Fasting-Induced Myocardial Lipid Accumulation in Long-Chain Acyl-CoA Dehydrogenase Knockout Mice Is Accompanied by Impaired Left Ventricular Function

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Background—Lipotoxicity may be a key contributor to the pathogenesis of cardiac abnormalities in mitochondrial long-chain fatty acid β-oxidation (FAO) disorders. Few data are available on myocardial lipid levels and cardiac performance in FAO deficiencies. The purpose of this animal study is to assess fasting-induced changes in cardiac morphology, function, and triglyceride (TG) storage as a consequence of FAO deficiency in a noninvasive fashion.

Methods and Results—MRI and proton magnetic resonance spectroscopy (1H-MRS) were applied in vivo in long-chain acyl-CoA dehydrogenase (LCAD) knockout (KO) mice and wild-type (WT) mice (n=8 per genotype). Fasting was used to increase the heart’s dependency on FAO for maintenance of energy homeostasis. In vivo data were complemented with ex vivo measurements of myocardial lipids. Left ventricular (LV) mass was higher in LCAD KO mice compared with WT mice (P<0.05), indicating LV myocardial hypertrophy. Myocardial TG content was higher in LCAD KO mice at baseline (P<0.001) and further increased in fasted LCAD KO mice (P<0.05). Concomitantly, LV ejection fraction (P<0.01) and diastolic filling rate (P<0.01) decreased after fasting, whereas these functional parameters did not change in fasted WT mice. Myocardial ceramide content was higher in fasted LCAD KO mice compared with fasted WT mice (P<0.05).

Conclusions—Using a noninvasive approach, this study reveals accumulation of myocardial TG in LCAD KO mice. Toxicity of accumulating lipid metabolites such as ceramides may be responsible for the fasting-induced impairment of cardiac function observed in the LCAD KO mouse. (Circ Cardiovasc Imaging. 2011;4:558-565.)

Key Words: hypertrophy ■ lipids ■ metabolism ■ MRI ■ spectroscopy

The heart has a high energy demand, and ATP must be continuously available for the myocardium in order to maintain its contractile function. The main fuel for cardiac ATP production are long-chain fatty acids, which are degraded through mitochondrial fatty acid β-oxidation (FAO).1 Recessively inherited defects are known for most of the enzymes involved in FAO. Neonates and infants affected by long-chain FAO disorders may present with hypoketotic hypoglycemia, hypertrophic cardiomyopathy, arrhythmia, and sudden death.2-3 Neonatal screening for FAO disorders can be effective in reducing death and serious adverse events in patients because treatment plans to avoid periods of fasting generally prevent hypoketotic hypoglycemia.4 Current interventions used to prevent cardiac disease in FAO disorders are based on expert opinion and are not evidence-based.5 To critically assess these interventions and to design novel therapeutic strategies, better understanding of the etiology of cardiac disease in FAO disorders is instrumental.

Clinical Perspective on p 565

In obese and diabetic patients, an increased supply of fatty acids is responsible for an imbalance between fatty acid import and utilization. The resulting elevated myocardial lipid levels are associated with decreased cardiac function,5,7 suggesting that myocardial lipid accumulation may be detrimental for the heart. Because of a genetic defect in FAO, lipids may accumulate as well, and their toxicity may be a key contributor to the pathogenesis of cardiac abnormalities found in FAO disorders. Previously, histological and bio-

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chemical analyses revealed elevation of myocardial lipid levels and cardiac hypertrophy in mouse models of FAO disorders.8–11 Until now, few data are available on myocardial lipid levels in combination with measurements of cardiac performance in models for FAO defects. MRI is the modality of choice to accurately assess cardiac morphology and function because of its excellent soft tissue contrast combined with the relatively high spatial and temporal resolution needed for small animal studies. Proton magnetic resonance spectroscopy \(^{1}H\)-MRS) is a powerful tool to noninvasively assess mobile intracellular lipid pools, predominantly present as triglyceride (TG) droplets in tissues such as skeletal muscle and liver.12 \(^{1}H\)-MRS of the mouse heart was introduced as a tool to study myocardial creatine levels in mice.13 Recently, this approach was used to examine myocardial lipid levels in mice predisposed to cardiac steatosis. Remarkably, the lipid signal as detected by \(^{1}H\)-MRS in this study was not exclusively attributable to TG.14,15 The purpose of this animal study is to examine, for the first time, changes in cardiac morphology, function, and TG storage as a consequence of FAO deficiency. MRI and \(^{1}H\)-MRS were applied in vivo in long-chain acyl-CoA dehydrogenase (LCAD) knockout (KO) mice and wild-type (WT) C57BL/6 mice. In the mouse, LCAD plays an essential role in human very long-chain acyl-CoA dehydrogenase deficiency (VLCADD).8,9,16 Fasting was used to further increase the heart’s dependency on FAO for ATP production. We hypothesized that due to the deficient FAO in LCAD KO mice, fasting would increase myocardial TG content and decrease cardiac performance.

