and D). However, nearly all VLCAD<sup>−/−</sup> mice developed severe and diffuse macrovesicular steatosis (grade 3) in the liver, with accumulation of large lipid droplets that displaced and condensed the nuclei of hepatocytes (Figure 4, G–I). Other features were observed with variable frequency and included mild lobular inflammation consisting of lymphocytes and other mononuclear cells and an occasional small collection of polymorphonuclear leukocytes (Figure 4, K and L). Furthermore, moderate ballooning of hepatocytes, stage 1 pericellular/perisinusoidal fibrosis in particular around cells with large fat vacuoles and single acidophil bodies, were also detected (Figure 4J). A significant increase in the triglyceride content was observed in both groups fed with MCT (P < 0.05), but the VLCAD<sup>−/−</sup> mice had a much more pronounced accumulation of triglycerides than did the WT mice with the same treatment (346 ± 31.8 compared with 253.8 ± 25.7 mmol/mg; P < 0.05; n = 5–7). In addition, we analyzed serum markers of liver function and found the activity of both serum transaminases—AST and ALT—in VLCAD<sup>−/−</sup> mice fed the MCT diet to be significantly enhanced compared with the activity in WT and VLCAD<sup>−/−</sup> mice fed the control diet (Table 2; P < 0.05).

MCT diet increases circulating FFA concentrations

Blood lipid analysis indicated no differences in the lipoprotein content between genotypes or diets. However, pronounced differences were found in the amount of FFAs between the individual groups. Although the FFA content was already substantially higher in the VLCAD<sup>−/−</sup> mice than in the WT mice fed the LCT diet (1.85 ± 0.4 compared with 0.22 ± 0.05 mmol/L), the MCT diet led to an additional increase in FFAs, with concentrations up to 1.20 ± 0.3 and 3.64 ± 0.4 mmol/L in the WT and VLCAD<sup>−/−</sup> mice, respectively (P < 0.05; n = 5–7). Furthermore, total cholesterol concentrations were significantly higher in the VLCAD<sup>−/−</sup> mice than in the WT mice under both dietary regimens (Table 2; P < 0.05). However, the long-term MCT diet also

FIGURE 3. Quantification of intrahepatic lipid content. A: Relative contribution of lipid signals to the total magnetic resonance (MR) signal from non-water-suppressed <sup>1</sup>H-MRS of the liver. Values are expressed as means ± SDs (n = 5–7). *Significantly different from wild-type (WT) mice within the same diet group, P < 0.05 (2-factor ANOVA and Student’s t test). †Significant differences between mice within the same genotype under different dietary conditions, P < 0.05 (2-factor ANOVA and Student’s t test). B: Localized in vivo <sup>1</sup>H-MRS acquired from the middle of the right liver lobe (voxel size: 3 × 3 × 3 mm<sup>3</sup>) showing the massive increase in liver fat and an almost complete disappearance of polysaturated fatty acid signals (A − 1), in mice with very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCAD<sup>−/−</sup>) after prolonged supplementation with medium-chain triglycerides (MCT) as compared with long-chain triglycerides (LCT; control). For signal assignments, refer to the structure and nomenclature of the triglycerides given in Figure 2A (top).
led to a further increase in total serum cholesterol concentrations in both genotypes.

MCT-induced lipid deposition is associated with oxidative stress in the liver

Because intrahepatic lipid accumulation is known to be accompanied by the enhanced formation of reactive oxygen species (ROS), which may further amplify hepatic damage (28), we verified in the next step the presence of established markers of oxidative stress (Figure 5). The MCT diet led to a massive increase in GPX activity in VLCAD<sup>−/−</sup> mice (P < 0.05). In contrast, the WT mice had a less pronounced but also significantly higher GPX activity with the MCT diet than with the control diet (P < 0.05). As shown in Figure 5A, the VLCAD<sup>−/−</sup> mice had a 2-fold higher GPX activity (117.1 ± 9.1 U/mg) than

![Figure 4](https://example.com/fig4.png)

