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Sexual dimorphism of lipid metabolism in very long-chain acyl-CoA dehydrogenase deficient (VLCAD$^{-/-}$) mice in response to medium-chain triglycerides (MCT)

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ABSTRACT

Medium-chain triglycerides (MCT) are widely applied in the treatment of long-chain fatty acid oxidation disorders. Previously it was shown that long-term MCT supplementation strongly affects lipid metabolism in mice. We here investigate sex-specific effects in mice with very-long-chain-acyl-CoA dehydrogenase (VLCAD) deficiency in response to a long-term MCT modified diet. We quantified blood lipids, acylcarnitines, glucose, insulin and free fatty acids, as well as tissue triglycerides in the liver and skeletal muscle under a control and an MCT diet over 1 year. In addition, visceral and hepatic fat content and muscular intramyocellular lipids (IMCL) were assessed by *in vivo* ^{1}H magnetic resonance spectroscopy (MRS) techniques. The long-term application of an MCT diet induced a marked alteration of glucose homeostasis. However, only VLCAD$^{-/-}$ female mice developed a severe metabolic syndrome characterized by marked insulin resistance, dyslipidemia, severe hepatic and visceral steatosis, whereas VLCAD$^{-/-}$ males seemed to be protected and only presented with milder insulin resistance. Moreover, the highly saturated MCT diet is associated with a decreased hepatic stearoyl-CoA desaturase 1 (SCD1) activity in females aggravating the harmful effects of a saturated MCT diet. Long-term MCT supplementation deeply affects lipid metabolism in a sexual dimorphic manner resulting in a severe metabolic syndrome only in female mice. These findings are striking since the first signs of insulin resistance already occur in female VLCAD$^{-/-}$ mice during their reproductive period. How these metabolic adaptations are finally regulated needs to be determined. More important, the relevance of these findings for humans under these dietary modifications needs to be investigated.

1. Introduction

Lipid metabolism is differently regulated between the sexes resulting in various phenotypes with regard to body composition, body fat distribution and substrate metabolism. It is known that lipid metabolism and expression of genes coding for fatty acid oxidation (FAO) enzymes are regulated by different means such as dietary fatty acids [1] or the transcription factor peroxisome proliferator-activated receptor α (*PPARα*). In addition, sex hormones do play an important role and determine regulation in a sexually dimorphic manner [2,3]. Therefore, the degree of disturbance in fatty acid metabolism and lipid homeostasis as they occur in inherited fatty acid oxidation disorders (FAOD) may also be strongly gender-specific.

FAOD are a group of diseases comprising defective enzymes of the mitochondrial β-oxidation [4]. Symptoms occur mainly during catabolic situations such as prolonged fasting or illnesses presenting with hypoketotic hypoglycemia, hepatic encephalopathy, cardiomyopathy and skeletal myopathy [5]. Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is considered the most common defect of the mitochondrial oxidation of long-chain fatty acids with an incidence of 1:50,000–1:100,000 [5–7]. VLCADD is characterized by different clinical phenotypes as well as severity and age at onset [8,9]. The recommended therapeutical approach includes the replacement of long-chain triglycerides by medium-chain triaglycerides (MCT), which can be fully oxidized in the mitochondrial β-oxidation. The clinical efficacy of MCT is widely recognized especially with respect to the prevention and treatment of cardiomyopathy and muscular symptoms [10,11]. Although an MCT diet is considered a safe dietary intervention and is applied in different FAOD for longer periods of time, recent reports highlight the adverse effects of an MCT diet in the murine model of VLCADD [12–16]. Long-term supplementation over one year contributed to the development of an unexpected clinical phenotype with an increased body fat content and a disturbance in body fat composition in the mouse model of VLCADD [13,16]. Because of these significant changes due to dietary interventions in the VLCAD mouse, we here assessed possible additional sex-specific effects.

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Biochemical parameters such as blood lipids, glucose, insulin and free fatty acids were measured, while the concentration of triglycerides (TAGs) was assessed in the liver and skeletal muscle of WT and VLCAD$^{-/-}$ mice under a control diet and after MCT supplementation. Acylcarnitine profiles were analyzed in dried blood spots to evaluate the metabolic state of WT and VLCAD$^{-/-}$ mice. Visceral and hepatic fat as well as the muscular IMCL content were assessed by *in vivo* ^{1}H (MR) techniques.

2. Materials and methods

2.1. Animals

Experiments were performed on the fourth- to fifth-generation intercrosses of C57BL6 + 129sv VLCAD genotypes. Littermates served as controls and genotyping of mice was performed as described previously in Exil et al. [17]. Serum parameters were determined under standard conditions and blood was taken 5 h after food intake at the age of six months. Mice at the age of 12 months were sacrificed immediately after MR investigation by CO_2 asphyxiation.

Blood samples were collected by heart puncture. Serum was obtained by centrifugation at 16,000 *g* for 10 min and stored at −80 °C for further analysis. The liver and skeletal muscle were rapidly removed and immediately frozen in liquid nitrogen. The right anterior and medial lobes of the liver with the gall bladder were transferred in 10% formaldehyde for histopathology.

All animal studies were performed with the approval of the University's Institutional Animal Care and Use Committee and in accordance with the Committees' (LANUV) guidelines.

2.2. Diet composition and supplementation

At 5 weeks of age, mice of each genotype were divided in two groups and fed with different diets for one year. The first group received a normal purified mouse diet containing 5% crude fat in the form of LCT, 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. Here, 4.4% from a total of 5% fat was MCT (Ceres®MCT-oil, basis GmbH, Oberpfaffenhofen, Germany) while the remaining 0.6% was derived from soybean oil providing the required essential long-chain fatty acids. The necessary amount of essential long-chain fatty acids was calculated in accordance to the Nutrient Requirements of Laboratory Animals (Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council). 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, 14.8% dextrin and 11% sugar. The detailed fatty acid composition of the diets was previously reported [13]. In both diets the carbohydrate and protein contents corresponded to 69% and 19% of metabolizable energy, respectively. All mouse groups received water *ad libitum*.

