Quantification of Liver Fat in Mice: Comparing Dual-Echo Dixon Imaging, Chemical Shift Imaging and $^1$H-MR Spectroscopy

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Original research

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**Abbreviations used:**

$^1$H = hydrogen
BSCL2 = Berardinelli-Seip congenital lipodystrophy type 2
CGL = Congenital generalized lipodystrophy
CSI = chemical shift imaging
CT = computed tomography
FC = fat content
HIS-S = semi-automatic vacuole segmentation procedure
IP-OP = in-phase and out-of-phase
LI = liver index
LL = liver lipid
MR = magnetic resonance
MRS = magnetic resonance spectroscopy
NAFLD = non-alcoholic fatty liver disease
$ob/ob$ = obese/obese
ROI = region of interest
SD = standard deviation
SI = signal intensity
SKO = human BSCL2/seipin gene knock-out
TR = repetition time
TE = echo time
WT = wild type
ABSTRACT

We evaluate dual-echo Dixon (in-phase and out-of-phase, IP-OP), chemical shift imaging (CSI), and $^1$H MRS in estimating fat content (FC) in phantoms and livers of mice. Phantoms were made according to the volume percentage of fat ranging from 0% to 100%. Three MR methods were performed to measure FCs in phantoms, livers of $ob/ob$, human BSCL2/seipin gene knock-out (SKO) and wild type (WT) mice. The results were compared to known FCs in phantoms and to reference standard from mice by histological semi-automatic vacuole segmentation procedure (HIS-S) and liver lipid (LL) chemical analysis. In phantoms, CSI underestimated FC in range of 50-100%, to a smaller extent than IP-OP. In vivo, liver FCs in $ob/ob$ and SKO mice measured by three MR methods were all significantly higher than that of WT mice. Liver FC measured by IP-OP are significantly lower than that measured by CSI and MRS with no significant difference between CSI and MRS. CSI and MRS showed a linear correlation with LL analysis and with each other. IP-OP underestimated FC, while CSI and MRS are more accurate for quantifying fat in both phantoms and liver. CSI and MRS have the potential to replace HIS-S and LL analysis in longitudinal studies.

Supplementary key words: non-alcoholic fatty liver disease; obesity; triglyceride; spin-spin relaxation time T2; spectroscopy; in vivo
INTRODUCTION

During the past decade, there has been evidence of an epidemic increase in the non-alcoholic fatty liver disease (NAFLD), which affects 10%–30% of adults (1-3) and 13% of children (4) in the general population. NAFLD has a strong association with type II diabetes mellitus, obesity, hyperlipidemia, and other diseases of the metabolic syndrome (5). The hallmark of NAFLD is fatty infiltration of hepatocytes. Hepatic steatosis is also reflective to the toxicity of drugs such as amiodarone, tamoxifen, and antiretrovirals (6).

Thus, liver fat quantification has generated considerable interest; it may be of clinical importance to be able to reliably measure liver fat content (FC). Liver biopsy and histological analysis are considered as the diagnostic reference standard. But in human, the biopsy procedure is both invasive and painful, and presents risks for the patients (7, 8). In addition, the very small liver sample may not be representative of the heterogeneous fat distribution (9). The analogous procedure in animals involves killing the animals and directly analyzing liver FC, which is not ideal for longitudinal follow-up studies.

Hydrogen (1H) magnetic resonance (MR) imaging offers several non-invasive methods to obtain separate fat and water images for the liver FC quantification. In 1984, the Dixon technique for water and fat imaging was described (10). The authors presented two imaging sequences, one of them was a conventional spin echo imaging with water and fat signals in-phase, and the second one had the readout gradient slightly shifted to create 180° out-of-phase of the water and fat signals. Over the last decade, the Dixon technique has been improved extensively in the aspects of phase errors, noise and artifacts. Triple-echo and multiecho in- and out-of-phase MR imaging were proposed, which allow correction for T2* decay and lipid quantification (11, 12). Iterative decomposition of water and fat with echo asymmetry and least-squares estimation (IDEAL) has also improved fat quantification and fat suppression (13, 14). In addition to the Dixon technique, in 1985, Haase et al. proposed chemical shift selective imaging technique, which relies on a single frequency-selective excitation pulse with a flip angle of 90° followed by a dephasing gradient to distinguish H2O and CH2 difference (15). Sbarbati et al. and Lunati et al. then applied chemical shift imaging to in vivo quantify lipid of brown adipose tissue (16, 17).

Animal models of hepatic steatosis and steatohepatitis have improved our understanding
of the pathogenesis of NAFLD. Continued studies in animals will further clarify the pathogenesis of these disorders and, therefore, will probably improve the diagnosis and treatment of human NAFLD. The \textit{ob/ob} mouse is an excellent model of hepatic steatosis because of its expression of many NAFLD traits (18). These mice are genetically leptin deficient, which causes excessive overeating and development of obesity, steatosis, steatohepatitis, and diabetes, among other symptoms of NAFLD (19).