**Methods**

**Animals**

Heterozygous LCAD KO mice (B6.129S6-Acald\(^{+/−}\)/Mmmb) on a pure C57BL/6 background were obtained from Mutant Mouse Regional Resource Centers. Male LCAD KO and WT mice were generated through heterozygote as well as homozygote breeding pairs and were backcrossed 15 times with C57BL/6. Mice (n=20) were housed at 21±1°C, 40% to 50% humidity, on a 12 hours light-dark cycle, with ad libitum access to water and a standard rodent diet (ssniff Spezialdiäten, Soest, Germany). At 13 weeks of age, baseline data were acquired in the fed state as described below. Within 2 weeks, mice were weighed, placed solitary in a clean cage without food, but with access to water, and fasted for 24 hours, followed by measurements in the fasted state. Immediately thereafter, anesthetized mice were euthanized by exsanguination from the vena cava inferior. The heart was rapidly excised, weighed, and processed for biochemical and histological analysis. All procedures were approved by the Animal Ethics Committee of Maastricht University (Maastricht, The Netherlands).

**MR Protocol**

Mice were anesthetized with 2% isoflurane in 0.4 L/min medical air and positioned supine in a purpose-built support cradle. Anesthesia was maintained by administering 1.6% isoflurane in a continuous flow of 0.4 L/min medical air through a nose cone. Body temperature was maintained at 37±0.5°C using a warm water flow integrated in the setup, and monitored with a rectal fiber optic probe. ECG electrodes were placed on the front paws and a respiratory sensor balloon was taped onto the abdomen. Vital signs were monitored and used for MR gating/triggering by the SA Monitoring and Gating System (Model 1025, Small Animal Instruments, Stony Brook, NY). The cradle was positioned into a horizontal-bore 9.4 T magnet (Magnex Scientific, Oxon, UK) interfaced to a Bruker Avance III console (Bruker Biospin MRI, Ettlingen, Germany) and controlled by the ParaVision 5.0 software package (Bruker Biospin). A quadrature-driven birdcage coil (Ø 35 mm, Bruker Biospin) was used for radiofrequency transmission and signal reception.

A segmented, prospective cardiac-triggered respiratory-gated FLASH sequence was used to acquire cinematographic MR image series of 14 to 16 frames per cardiac cycle. Six 1-mm contiguous left ventricular (LV) short-axis slices were complemented with 4- and 2-chamber long-axis views. Imaging parameters were field of view=30×30 mm\(^2\); matrix=192×192; echo time (TE)=1.8 ms; repetition time (TR)=7 ms; flip angle=15°; number of averages (NA)=6. To assess diastolic function, 22 to 26 frames of the equatorial short-axis view were acquired with a higher temporal resolution (TR=4.8 ms; matrix=128×128) and an increased flip angle (30°) for enhanced contrast between LV cavity and myocardial wall.11

Immediately after MR imaging, localized \(^{1}H\)-MRS was performed using a cardiac-triggered respiratory-gated point resolved spectroscopy (PRESS) sequence.13 A 4-µL voxel was positioned in the diastolic interventricular septum, avoiding contamination of the spectra with signal originating from pericardial lipid deposits. PRESS parameters were TE=9.1 ms; TR ~2 seconds; 0.41 ms 90° Hermite-shaped excitation pulse; 0.9 ms 180° Maa-type refocusing pulses. Triggering was timed at ~75% of the cardiac period after ECG R-wave upslope detection. Volume selective shimming of the voxel of interest was done manually using the same PRESS sequence, until a water line width less than 40 Hz was achieved. Water suppression was performed by preceding the PRESS sequence with a cardiac-triggered respiratory-gated chemical shift selective (CHESS) module consisting of 3 frequency-selective radiofrequency pulses (bandwidth=250 Hz) each followed by spoiler gradients in orthogonal directions. Total CHESS duration was 90.8 ms. Steady state of magnetization, required for accurate quantification of metabolites,13 was maintained by applying dummy excitations during respiratory gates, allowing a window of <30 ms for trigger detection. Water-suppressed spectra were acquired on-resonance on creatine (1.71 parts per million (ppm)) upfield of water; NA=256; acquisition time =13 minutes. As a quantification reference, water spectra (NA=32) were acquired on-resonance on water with the radiofrequency pulses for water suppression turned off.