2.3. Magnetic resonance imaging (MRI)

2.3.1. General setup

Data were recorded on a Bruker Avance^{III} 9.4 Tesla Wide Bore (89 mm) nuclear magnetic resonance (MR) spectrometer operating at frequencies of 400.13 MHz for ^{1}H as previously described [13,18].

2.3.2. Intra- and extramyocellular lipids (IMCL and EMCL)

The mice were positioned on their left side within the animal handling system and the right hind leg was fixed in a 10-mm saddle coil with the *Tibialis anterior* (TA) muscle aligned along the main magnetic field direction ensuring maximal spectral separation of IMCL and EMCL resonances [19]. Image acquisition and IMCL quantification have been performed as previously described [20].

2.4. Histological evaluation

Liver tissue was excised from the eviscerated animals and fixed in 10% formalin. For light microscopy examination, the tissues were embedded in paraffin and sectioned at 5 μm. Liver slices of all analyzed mice were evaluated with a magnification of ×33 and stained with hematoxylin and eosin (H&E) for assessment of steatosis, inflammation, and necrosis or 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, degree of inflammation and stage of fibrosis were assessed as previously described [13,21].

2.5. Liver homogenate, triglyceride (TAG) and lipid peroxide content

Tissues were homogenized in CelLytic MT Buffer (Sigma-Aldrich, Steinheim, Germany) in the presence of 1 mg·mL^{-1} protease inhibitors and centrifuged at 4 °C and 16,000 *g* for 10 min to pelletize any cell debris. The clear supernatant was immediately used for the enzyme assays or stored at −80 °C.

The concentration of thiobarbituric acid reactive substance (TBARS) resulting from decomposition of lipid peroxide products was determined fluorimetrically in serum and liver tissue as previously described [14]. TAG concentrations were measured in the liver as duplicates by using enzymatic kits (EnzyChrom triacylglyceride Assay Kit, BioTrend, Cologne, Germany) following the manufacturer's instructions. Briefly, TAGs were extracted from the liver and the concentration was measured enzymatically. Extracted TAGs were hydrolyzed to free fatty acids and glycerol. This reacted with a dye to generate a colored compound visible at 570 nm (spectrophotometry) or at Ex/Em = 535/587 nm (fluorescence).

2.6. Analysis of serum variables and transaminases

Free fatty acid (FFA), TAG and lipoprotein concentrations were measured as duplicates in serum samples as described previously [15]. Glucose and ketone bodies were determined with a Precision Xceed blood sugar meter (Abbott, Wiesbaden, Germany).

Insulin was measured in duplicate by using the Ultrasensitive Mouse Insulin ELISA Kit (Mercodia AB, Uppsala Sweden). Oxidized low density lipoproteins (ox-LDL) were quantified in duplicates by using the Enzyme-linked Immunosorbent Assay Kit for ox-LDL (Hölzel Diagnostika, Cologne, Germany). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined at 37 °C accordingly to the ICFF procedures [22,23]. Insulin resistance was calculated by the homeostasis monitoring assessment (HOMA) formula [24]. The homeostasis model assessment (HOMA) of insulin resistance index, as described by Matthews et al. [24], is the most easily obtained measurement of insulin resistance which can be used as a reliable surrogate measure of *in vivo* insulin sensitivity since this method correctly differentiated between insulin sensitivity and insulin resistance [25]. HOMA index was calculated with glucose and insulin concentrations obtained after 5 h of fasting using the following formula: fasting blood glucose (md/dL) × fasting insulin (μU/mL) / 22.5.

2.7. Analysis of acylcarnitines

Analysis of acylcarnitines was performed as described previously [26–28]. Briefly, acylcarnitines were extracted from dried blood spots and tissues with acetonitrile/water (80/20% v/v) in the presence of [$^{2}H_3$] free carnitine, [$^{2}H_3$] octanoyl-carnitine and [$^{2}H_3$] palmitoyl-carnitine as internal standards. The extracted supernatant was dried and the butylated acylcarnitines were analyzed by electron spray

ionization tandem mass spectrometry (ESI-MS/MS). All even-chain C4–C18 acylcarnitines (saturated and unsaturated) were measured.

2.8. Statistical analysis

MRI data are reported as mean values ± standard deviation (SD). All other reported data are presented as means ± standard error of the mean (SEM). *n* denotes the number of animals tested. Analysis for the significance of differences was performed using Student's t-tests for paired and unpaired data. To test the effects of the variables, diet, genotype and sex two-way analysis of variance (ANOVA) with Bonferroni post-test was performed (GraphPad Prism 5.0, GraphPad Software, San Diego, California, USA). Differences were considered significant if $p < 0.05$.

3. Results

3.1. Clinical phenotype

As shown in Table 1, six month-old VLCAD$^{-/-}$ mice of both sexes showed significantly higher mean body weights compared to the WT group under normal LCT mouse diet. Interestingly, mice upon an MCT diet showed significantly lower body weights compared to mice under the LCT diet ($p < 0.05$), however, a significantly increased liver/body weight ratio was observed, which was especially evident in VLCAD$^{-/-}$ female mice (Table 1) indicative of a significant intrahepatic lipid accumulation.

3.2. MCT diet severely affects glucose homeostasis

Since one year old VLCAD$^{-/-}$ mice under an MCT diet displayed a phenotype similar to the metabolic syndrome [13], we tested whether mutants develop sex specific insulin resistance already at the age of six months and analyzed blood glucose and insulin concentration after 5 h of fasting.