**FIGURE 4.** Histologic evaluation of liver tissue. Representative liver slices from wild-type (WT) mice and mice with very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCAD<sup>−/−</sup>) supplemented with either long-chain triglycerides (LCT) or medium-chain triglycerides (MCT). Liver slices were stained with hematoxylin and eosin for assessment of steatosis and inflammation (A–I, K, L) and with Sirius red for assessment of fibrosis (J). To determine the degree of steatosis, the liver slices were additionally stained with Sudan III (C, D, G, H). In LCT-fed WT and VLCAD<sup>−/−</sup> mice, no abnormalities were detected (A, B, E, F). With the MCT diet, the WT mice had no clinical findings (E) or microvesicular lipid droplets (F). With the same dietary regimen in the liver tissue of VLCAD<sup>−/−</sup> mice, prominent steatosis (G, H) with nuclei degeneration (I), stage 1 pericellular/perisinusoidal fibrosis (J), and inflammatory cell infiltration (K, L) were detected.

**TABLE 2**

<table>
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<th>Liver variables, serum lipids, and serum transaminases in wild-type (WT) and very-long-chain acyl-coenzyme A dehydrogenase deficient (VLCAD&lt;sup&gt;−/−&lt;/sup&gt;) mice fed a diet supplemented with either long-chain triglycerides (LCTs) or medium-chain triglycerides (MCTs)&lt;sup&gt;i&lt;/sup&gt;</th>
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<th>MCTs</th>
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<td>WT</td>
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<td>Liver weight (g)</td>
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<td>Liver TGs (nmol/mg)</td>
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<td>204.7 ± 11.1&lt;sup&gt;•&lt;/sup&gt;</td>
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<sup>i</sup>All values are means ± SEMs; n = 5–7. TGs, triglycerides; FFA, free fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase. <sup>•</sup>Significantly different from WT or VLCAD<sup>−/−</sup> mice under different dietary conditions, P < 0.05 (2-factor ANOVA and Student’s t test). *Significantly different from WT mice under the same dietary conditions, P < 0.05 (2-factor ANOVA and Student’s t test).
did the MCT-fed WT mice (61.8 ± 6.2 U/mg; P < 0.05; n = 5–7). Quantification of GSH as substrate for GPX showed a direct correlation between increased GPX activity and reduced GSH content in mice fed the MCT diet (Figure 5B). In fact, the MCT diet induced a significant decrease in GSH concentrations to a value of 16.3 ± 6.1 nmol/mg in the VLCAD<sup>−/−</sup> mice compared with LCT-fed VLCAD<sup>−/−</sup> mice (24 ± 2.6 nmol/mg; P < 0.05). Furthermore, in both genotype groups, the MCT diet resulted in increased hepatic concentrations of TBARS (171 ± 6.1 nmol/mg in WT mice compared with 215.9 ± 11.7 nmol/mg in VLCAD<sup>−/−</sup> mice; P < 0.05; n = 5–7), although the livers of VLCAD<sup>−/−</sup> mice fed the LCT diet had a TBARS concentration >2-fold that of WT mice (207.5 ± 17.7 compared with 77.6 ± 4.9 nmol/mg; P < 0.05; Figure 5C).

**Effect of MCT diet on genes regulating lipid metabolism**

In a final step, we investigated whether MCT supplementation resulted in an up-regulation of lipogenesis at the mRNA level. We therefore, analyzed the expression of SREBP-1c, which directly activates FASN, which is involved in the biosynthesis and elongation of short- and MCFAs, respectively. In addition, we also verified the expression of SCD1, which is responsible for the biosynthesis of unsaturated fatty acids (29). As shown in Figure 6, RT-PCR showed that the expression of SREBP-1c and FASN, but not of SCD-1, was strongly up-regulated in WT mice fed the MCT diet. In contrast, all of the lipogenic genes analyzed in VLCAD<sup>−/−</sup> mice—SREBP-1c, FASN, and SCD1—were already up-regulated under control conditions. Surprisingly, the long-term MCT diet resulted in a down-regulation of these genes comparable with levels in the WT mice fed the normal LCT diet.