**Image Analysis**

LV cavity and wall volumes were determined by semiautomatic segmentation (PIE Medical Imaging, Maastricht, The Netherlands).18 LV mass was calculated by multiplying the end-diastolic myocardial wall volume with a myocardial density of 1.05 g/mL.19 End-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), and ejection fraction (EF) were calculated as described previously.19 cardiac output (CO) was calculated by multiplying the SV with the average heart rate. Peak ejection rate and peak filling rate were determined from the high temporal resolution cine series as, respectively, the minimum and maximum in the time-derivative of the LV cavity volume.17

**Spectral Analysis**

Spectral fitting was performed using AMARES in jMRUI.20 The unsuppressed water spectrum was corrected for eddy-current distortions, after which DC offset was removed. The water peak was fitted to a Lorentzian line shape. The water-suppressed spectrum was phased using the corresponding unsuppressed water spectrum as a reference. The TG methylene resonance at 1.28 ppm was used as internal chemical shift reference. Peaks of metabolites were assigned according to reports in literature21 and fitted to Gaussian line shapes. The metabolite concentrations were expressed as a percentage of the unsuppressed water signal measured in the same voxel.
Ex Vivo Myocardial Lipid Measurements

Total TG content was determined biochemically from myocardial tissue collected immediately after the MR measurements in the fasted state. Tissue (50 to 100 mg wet weight) was homogenized on ice in 2.5 mL Dole extraction mixture (isopropanol:heptane:1 mol/L H2SO4; 40:10:1), using a dispersion tool (Ika T10 basic). Next, 1.5 mL heptane and 0.6 mL 0.4 mol/L MOPS buffer (pH 6.4) was added, followed by thorough mixing and centrifugation (4°C, 3000 g). The upper phase was collected and the lower phase was extracted again with 1.5 mL heptane. Upper phases were pooled and evaporated to dryness. The residue was dissolved in 1 mL chloroform containing 1% Triton-X100, followed by evaporation to dryness and resuspension in 0.25 mL water. This solution was used for colorimetric quantification of total TG (Triglycerides LiquiColor, Instruchemie, Delfzijl, The Netherlands).

Because the in vivo 1H-MRS measurements do not allow for a detailed analysis of lipid composition, we measured the total fatty acid profile. Myocardial tissue was homogenized in PBS using a dispersion tool. After sonication (twice at 8-W output, 40 J, on ice), the protein concentration was measured using the bicinchoninic acid assay. All samples were diluted to a protein concentration of 10 mg/mL. Fatty acids from a 500 mg protein sample were directly transesterified and analyzed by gas chromatography with flame ionization detection (GC-FID). Myocardial ceramide levels were determined as described previously.

Histology

To detect neutral lipids, 4-μm-thick cryostat sections of frozen tissue were stained with 0.3% Oil Red O (BDH, Poole, United Kingdom). After washing, sections were counterstained with hematoxylin. The sections were analyzed by light microscopy. Multispectral imaging was performed using a Nuance N-MSI-420 20 camera with Nuance 3.0 software (Cambridge Research & Instrumentation, Woburn, MA). Data sets were acquired at 420 to 720 nm at 10-nm intervals. Spectral libraries for hematoxylin and Oil Red O, each obtained from single-stained cells, were used to unmix the staining patterns into the individual components and to separate these from background. The fraction of Oil Red O–stained surface of tissue section surface was quantified using Image-Pro 7.0 software (Media Cybernetics, Bethesda, MD).