As shown in Table 1, WT mice presented with sex specific differences in glucose an insulin concentration with respect to the genotype, in that the glucose concentration was significantly higher in VLCAD$^{-/-}$ males as compared to WT under the dietary regimen. Moreover, female VLCAD$^{-/-}$ mice showed significantly lower concentrations of glucose and insulin as compared to males. Furthermore, we could observe an overall high impact of the MCT diet on these parameters. Indeed, all mice showed significantly higher glucose and insulin concentrations in all groups independent of sex and genotype. This effect was very evident in females of both genotypes but it was especially striking in VLCAD$^{-/-}$ females resulting in a markedly increased HOMA index. In that, WT females supplemented with MCT displayed a two-fold higher HOMA index as compared to WT females upon LCT diet (19.1 ± 3.2 *vs* 9 ± 1.8). This parameter was even higher in VLCAD$^{-/-}$ females (22.2 ± 3.1 in VLCAD$^{-/-}$ females upon MCT *vs* 6 ± 1.4 in VLCAD$^{-/-}$ females upon LCT) which was strongly suggestive of insulin resistance. In contrast, males were not strongly affected as only VLCAD$^{-/-}$ males upon MCT showed a significantly higher HOMA index as compared to mice of the same genotype upon LCT diet (Table 1).

3.3. Alteration of blood lipids due to MCT supplementation

Blood lipid analysis revealed no sex specific but genotype dependent differences upon LCT diet (Table 1). In accordance with previous data [13,15], diet serum triacylglycerides (TAGs) and lipoprotein content was significantly higher in mutants as compared to WT littermates. Sex-specific differences were only observed with respect to the highly atherogenic ox-LDL. Already under this normal mouse diet VLCAD$^{-/-}$ female mice were characterized by significantly higher oxidized-LDL (ox-LDL) lipoprotein levels (825.8 ± 42.3 *vs* 630.7 ± 27.8 ng/mL) which have been recently associated with the development of metabolic syndrome [29].

As shown in Table 1, MCT supplementation strongly affected lipoprotein content in VLCAD$^{-/-}$ females with special regard to low-density lipoproteins. Indeed, the concentration of VLDL/LDL was significantly higher as compared to VLCAD$^{-/-}$ males under the same dietary regimen as well as the content of the atherogenic ox-LDL (1003.2 ± 25.4 *vs* 821.3 ± 35.7 ng/mL).

3.4. Ectopic TAG increase in female VLCAD$^{-/-}$ mice under MCT diet

An ectopic TAG accumulation has been associated with insulin resistance [30–32]. Therefore we tested whether a long-term MCT supplementation induces a sex-specific accumulation of ectopic lipids.

Table 1
Clinical parameters in VLCAD$^{-/-}$ mice either under control or MCT diet at the age of six months. The values are mean ± SEM. ($n = 6$–8). Values denoted by *, # and § were considered significant if $p < 0.05$ (Two way ANOVA and Student's t-test). * indicates significant differences between male and female mice of the same genotype under the same dietary regimen. # indicates significant differences between mice of the same sex but different genotypes under the same dietary regimen. § indicates significant differences between mice of the same sex and phenotype under different dietary regimens.

	LCT				MCT			
	WT		VLCAD$^{-/-}$		WT		VLCAD$^{-/-}$	
	Male	Female	Male	Female	Male	Female	Male	Female
Clinical phenotype								
Body weight [g]	27 ± 0.8	21.4 ± 1	30 ± 0.5#	23.2 ± 0.4#	25.6 ± 0.6	21.7 ± 0.4	26.5 ± 1§	20.8 ± 0.6§
Ratio liver/body weight [g/mg]	4.8 ± 0.07	4.3 ± 0.03*	5.1 ± 0.1	5.0 ± 0.15	4.7 ± 0.18	4.6 ± 0.21	4.5 ± 0.09§	5.3 ± 0.22*
Serum variables								
Glucose [mg/dL]	222 ± 11.4	240 ± 13.23	264 ± 17.5#	218.5 ± 8.85*	269 ± 18.61§	296.5 ± 35.42*§	282.5 ± 15.99	290 ± 29.13§
Insulin [pmol/L]	115.4 ± 29.3	105.9 ± 21.2	83.9 ± 17.4	76.5 ± 19.1*	123.7 ± 17.9	173.5 ± 25.8	179.1 ± 15.5§	207 ± 20.8§
HOMA index	9.1 ± 2.1	9 ± 1.8	9.1 ± 1.7	6 ± 1.4	11.8 ± 2	19.1 ± 3.2§	15.4 ± 1.6§	22.2 ± 3.1§
AST [U/L]	86.2 ± 5.7	83.2 ± 7.9	81.2 ± 9.3	87.6 ± 10.2	110.6 ± 9.7	81.2 ± 6.0	140.5 ± 12.4§	129.8 ± 19.33
ALT [U/L]	29.7 ± 3.6	20.0 ± 1.1*	19.6 ± 1.0	15.2 ± 1.2	22.8 ± 3.4§	15.20 ± 1.2*§	32.2 ± 1.4§	26.2 ± 4.3§
Serum lipids								
FFA [μM]	319.4 ± 51.6	320.7 ± 50.6	369 ± 67.7	408.6 ± 51.2	436.2 ± 56.6§	337.1 ± 42.1*	655.2 ± 87.7§	709.2 ± 29.6#§
TAG [mg/dL]	57.5 ± 10.6	40.6 ± 7	92 ± 6.2#	70.3 ± 4.4*#	70.1 ± 9.7	66.6 ± 6.8§	87.3 ± 6.3	87.6 ± 7.2§
Cholesterol total [mg/dL]	66.8 ± 11.5	72.8 ± 7.8	135.5 ± 13.9#	110 ± 2.4#	126 ± 9.8§	111.7 ± 10§	116.3 ± 1.7	133.2 ± 4.4*#§
HDL [mg/dL]	55.5 ± 8.5	44.7 ± 8.3	75.8 ± 4	66.4 ± 3*	76.1 ± 6.2	63.6 ± 3.2	66.9 ± 2.8	64.8 ± 1.7
VLDL/LDL [mg/dL]	18.4 ± 2.7	11.4 ± 0.7*	30 ± 3#	32 ± 0.9#	29.6 ± 0.8§	34.7 ± 0.7*§	33.5 ± 0.9#	47.7 ± 2.3*#§
ox-LDL [ng/mL]	611.8 ± 25.7	630.7 ± 25.7	605.2 ± 27.8	825.8 ± 42.3*#	699.3 ± 67.6	898 ± 36.3*§	821.3 ± 35.7§	1003.2 ± 25.4*§