**DISCUSSION**

A diet based on MCTs is a recommended treatment in symptomatic VLCAD deficiency. Whereas an MCT-modified diet is currently considered extremely safe, in the current study we showed that the replacement of LCTs by MCTs resulted in a dramatic accumulation of visceral fat and liver lipids in VLCAD<sup>−/−</sup> mice when applied over 1 y. Furthermore, not only did the abdominal lipid content increase, but the tissue fat composition changed significantly. The contents of SFAs and MUFAs were elevated, whereas the content of physiologically important PUFAs decreased by >60% with MCT therapy as compared with the normal diet. Concomitantly, the long-term MCT diet induced severe liver damage in VLCAD<sup>−/−</sup> mice and substantial signs of oxidative stress and steatohepatitis. These findings are of significant clinical relevance, because both symptomatic and many asymptomatic patients with LCFA oxidation...
defects are supplemented, beginning in the neonatal period, with a long-term MCT diet.

The detrimental effects induced by the altered triglyceride composition of the diet are particularly striking because the total fat content of the MCT preparation was equal to that of the LCT diet. However, the metabolism of MCTs differs in many aspects from that of normal LCTs. In fact, MCTs are rapidly hydrolyzed to FFAs and quickly absorbed by the gastrointestinal tract. In contrast with LCFAs, only a minor amount of MCFAs are incorporated into chylomicrons (30) but exit the enterocyte via the portal circulation as nonesterified FFAs and thus may be easily accessed by cardiac and skeletal muscle. Of note, spillover MCFAs are not stored as MCTs in adipocytes, as shown by 13C-MRS analysis of the average fatty acid chain length in abdominal tissue, which—even after 1 y of the MCT diet—showed only a slight shortening of the carbon chain as compared with the LCT diet. This finding agrees with previous findings of an impaired uptake of MCFAs into adipocytes (31). As a consequence, excess MCFAs are primarily transported to the liver (32), where they can be converted via β-oxidation into C2-fragments and ketone bodies, respectively, or elongated to LCFAs and subsequently esterified to LCTs (13, 33). As evidenced by the 13C-MRS data, the latter pathway clearly dominates under well-fed conditions in both WT and VLCAD−/− mice. Because elongated FAs cannot be used for energy production in VLCAD-deficient mice, this results in a massive lipid accumulation in the mutant as shown by localized 1H-MRS of the liver and, in the long run, constitutes a vicious cycle because of the continuously enhanced enrichment and turnover of MCFAs in the liver.

Elevated lipid concentrations in the liver are known to be associated with elevated lipid peroxidation and oxidative stress (34). In line with this, we found GPX—a major player in detoxification of mitochondrial ROS (35)—to be up-regulated in MCT-treated VLCAD−/− mice. Interestingly, in both MCT-supplemented and -nonsupplemented VLCAD−/− mice, decomposition products of lipid peroxides were increased, which suggests that, independent of the degree of steatosis, VLCAD-deficient livers are exposed to enhanced oxidative stress. Elevated hepatic lipid concentrations in MCT-fed VLCAD−/− mice were accompanied by a pronounced increase in both serum FFAs and visceral fat deposition. These data emphasize the adverse effects of long-term MCT supplementation because high serum FFAs and visceral fat are strongly related with the development of cardiovascular disease, insulin resistance, and the metabolic syndrome (36–38). On the other hand, the content of physiologic important PUFAs was massively reduced in mice fed with MCTs. PUFAs have cardioprotective properties through several mechanisms (39–42), whereas elevated SFA were reported to activate a signal cascade leading to apoptosis (43). PUFAs, but not MUFAs or SFAs, down-regulate the expression of SREBP-1c—a key regulator of lipogenesis (44). Therefore, the initial up-regulation of liver lipogenesis in WT and VLCAD−/− mice fed the MCT diet for 5 wk, as observed previously (13, 33), was not surprising. Of note, these alterations persisted only in WT mice after 1 y of the MCT diet, whereas in VLCAD−/− mice the lipogenic transcription was no longer up-regulated. This was most likely associated with the severe pathologic liver phenotype in the mutant with macrovesicular lobular steatosis accompanied by fibrosis and inflammation as consequence of prolonged MCT therapy.