Statistical Analysis

Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL). Statistical significance of fasting and genotype effects on in vivo MR data were assessed using ANOVA for repeated measures, with 1 between-subjects factor (genotype) and 1 within-subjects factor (fasting). If the interaction term between the factors was significant, the effects of genotype and fasting were analyzed separately using 2-sided unpaired and paired t tests, respectively. The effect of genotype on ex vivo determined lipid content was determined using a 2-sided t test. Relations between variables were assessed by Pearson r correlation coefficients. All data are presented as mean±SD, with the level of significance set at P<0.05.

Results

LV Morphology and Function

We assessed cardiac LV morphology and function from cine MR images acquired in fed and fasted LCAD KO mice and controls (Figure 1 and Table 1). At baseline, body weight was similar for both genotypes. During 24 hours of fasting, body weight decreased by 17% compared with body weight at the start of fasting. LV mass was 10% higher in fed LCAD KO mice compared with fed WT mice (P<0.05), indicating mild LV myocardial hypertrophy. Minor loss of LV mass (~7%, P<0.01) was observed in fasted WT mice, whereas LV mass of LCAD KO mice was unaffected by fasting. EDV was 14% lower in fed LCAD KO mice than in fed WT mice (P<0.05).

After fasting, EDV decreased (~10%, P<0.01) in WT mice, whereas ESV remained the same in fasted WT mice. In contrast, ESV increased in fasted LCAD KO mice (~54%, P<0.05), whereas EDV did not change in fasted LCAD KO mice. Peak ejection rate, SV, and CO were similar in both genotypes and were not affected by fasting. In the fed state, EF was not different between genotypes. After fasting, EF was preserved in WT mice, but decreased in LCAD KO mice (~13%, P<0.01). Additionally, in LCAD KO mice, peak
filling rate was significantly reduced by fasting (−23%, \(P<0.01\)), indicating decreased LV diastolic function. Combined, these data reveal impaired LV performance in the fasted LCAD KO mouse.

**In Vivo Myocardial Metabolite Levels**

To assess myocardial metabolite levels, we applied in vivo localized \(^1\)H-MRS to the mouse heart. Several resonances can be distinguished in the water-suppressed \(^1\)H-MR spectra (Figure 2). Concentrations of nine metabolites were quantified relative to the unsuppressed water signal obtained from the same voxel (Table 2). At baseline, the prominent TG methylene signal at 1.28 ppm was higher in LCAD KO myocardium than in controls, indicating elevated myocardial TG levels in fed LCAD KO mice. Importantly, the TG methylene signal strongly increased in fasted LCAD KO mice (\(+63\pm 56\%\), \(P<0.05\)), whereas this signal decreased after fasting in WT mice (−40±33\%, \(P<0.05\)). Other TG associated peaks showed a similar pattern, such as those from the \(\alpha\)-methylene protons (\(\text{C}_n\text{H}_m\text{COO}\), 2.21 ppm) and the allylic methylene protons (\(\text{CH}_2\text{CH}=\text{CH}_{2}\), 1.99 ppm). The residual water peak at 4.7 ppm hampered accurate quantification of nearby peaks. Consequently, the resonances originating from the glycerol backbone at 4.1 ppm and the

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**Table 1. Animal Characteristics, LV Morphology, and LV Function of Wild-Type and LCAD KO Mice in Fed Conditions and After 24 Hours of Fasting**