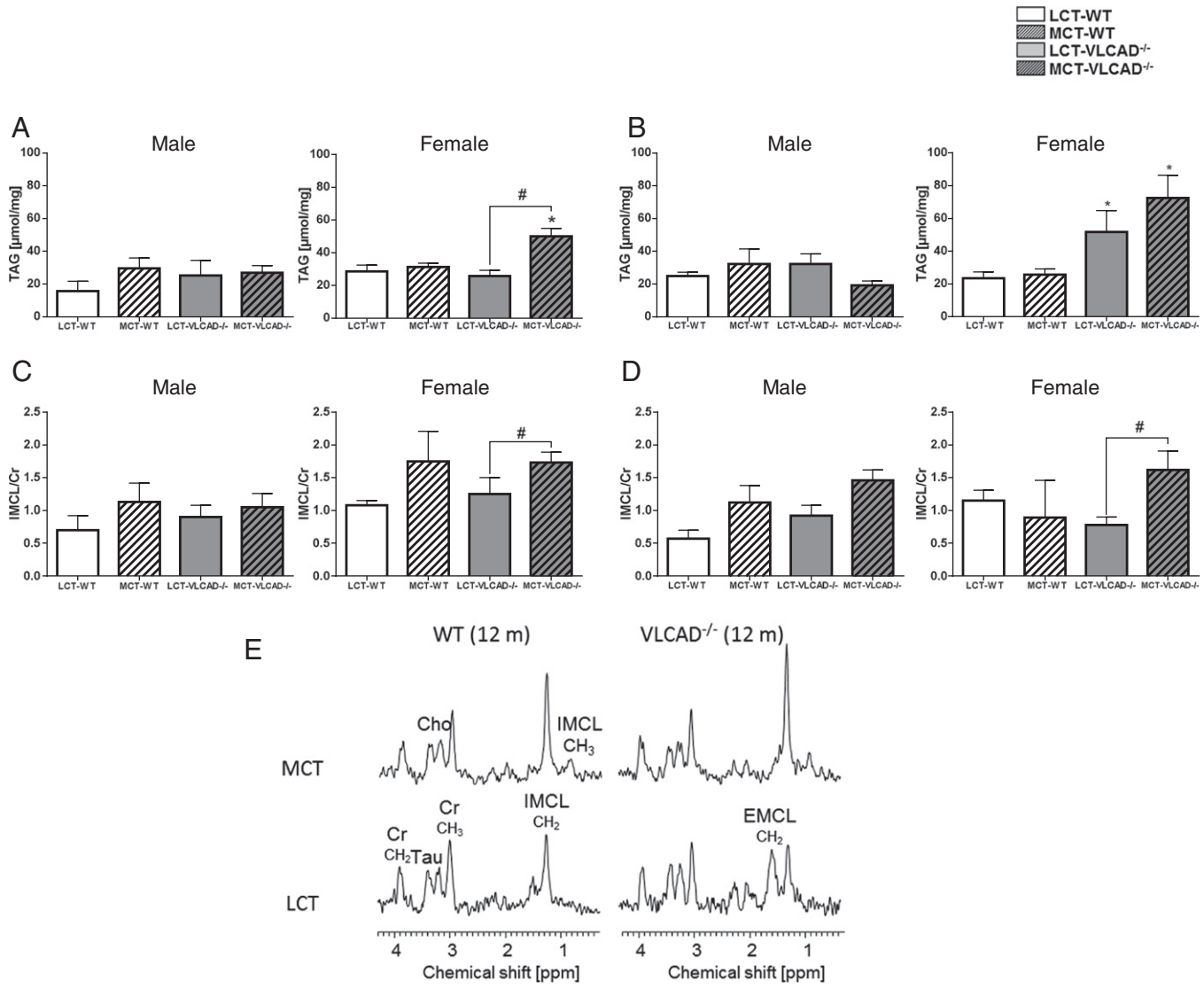

Fig. 1. Time-dependent triglyceride (TAG) and IMCL accumulation in the skeletal muscle of female VLCAD$^{-/-}$ mice under MCT diet. A) and C) represent mice at the age of 6 months. B) and D) represent mice at the age of 12 months. E) shows representative ^{1}H MR spectra acquired from the *Tibialis anterior* muscle of WT and VLCAD$^{-/-}$ mice under MCT (top) and LCT (bottom) diet (Cho, choline; Cr, creatine; Tau, taurine). TAG concentration is expressed in µmol/mg tissue. White bars and black bars represent WT mice and VLCAD$^{-/-}$ mice, respectively. The values are mean ± SEM ($n = 6$–8). LCT, long-chain triglycerides. MCT, medium-chain triglycerides. Values denoted by * and # were considered significant if $p < 0.05$ (Two way ANOVA and Student's t-test). * indicates significant differences between genotypes under the same dietary regimen. # indicates significant differences between mice of the same genotype under different dietary regimens.