Despite the finding that all MCT-induced effects were exceptionally pronounced in VLCAD−/− mice, WT mice also showed a trend to a “fatty” phenotype similar to that of VLCAD-deficient mice. After 1 y of the MCT diet, a tendency to enhanced lipid deposition in the liver (in both 1H MRS and histology data) and elevated AST and ALT concentrations in the blood were observed. These effects were accompanied by enhanced oxidative stress (elevated TBARS levels and GPX activity), increased concentrations of circulating FFAs, and up-regulation of genes involved in lipogenesis, although alterations in visceral fat content were not observed. Furthermore, the alterations induced by the MCT diet on body fat composition were as striking as in VLCAD−/− mice. The development of this tendency to a fatty liver into a serious pathological phenotype in VLCAD−/− mice, similar to the clinical characteristics of nonalcoholic steatohepatitis (NASH), might be favored by 1) the presence of enhanced oxidative stress as described above and 2) the significantly shorter chain size (~16 carbons) of stored triglyceride fatty acids, because palmitic acid is considered to be one of the key factors in promoting insulin resistance and the metabolic syndrome (45–48).

From a clinical point of view, supplementation with MCTs has been proven to be effective in preventing the development of cardiomyopathy and skeletal myopathy in VLCADD in that they bypass the metabolic bottlenecks and provide organs relying on fatty acids as a major energy source with the required substrates (12, 13). Furthermore, several short-term studies in both animals and humans suggest that an MCT diet might also be beneficial in the treatment of obesity because of the limited deposition of MCFAs in adipocytes (31, 49). The enhanced energy requirement for MFA elongation indeed may contribute to weight loss in the short term (50); however, in the long term, the permanently enhanced fat supply to the liver obviously increases the risk of the development of steatosis, as was also observed in the current study for healthy WT animals. Thus, it was not surprising that there are reports about the occurrence of fatty liver on a ketogenic MCT diet during untreatable epilepsy (51). Importantly, the development of significant fatty liver was also observed in a patient with an LCFA oxidation defect after the start of continuous MCT supplementation for muscular pain (U Spiekerkoetter, unpublished observations, 2010), which in the long run is associated with the risk of steatosis and with the clinical characteristics of NASH, as shown for our murine model of FAOD.

In summary, this study showed in a murine model of VLCADD that a long-term MCT-based diet (1 y) results in severe deterioration of whole-body lipid homeostasis, with strongly elevated serum FFA concentrations, massive visceral fat infiltration, impaired body fat composition with decreased PUFAs, and the development of steatohepatitis. All of these changes are comparable with those reported for NASH and the metabolic syndrome (52, 53). Although the recommended dietary treatment in asymptomatic newborns with VLCADD is less MCTs, as used in this study (3, 8), patients with a disorder of the mitochondrial trifunctional protein should receive a strictly MCT-modified diet, as used in this study. Similar, but milder, effects may be expected by a lower MCT content and need to be carefully investigated and monitored. As a noninvasive strategy for surveillance of changes in body fat distribution and composition in treated patients, we argue for the use of the MR techniques used in this study which could be readily transferred to clinical scanners. According to the data
presented here, the use and dose of MCT supplementation has to be reevaluated not only for the treatment of VLCADD but also for all other applications.

The authors’ responsibilities were as follows—ST, UF, and US: designed the research; ST: conducted the research and drafted the manuscript; UF: performed the MR analysis and drafted the manuscript; MS: contributed to the data collection and analysis; EB: performed the histological evaluation; and US: had primary responsibility for the final content. All authors read and approved the final manuscript. The authors had no conflicts of interest to disclose.

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