<table>
<thead>
<tr>
<th></th>
<th>Fed (n=8)</th>
<th>LCAD KO (n=8)</th>
<th>Fasted (n=8)</th>
<th>LCAD KO (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, wk</strong></td>
<td>13.8±0.5</td>
<td>13.0±0.4</td>
<td>15.4±0.2</td>
<td>14.9±0.2</td>
</tr>
<tr>
<td><strong>Body weight, g</strong></td>
<td>29.9±1.7</td>
<td>29.7±0.8</td>
<td>25.3±0.8</td>
<td>24.8±1.1</td>
</tr>
<tr>
<td><strong>Heart mass at autopsy, mg</strong></td>
<td>122.9±5.3</td>
<td>151.6±7.3§</td>
<td>112.3±11.7¶</td>
<td>138.5±9.2$§</td>
</tr>
<tr>
<td><strong>LV mass, mg</strong></td>
<td>120.5±10.2</td>
<td>132.3±12.2†</td>
<td>112.3±11.7¶</td>
<td>138.5±9.2$‡</td>
</tr>
<tr>
<td><strong>End-diastolic volume, (\mu)L</strong></td>
<td>77.5±5.9</td>
<td>66.4±9.4†</td>
<td>70.1±8.1¶</td>
<td>76.6±12.1</td>
</tr>
<tr>
<td><strong>End-systolic volume, (\mu)L</strong></td>
<td>24.9±4.5</td>
<td>19.4±3.3†</td>
<td>21.8±4.4</td>
<td>29.9±7.5†‡</td>
</tr>
<tr>
<td><strong>Stroke volume, (\mu)L</strong></td>
<td>52.6±3.1</td>
<td>47.0±7.4</td>
<td>48.3±5.0</td>
<td>47.1±6.6</td>
</tr>
<tr>
<td><strong>Ejection fraction, %</strong></td>
<td>68.0±3.9</td>
<td>70.6±3.7</td>
<td>69.1±3.7</td>
<td>61.7±4.7¶‡</td>
</tr>
<tr>
<td><strong>Heart rate, min(^{-1})</strong></td>
<td>526±49</td>
<td>512±57</td>
<td>511±57</td>
<td>509±43</td>
</tr>
<tr>
<td><strong>Cardiac output, mL/min</strong></td>
<td>27.7±3.1</td>
<td>24.0±4.0</td>
<td>24.5±2.4</td>
<td>23.9±3.3</td>
</tr>
<tr>
<td><strong>Peak ejection rate, ml/s</strong></td>
<td>0.30±0.04</td>
<td>0.31±0.03</td>
<td>0.31±0.04</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td><strong>Peak filling rate, ml/s</strong></td>
<td>0.25±0.01</td>
<td>0.26±0.02</td>
<td>0.22±0.02</td>
<td>0.20±0.01¶§</td>
</tr>
</tbody>
</table>

LV indicates left ventricle; LCAD, long-chain acyl-CoA dehydrogenase; and KO, knockout; WT, wild-type.

Data are expressed as mean±SD.

General fasting effect: *\(P<0.001\). If interaction was significant, the results of 2-sided t tests are given: †\(P<0.05\); ‡\(P<0.01\); §\(P<0.001\) versus WT, ¶\(P<0.001\) versus fed.

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**Figure 2.** Examples of myocardial \(^1\)H-MR spectra obtained in vivo from a wild-type (WT) mouse (A) and a long-chain acyl-CoA dehydrogenase (LCAD) knockout (KO) mouse (B) in fed condition and after 24 hours of fasting. Spectra are scaled equally. Metabolite peaks were assigned according to literature. 1, Taurine, 3.39 ppm; 2, choline/carnitine, 3.21 ppm; 3, creatine -C\(\text{H}_2\text{N},\) 2.99 ppm; 4, TG CH=CH-C\(\text{H}_2\text{CH}=[\text{CH}_2]\)CH=CH, 2.72 ppm; 5, TG C\(\text{H}_3\text{H}_2\text{COO},\) 2.21 ppm; 6, TG CH=CH-C\(\text{H}_2\text{CH}=[\text{CH}_2]\)CH=CH, 1.99 ppm; 7, TG C\(\text{H}_3\text{H}_2\text{COO},\) 1.57 ppm; 8, TG -(CH\(\text{H}_2\text{O}))_n\), 1.28 ppm; 9, TG -CH\(\text{H}_2\text{O},\) 0.84 ppm. Glc, composite resonances from the glycerol backbone, glucose, and other carbohydrates. TG indicates triglyceride. C, Myocardial TG derived from the methylene peak. \(***P<0.001\) versus WT, §\(P<0.05\) versus fed.
olefinic protons (\(\text{CH} = \text{CH}\)) at 5.3 ppm could not be quantified. Nevertheless, the peak originating from olefinic protons was more prominent in the spectra obtained from LCAD KO myocardium, illustrating that the increased TG methylene signal was paralleled by increased signal associated with unsaturated bonds in LCAD KO myocardial TG. This observation is corroborated by a higher signal of aliphatic methylene in LCAD KO mice, which increased further on fasting. Taurine, choline/carnitine, and total creatine levels were similar in both genotypes and were not affected by fasting. These data show that in WT mice, myocardial TG pools are depleted during fasting, whereas in LCAD KO mice, myocardial TG levels are elevated in fed conditions and further increase on fasting.