Under control diet we observed neither genotype nor sex specific differences in the content of muscle TAG at the age of 6 months, as shown in Fig. 1A. Six month-old female VLCAD$^{-/-}$ mice upon MCT, however showed a significantly higher TAG content as compared to VLCAD$^{-/-}$ females under control diet (50 ± 10.5 *vs* 25.7 ± 8.5 µmol/mg). These data correlated with the accumulation of intramyocellular (IMCL) lipids in the *Tibialis anterior* (TA) muscle. Characteristic ^{1}H MR spectra acquired *in vivo* for WT and VLCAD$^{-/-}$ mice at the age of 12 months fed with either an LCT or an MCT diet are shown in Fig. 1E. The spectra showed a very striking increase in signal intensity for IMCL protons at 1.3 ppm, in both genotypes fed with MCT (top) as compared to mice under normal diet (bottom). As shown in Fig. 1C, quantification of the spectra in 6 month-old females revealed a nearly 40% increase in IMCL under the MCT diet in both genotypes as compared to the LCT diet indicating that the replacement of LCT by MCT may contribute to the development of insulin resistance long-term [33].

Upon LCT VLCAD$^{-/-}$ females at the age of 12 months displayed a significant increase in muscle TAG with a two-fold higher content as compared to WT females at the same age (Fig. 1B). In accordance to the data obtained in 6 month-old VLCAD$^{-/-}$ females, a continuous supplementation with an MCT diet over 12 months further increased muscle TAG content up to 72.5 ± 14 µmol/mg. However, this increase was not associated with a higher IMCL content since quantification of spectra showed no further accumulation in VLCAD$^{-/-}$ females. Of note, IMCL content in VLCAD$^{-/-}$ females was two-fold higher as compared to VLCAD$^{-/-}$ females under LCT diet at 12 months (Fig. 1D), whereas MCT supplementation did not result in sex-specific differences with respect to the IMCL content after this time period.

3.5. Altered fat distribution and fatty acid composition accompanied by hepatic steatosis in VLCAD$^{-/-}$ females under MCT diet

Because of the increased muscular IMCL content suggestive of insulin resistance, we analyzed the sex-specific effects of a prolonged MCT diet on abdominal fat distribution and composition using *in vivo* ^{1}H MR imaging (MRI). Analysis of fat-only images in mice at the age of

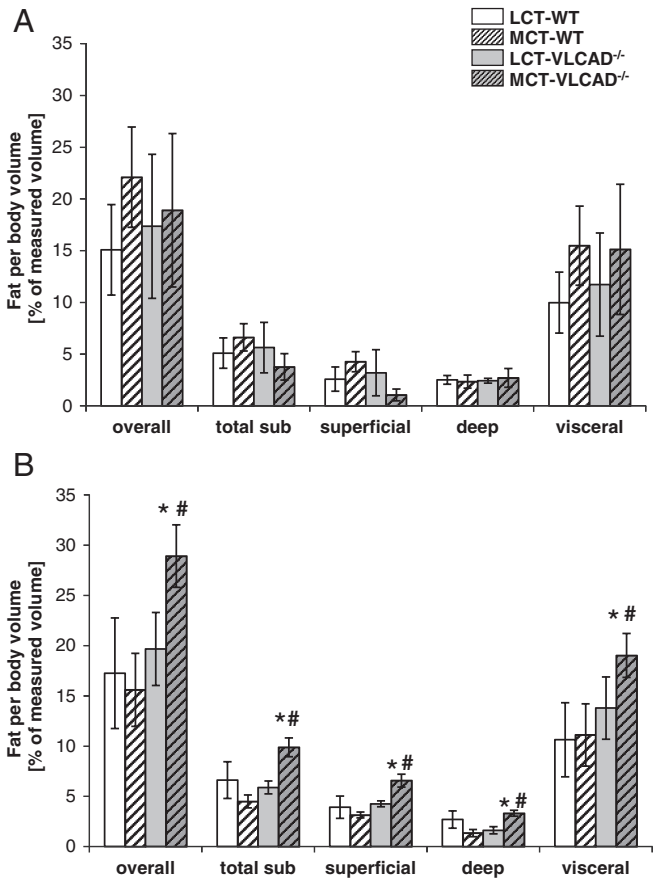

Fig. 2. Quantification of abdominal fat distribution by ^{1}H MRI. A) represents male and B) represents female mice under either LCT or MCT diet. LCT, long-chain triglycerides. MCT, medium-chain triglycerides. Values are expressed as fat per body volume measured as % of measured volume. Values are mean ± SEM (n = 6–8). Values denoted by * and # were considered significant if $p < 0.05$ (Two way ANOVA and Student's t-test). * indicates significant differences between WT and VLCAD$^{-/-}$ mice under the same dietary regimen. # indicates significant differences between mice of the same genotype under different dietary regimens.

one year showed no diet-dependent differences of fat distribution in males (Fig. 2A). In strong contrast, VLCAD$^{-/-}$ females under MCT diet exhibited a significantly higher overall fat content per measured body volume (BV) as compared to WT mice under the same dietary regimen (28.9 ± 3.1 *vs* 19.7 ± 5.2% BV; Fig. 2B). The classification of abdominal fat in visceral and deep/superficial subcutaneous fat revealed a predominant accumulation of visceral fat as compared to mutants upon the LCT diet followed by alterations in subcutaneous fat to a minor extent. In parallel, we observed a striking intrahepatic lipid accumulation upon MCT as measured by *in vivo* ^{1}H MRS (Fig. 3A). Indeed, VLCAD$^{-/-}$ females receiving MCT displayed a two-fold higher hepatic lipid content as compared to VLCAD$^{-/-}$ females under the LCT diet. These findings were confirmed on liver histology with severe and diffuse macrovesicular steatosis (grade 3) in VLCAD$^{-/-}$ females and accumulation of large lipid droplets as shown in the representative picture of Fig. 3B (right). In contrast, although a long-term MCT diet increased the intrahepatic lipid content also in VLCAD$^{-/-}$ males, we observed in only few cases cloudy swelling of hepatocytes with rarely microvesicular vacuolization (Fig. 3B, right). Moreover, we observed a positive correlation between serum thiobarbituric acid reactive substances (TBARS), serum cholesterol and visceral fat accumulation which has been associated with the presence of metabolic syndrome [51]. Indeed, as shown in Fig. 3C, the amount of visceral fat and the serum TBARS:cholesterol ratio significantly correlated ($r^2 = 0.5095$; $p < 0.0001$) and the highest ratio was detected in VLCAD$^{-/-}$ females upon MCT indicating that insulin resistance occurring already at the age of 6 months degenerated into a metabolic defect strongly resembling the human metabolic syndrome over time.

3.6. MCT supplementation contributes to the accumulation of long-chain acylcarnitines in blood in mice at the age of one year

Acylcarnitines are considered a faithful parameter reflecting the efficiency of β-oxidation of fatty acids in providing the required energy. Higher blood concentrations of acylcarnitines have been associated with catabolic conditions and also with type 2 diabetes (T2D) [34]. In contrast to previous reports [35–37], we did not observe a higher acylcarnitine accumulation in male VLCAD$^{-/-}$ mice under LCT diet (Fig. 4). However, this effect was evident when mice were supplemented with MCT. Moreover, we observed a higher acylcarnitine concentration in male mice probably due to the higher sex specific muscle mass in males associated with the regulation of plasma carnitine and thereby acylcarnitines by sex hormones [38] as already reported in adult female rats [39]. Of interest was also the significantly higher concentration of the monounsaturated acylcarnitine species C16:1 (0.6 ± 0.01 *vs* 0.33 ± 0.03 μmol/L; $p < 0.05$) and C16 (2.56 ± 0.04 *vs* 1.6 ± 0.13 μmol/L; $p < 0.05$) in mutant males as compared to females under the same dietary regimen (Fig. 4) (desaturation index: 0.291 *vs* 0.268; $p < 0.05$). Whereas, the content of saturated C16:0 and C18:0 acylcarnitines was significantly higher in VLCAD$^{-/-}$ female mice as compared to VLCAD$^{-/-}$ males. Of interest, was the dramatic reduction of C18:2 acylcarnitines in both genotypes of VLCAD$^{-/-}$ mice under MCT diet as compared to mice under control diet.

4. Discussion

In this work, we demonstrate for the first time that the long-term application of dietary MCT without increasing the total fat intake results in a severe metabolic defect strongly resembling the human metabolic syndrome over time in a sex specific manner. Indeed, female VLCAD$^{-/-}$ mice present with marked insulin resistance, dyslipidemia, abdominal obesity and steatohepatitis whereas VLCAD$^{-/-}$ males remain generally protected. The sex-specific response to the MCT diet is also reflected by the accumulating acylcarnitine species which differ in the degree of desaturation between male and female mice.

Ectopic lipid accumulation is a critical feature for the development of insulin resistance [40–42]. In addition, the intramyocellular lipids (IMCL) in the skeletal muscle are shown to play an important role in this respect. Moreover, other factors such as fatty acid availability, uptake and oxidation are crucial [43]. Because of the great bulk of reports that link impaired fatty acid oxidation to insulin resistance [44–47] we proposed that VLCAD$^{-/-}$ mice would develop a metabolic defect strongly resembling the human metabolic syndrome. However, in accordance with a very recent report on patients with long-chain fatty acid oxidation disorders [48], VLCAD$^{-/-}$ mice of both sexes did not display excessive accumulation of IMCL despite increased long-chain acylcarnitines, increased serum TAG and lipoprotein concentrations. In fact, we observed in both genotypes lower circulating insulin levels suggesting either higher peripheral insulin sensitivity [47] or lower insulin secretion. Only upon dietary modification, female VLCAD$^{-/-}$ mice did display a significant increase in muscular IMCL as early as during their reproductive period at the age of 6 months associated with a marked insulin resistance as demonstrated by the significant increase in HOMA index. These findings were also reflected by the high blood concentrations of free fatty acids, the serum VLDL and ox-LDL profile [29,49]. At 12 months of age a full metabolic defect strongly resembling the human metabolic syndrome was observed in female VLCAD$^{-/-}$ mice upon MCT with altered abdominal fat distribution and a massive increase in visceral fat accompanied by steatohepatitis. This effect is likely due to sex-specific differences in handling hepatic fatty acids resulting in a more severe injury and systemic oxidative stress in females as a