### Ex Vivo Myocardial Lipid Analysis

We compared the results obtained with \(^1\)H-MRS to biochemical TG measurement. As shown in Figure 3, TG methylene levels measured in vivo with \(^1\)H-MRS correlated with the biochemical measurements of total TG (\(r=0.91, P<0.0001\)). Corresponding to in vivo observations, total myocardial TG in fasted LCAD KO mice was higher than in fasted WT mice (\(P<0.01\)). Histology confirmed the accumulation of lipid droplets in cardiomyocytes of fasted LCAD KO mice as compared with fasted WT mice, indicating development of microvesicular steatosis (Figure 4).

Next, GC-FID was used to obtain total fatty acid profiles. Total myocardial fatty acid content was significantly higher in fasted LCAD KO mice compared with fasted WT mice (LCAD KO, 570.9 ± 104.8 nmol/mg protein versus WT, 407.5 ± 47.8 nmol/mg protein; \(P<0.01\)). The relative contributions of both saturated (SFA) and polyunsaturated fatty acids (PUFA) to the total myocardial fatty acid content were slightly lower, whereas the relative content of monounsaturated fatty acid (MUFA) was higher in LCAD KO mice (Figure 5A). Detailed analysis of the individual fatty acid species in the myocardium revealed a prominent, 2.5-fold higher level of oleate (C18:1[n-9]) in LCAD KO mice (\(P<0.001\)) and a mild elevation of linoleate (C18:2[n-6]) (\(P<0.05\)). Notably, of all fatty acid species, only docosahexaenoic acid (C22:6[n-3]) content was lower in the LCAD KO myocardium, both in absolute terms (\(P<0.05\)), and relative to the total myocardial fatty acid content (\(P<0.0001\), Figure 5B). Downstream of oleate and linoleate in the FAO pathway are C14:1[n-9] and C14:2[n-6], respectively. These fatty acids were strongly elevated in the LCAD KO myocardium (\(P<0.0001\), Figure 5B), although the absolute levels were low. These elevations are paralleled by prominently increased levels of the corresponding C14:1 and C14:2 acylcarnitines in blood, plasma, and tissue samples of LCAD KO mice as reported previously.8,9,16 Taken together, these data underscore the essential role of LCAD in the oxidation of oleate and linoleate.

| Table 2. Myocardial Metabolite Concentrations as a Percentage of the Total Water Signal |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | **Fed**         | **LCAD KO**     | **Fasted**      | **LCAD KO**     |
|                                | \(\delta\), ppm | WT (n=8)        | KO (n=8)        | WT (n=7)        | KO (n=7)        |
| Taurine (1)                    | 3.39            | 0.10±0.03       | 0.13±0.07       | 0.13±0.03       | 0.12±0.03       |
| Choline/carnitine (2)          | 3.21            | 0.13±0.06       | 0.15±0.08       | 0.20±0.05       | 0.15±0.07       |
| Creatine \(-\text{CH}_3\) (3)  | 2.99            | 0.08±0.04       | 0.12±0.08       | 0.12±0.05       | 0.08±0.03       |
| TG CH=CH-CH, CH=CH (4)         | 2.72            | 0.05±0.02       | 0.08±0.04       | 0.07±0.02       | 0.08±0.03       |
| TG C\(_2\)H\(_5\)COO (5)      | 2.21            | 0.08±0.03       | 0.13±0.04\(\dagger\) | 0.07±0.02       | 0.19±0.06\(\dagger\)** |
| TG CH\(_2\)-CH=CH\(_2\) (6)   | 1.99            | 0.12±0.03       | 0.21±0.06\(\dagger\)| 0.13±0.05       | 0.31±0.09\(\dagger\)** |
| TG C\(_3\)H\(_5\)COO (7)\(\dagger\) | 1.57        | 0.06±0.02       | 0.11±0.04       | 0.07±0.03       | 0.17±0.08       |
| TG \(-\text{CH}_3\) (8)        | 1.28            | 0.65±0.16       | 1.10±0.20\(\dagger\)| 0.39±0.16**      | 1.79±0.60****   |
| TG \(-\text{CH}_3\) (9)        | 0.84            | 0.14±0.05       | 0.24±0.11       | 0.14±0.04       | 0.27±0.08       |

LCAD indicates long-chain acyl-CoA dehydrogenase; WT, wild-type; KO, knockout; and TG, triglyceride.

Chemical shift (\(\delta\)) given in ppm (parts per million). Numbers in parentheses refer to the peaks assigned in Figure 2. Data are expressed as mean±SD.

General fasting effect: \(*P<0.05\). General genotype effect: \(\dagger P<0.05\; \dagger P<0.01\). If interaction was significant, the results of 2-sided \(t\) tests are given: \(\dagger P<0.05\; \dagger P<0.01\; \dagger P<0.001\) versus WT, **\(P<0.05\) versus fed.

Figure 3. Regression analysis shows good correlation (\(r=0.91, P<0.0001\)) between myocardial triglyceride levels as measured in vivo with \(^1\)H-MRS, and ex vivo with a standard biochemical assay in wild-type (WT) and long-chain acyl-CoA dehydrogenase knockout (KO) mice after 24 hours fasting. Solid line represents the linear fit.
To establish whether increased TG accumulation coincides with an increase in lipotoxic compounds, we measured myocardial ceramide levels. Ceramide content was elevated in fasted LCAD KO mice compared with fasted WT mice ($P < 0.05$, Figure 5C).

Discussion
An imbalance between fatty acid utilization and supply may lead to cardiac steatosis. As such, excessive lipid accumulation has been proposed as a contributor to the pathogenesis of cardiac abnormalities in long-chain FAO disorders. Until now, limited data were available on the effects of impaired FAO on myocardial performance and lipid accumulation. We assessed cardiac morphology, function, and myocardial TG storage of WT and LCAD KO mice, using noninvasive MR methods. Previous studies have shown that the LCAD KO mouse phenotype resembles human VLCADD and that it is currently the best preclinical model for long-chain FAO disorders. We show that fasting decreased myocardial TG content in WT mice, whereas cardiac performance was not affected. In contrast, elevated levels of myocardial TG in fasted LCAD KO mice were accompanied by decreased LV EF and reduced peak filling rate. Cardiac steatosis was confirmed...
by histology, revealing prominent accumulation of lipid droplets in cardiomyocytes of fasted LCAD KO mice.

The MR images obtained in fed conditions illustrate cardiac hypertrophy and maintenance of systolic cardiac performance in LCAD KO mice compared with WT mice, confirming previous M-mode echocardiography measurements. In addition, we show that, due to an increase of ESV, EF decreases in fasted LCAD KO mice, which suggests that fasting induces contractile dysfunction of the LCAD KO myocardium. No quantitative measures of diastolic function were previously reported. We show that diastolic performance, in terms of peak filling rate, is similar for both genotypes in the fed state but decreases after 24 hours fasting in the LCAD KO mice. Myocardial fibrosis, previously reported for 10% of 14- to 16-week-old male LCAD KO mice,8 may alter LV filling rates.25 It is unlikely that fibrotic tissue will develop within a 24 hours fasting period, because in mouse models of myocardial ischemia or progressive heart failure, fibrosis occurs only after several days or even weeks from disease onset.26 Furthermore, the LV hypertrophy already present in LCAD KO mice at baseline was not exacerbated after fasting. Indeed, histological analysis did not show prominent myocardial fibrosis in fed and fasted LCAD KO mice (data not shown). Moreover, expression of brain natriuretic peptide and atrial natriuretic factor, markers for hypertrophic myocardial adaptation, was not different between LCAD KO mice and controls and was not affected by (repeated) fasting (data not shown). Apparently, the heart of LCAD KO mice is able to compensate the relatively mild metabolic defect in the fed condition, and this compensation fails on fasting. A shift from FAO toward glucose metabolism for maintaining energy homeostasis may account for normal cardiac function as observed under fed conditions but may become inadequate when glucose levels drop after a prolonged period of fasting.8 Diastolic dysfunction often precedes systolic failure, which may develop over longer time, in more severe long-chain FAO deficiencies, or after a more strenuous nutritional challenge. Altogether, our findings suggest that the LV functional alterations in the LCAD KO mouse are not caused by fasting-induced fibrotic changes of the myocardium but more likely by a metabolic effect of fasting.