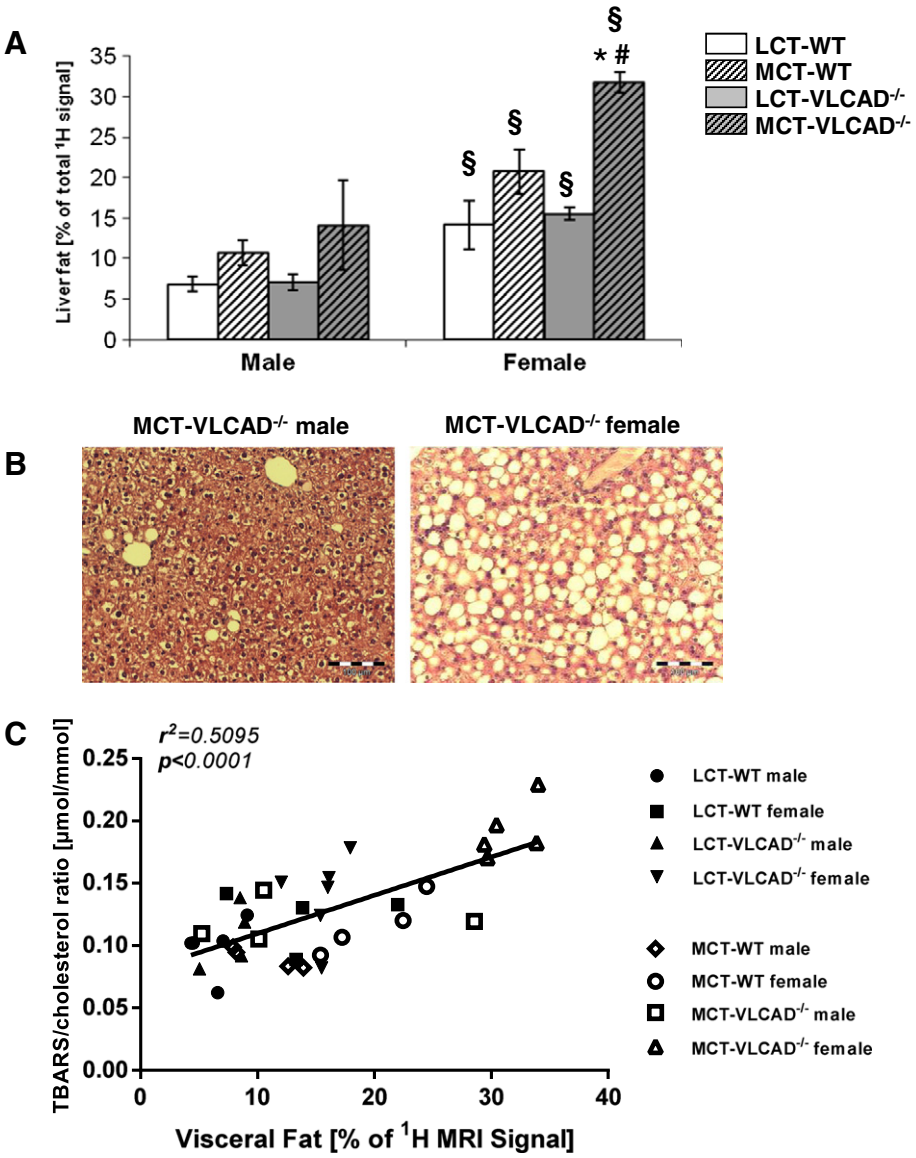

Fig. 3. Long-term MCT diet induces hepatic steatosis and systemic oxidative stress in VLCAD$^{-/-}$ female mice. A) Relative contribution of lipid signals to the total MR signal from non-water suppressed ^{1}H MR spectra of the liver (given in percent). B) Representative liver slices from one year old VLCAD$^{-/-}$ mice under MCT diet (×33). The degree of steatosis was assessed with Sudan III. C) Positive correlation between serum thiobarbituric acid reactive substances (TBARS):total cholesterol ratio and visceral fat in WT and VLCAD$^{-/-}$ mice fed with either LCT or MCT diet. LCT, long-chain triglycerides. MCT, medium-chain triglycerides. Values are mean ± SEM ($n = 6–8$). Values denoted by *, # and § were considered significant if $p < 0.05$ (Two way ANOVA and Student's t-test). * indicates significant differences between WT and VLCAD$^{-/-}$ mice under the same dietary regimen. # indicates significant differences between mice of the same genotype under different dietary regimens. § indicates significant differences between sex but of the same genotype and under the same dietary regimen.

result of it [50,51]. In accordance, we found a linear relationship between visceral fat content and TBARS:cholesterol ratio as previously reported [51–54]. Estrogens are known to protect against weight gain, obesity and dyslipidemia [55], however, a decline in estrogen levels after menopause or estrogen deficiency leads to a dysregulation of lipid and energy metabolism inducing metabolic changes that expose women to the same risk as men for the development of type 2 diabetes, metabolic syndrome and cardiovascular diseases [56,57]. MCT induces such metabolic dysregulation in VLCAD$^{-/-}$ female mice already during their reproductive period. Our data therefore strongly suggest that MCT indeed inhibits the likelihood protective effects of estrogens [50] acting on sex-linked gene expression such as signaling pathways and receptors.

Although congruent with the phenotype of a metabolic syndrome, our findings are in contrast to published data on the sex dimorphism in distribution of visceral fat in humans and the disposition to the development of insulin resistance in rodents. In humans, men are prone to accumulate visceral fat in the abdominal region, whereas premenopausal women accumulate more subcutaneous adipose tissue primarily localized in peripheral body areas [58,59], however, a fat redistribution occurs during and after menopause in response to sexual hormones [60]. In addition, reports on rodents have demonstrated that males are more prone to diet-induced obesity and insulin resistance and display a lower glucose tolerance accompanied by a higher expression of markers for oxidative stress [61–63]. An important observation of our study, however, is that the overall fat metabolism seems to be differently regulated in defects of mitochondrial β-oxidation resulting in a sex-specific response to MCT supplementation.