In healthy human volunteers, it was shown that short-term caloric restriction induces impaired diastolic LV function without altering systolic function. Myocardial phosphocreatine-to-ATP ratio was maintained, suggesting that energy shortage is not the main cause of impaired diastolic function after caloric restriction in healthy subjects. Increased levels of myocardial TG correlated negatively with diastolic function, suggesting that lipotoxicity plays a role.27 In line with these findings, Hammer et al showed that after a 3-day period of complete starvation, myocardial TG content increases up to 3-fold compared with baseline levels in healthy humans and that diastolic function decreases concomitantly.28 Insufficient FAO leads to accumulation of TG in nonadipose tissue such as the myocardium, as was demonstrated in a rodent model for extreme obesity.29 As expected, LCAD KO mice display elevated myocardial TG levels, which increase even further after fasting. In accordance with findings in lean healthy men27,28 and diabetic patients,7 increased myocardial TG levels were accompanied by decreased diastolic function in LCAD KO mice. In addition, total myocardial ceramide content was higher in fasted LCAD KO mice compared with fasted WT mice, showing that elevated myocardial TG levels may be accompanied by increased levels of lipotoxic compounds such as ceramide. Further investigations of the myocardial energy homeostasis in the LCAD KO mouse are required before the detrimental effects of fasting on cardiac function can be fully attributed to lipotoxicity.

In sharp contrast with LCAD KO mice, WT mice in the current study showed a decrease in myocardial TG content after 24 hours fasting. For these mice, a fasting period of 24 hours may be sufficient to shift the equilibrium between cardiac storage of fatty acids and their utilization toward the latter, resulting in a depletion of the myocardial TG pool. The loss of LV mass found in fasted WT mice is consistent with a previous study, and may be explained by starvation-induced autophagy.30

In apparent contrast to a recent 1H-MRS study of the mouse heart,14 we find a good correlation between myocardial lipid levels measured in vivo and the TG content determined biochemically. In addition, histology supports that the TG signal in the 1H-MR spectra originates from lipid droplets inside cardiomyocytes. Thus far, assessment of myocardial TG levels in laboratory animals has been limited to histology, biochemical assays on tissue samples, 1H-MRS of isolated intact or dissected hearts, or a combination of those techniques.6 The current work demonstrates that noninvasive, in vivo cardiac 1H-MRS at physiological heart rates can be applied to animal models of disease in a longitudinal study design, allowing monitoring changes in myocardial TG levels and other 1H-MRS observable metabolites.

In conclusion, the present study reveals accumulation of myocardial TG in LCAD KO mice, which increases even further on fasting. Concomitantly, LV EF and diastolic function in LCAD KO mice decrease after fasting, suggesting adverse effects of increased lipid metabolite levels. The results obtained here provide a basis for future longitudinal investigations in animal models of FAO defects to noninvasively assess the impact of therapeutic interventions on myocardial TG levels and cardiac function.

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Disclosures
None.

References
deficient heart are required before the detrimental effects of fasting can be fully attributed to lipotoxicity. Is induced by a metabolic effect of fasting. Further investigations of myocardial energy homeostasis in the long-chain FAO deficient heart, which examines the effect of long-chain FAO deficiency on cardiac function and myocardial lipid levels. We show that, in fed patients affected with inborn errors of long-chain FAO may present with hypertrophic cardiomyopathy. The pathogenesis of cardiomyopathy in long-chain FAO disorders is poorly understood, hampering the design of rational therapeutic strategies. Deficient oxidation of long-chain fatty acids may lead to accumulation of potentially lipotoxic metabolites. By applying in vivo cardiac MRI and 1H-MRS in the long-chain acyl-CoA dehydrogenase KO mouse model, the present study examines the effect of long-chain FAO deficiency on cardiac function and myocardial lipid levels. We show that, in fed conditions, the FAO-deficient heart is hypertrophied and harbors elevated levels of intracellular TG droplets. On fasting, myocardial TG levels further increased, accompanied by a reduced peak filling rate and a decreased left ventricular ejection fraction. These observations are in line with reports of decreased cardiac function on elevated myocardial TG levels in diabetic and obese humans. With the absence of signs for tissue fibrosis, our results suggest that contractile dysfunction is induced by a metabolic effect of fasting. Further investigations of myocardial energy homeostasis in the long-chain FAO deficient heart are required before the detrimental effects of fasting can be fully attributed to lipotoxicity.
Fasting-Induced Myocardial Lipid Accumulation in Long-Chain Acyl-CoA Dehydrogenase Knockout Mice Is Accompanied by Impaired Left Ventricular Function
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