It becomes very clear that the low desaturation degree of the MCT diet may play an important role in this context. This dietary effect is namely associated with a lower hepatic stearoyl-CoA desaturase 1 (SCD1) activity in females [64]. This enzyme regulates the desaturation degree of the total lipid fraction [65] and shows a hormonal regulation with higher activity in males [66]. These sex-specific differences are in our study clearly

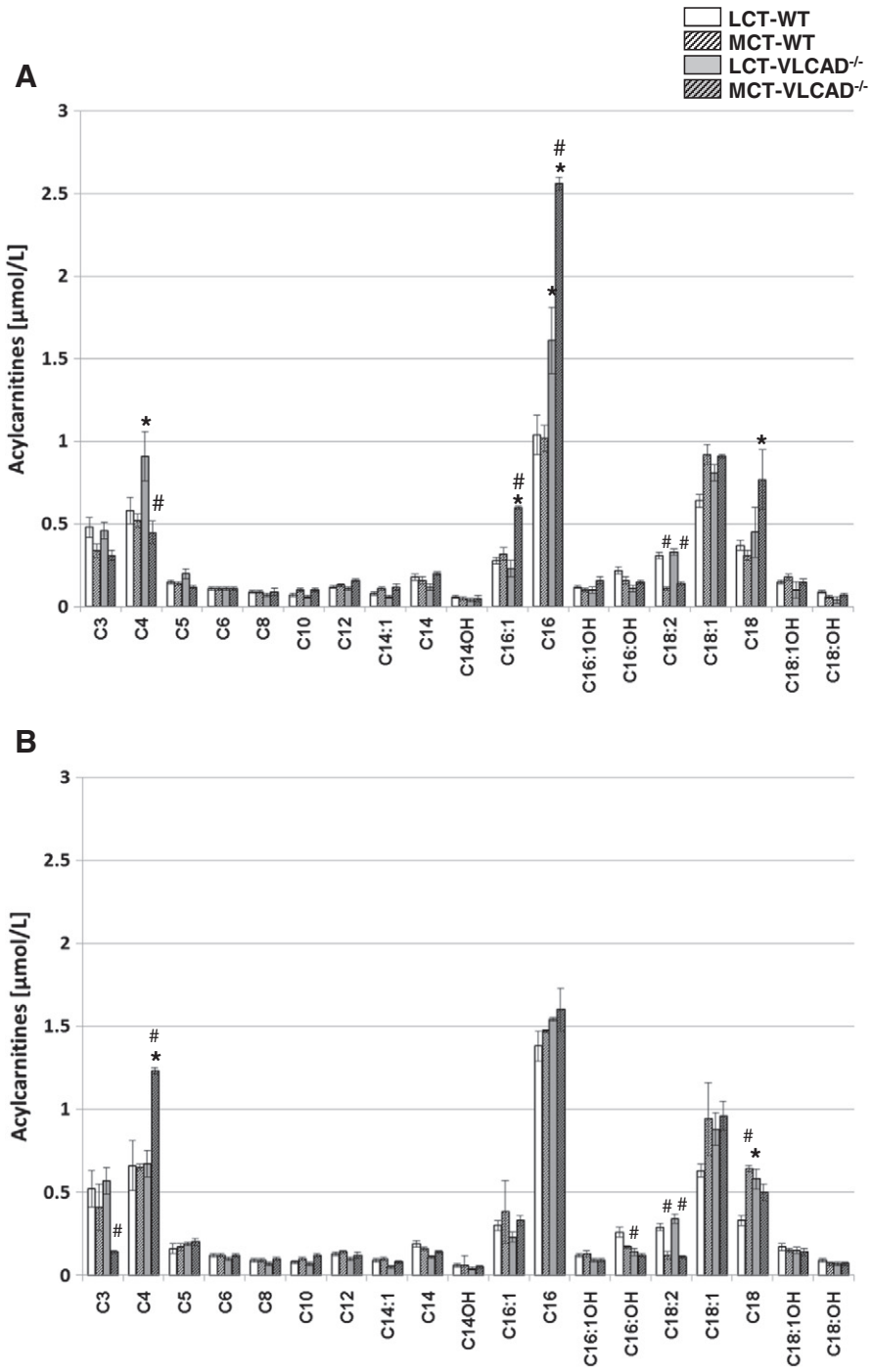

Fig. 4. Acylcarnitine profiles from dried blood spots of WT and VLCAD$^{-/-}$ mice fed with either LCT or MCT diet. LCT, long-chain triglycerides. MCT, medium-chain triglycerides. A) Male mice. B) Female mice. Values are mean ± SEM ($n = 6$–8). Values denoted by * and # were considered significant if $p < 0.05$ (Two way ANOVA and Student's t-test). * indicates significant differences between WT and VLCAD$^{-/-}$ mice under the same dietary regimen. # indicates significant differences between mice of the same genotype under different dietary regimens.

reflected by the desaturation index [67] of the acylcarnitine profile that serves as a marker of the mobilization of endogenous lipid stores.

Our findings were obtained in the VLCAD$^{-/-}$ mice and therefore they do not allow a full extrapolation to the human VLCAD-deficiency. However, many patients affected by defects of long-chain fatty acid oxidation are supplemented with MCT from the time of diagnosis [68,69]. Although MCT is widely applied also over longer periods of time there are no observations and neither short- nor long-term studies on the effects of MCT in patients with regard to glucose homeostasis and insulin resistance. Our results suggest a need to take into consideration that the sex-specific response to long-term MCT intake may be of relevance when patients with mitochondrial fatty acid β-oxidation defects are treated long-term.

4.1. Conclusions

In summary, we here show that the long-term application of an MCT diet deeply disturbs glucose homeostasis in a sex-specific manner resulting in a severe metabolic syndrome in female VLCAD$^{-/-}$ mice. In particular, the protective effects of estrogens seem to be inhibited

already during their reproductive period. The highly saturated MCT diet associated with a lower hepatic stearoyl-CoA desaturase 1 (SCD1) activity in females exaggerates the harmful effects of an MCT diet. The molecular mechanism behind this sex-specific metabolic response to MCT needs to be elucidated. More important, the relevance of these findings for humans under these dietary modifications needs to be investigated.

Transparency document

The Transparency document associated with this article can be found in the online version.

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