Berardinelli-Seip congenital lipodystrophy type 2 (BSCL2) is a recessive disorder characterized by an almost complete loss of adipose tissue, insulin resistance and fatty liver (20, 21). BSCL2 encodes a protein, seipin, the function of which is largely unknown (22, 23). Recently, Cui et al. created the first murine model of BSCL2 by targeted disruption of seipin and suggested a possible tissue-autonomous role of seipin in liver lipid storage (24). To better understand the effect of seipin levels on fatty liver, we have measured liver fat content in a human BSCL2/seipin gene knock-out (SKO) mouse model in vivo and in vitro.

The purpose of this study was to evaluate the dual-echo Dixon MR imaging (in-phase and out-of-phase, IP-OP), chemical shift imaging (CSI) selective for either fat or water protons imaging, and $^1$H MR spectroscopy (MRS) for fat measurement in phantoms and in the liver of \textit{ob/ob} and human BSCL2/seipin gene knock-out (SKO) mice, and to compare these MR-derived data with those from histological and chemical analysis.
MATERIALS AND METHODS

Animals

All animal experiments were approved by the institutional Committee on Animal Research. Obese leptin-deficient mice (C57BL/\(\times\)OlaHsd-Lep; \(ob/ob\)) and wild type (WT) C57BL/6J mice were obtained from Laboratory Animal Center of Academy of Military Medical Science and Shanghai Model Animal Research Center, respectively. They were fed normally before the experiment and kept at temperature of 20-24°C in 12 hour day/night periods. Six male \(ob/ob\) and WT mice each, of ten-week-old with an average weight of 47.5 ± 1.54 g and 26 ± 0.71 g respectively, were used in this study. Four human BSCL2/seipin gene knock-out (SKO) and four wild type (WT) C57BL/6J mice were presented by Peking University Health Science Center, ten-week-old with an average weight of 21.8 ± 1.61 g and 22.6 ± 1.25 g respectively. After MR scan, they were euthanized with an overdose of pentobarbital.

Fat/ Water Phantoms

According to Poon et al. (25), phantoms were made by mixing known amounts of water (doped with 0.2 mM MnCl\(_2\)) and vegetable oil (extra virgin olive oil, Olivoila, Italy). Percentages of oil by volume ranged from 0 to 100 % in steps of 10 %. To improve the stability of the mixture, 2 % of Tween 80 (polyoxyethylene sorbitan monooleate) by volume of oil was added. The mixture was blended homogeneously using an ultrasonic homogenizer (KQ-400KQE, Kunshan Ultrasonic Equipment CO, LTD, Kunshan, China). Plastic tubes, of 15 mm in diameter, containing the suspensions were placed longitudinally into the magnet. Phantoms were scanned and analyzed with the same experimental protocol as that in vivo.

MR Imaging and \(^1\)H MRS Protocol

For in vivo MR acquisition, anesthesia was induced by inhalation of a mixture of oxygen and 5% isoflurane and maintained by a mixture of oxygen containing 0.5 % to 1 % isoflurane. All MR experiments were carried out using a 7T small animal magnetic resonance system (Bruker PharmaScan, Ettlingen, Germany) that is interfaced to a Bruker console. The horizontal bore system is equipped with a 15 cm diameter gradient set capable of generating
375 mT/m gradient strengths in all three directions. A 31 mm inner diameter transmit-receive quadrature coil was used for MR data collection.

T1-weighted images were acquired with a respiratory-gated spin echo sequence, 500/15 ms; section thickness, 2 mm; matrix, 256×256; field of view, 3.5×3.5 cm; and number of excitations, 4. The T1-weighted images were used to study the distribution of fat stores and measure the volume of the liver using ImageJ software.

A point-resolved spectroscopy sequence for localized 1H MRS sequence was used with the following parameters: repetition time (TR)/echo time (TE), 2500/20 ms; voxel, 3 × 3 × 3 mm; and number of excitations, 128. To correct for T2 decay, seven consecutive spectra were acquired with echo times of 10, 20, 30, 40, 50, 70 and 90 msec. A 9-mm3 region of interest (ROI) was placed over the left lobe of the liver, avoiding intrahepatic blood vessels. Before measurement, the automatic shimming procedure FASTMAP was used to achieve optimal uniformity of the magnetic field across the voxel volume. Water suppression was never used in all spectroscopy sequences during the measurement. The free induction decay signals were Fourier-transformed. The phase and the baseline of the spectra were also corrected with great care using TOPSPIN (Bruker BioSpin MRI GmbH). Spectra were used only if homogeneity after shimming was better than 0.45 ppm, measured as the full width at 50 % peak height. Spin-spin relaxation times (T2) was determined for nine different peaks (in the range from 0.9 to 5.32 ppm) by fitting the monoexponential model function \( M_{TE} = M_0 \times \exp(-TE/T2) \) to the measured peak integrals at the different TEs, and the correction factors \( (M_0/M_{TE}) \) for the nine different proton resonances of triacylglycerols and the proton resonance of the water peak were calculated (26-28). The degree of FC was calculated using the following formula:

\[
FC_{\text{MRS}} = 100 \times \frac{\text{integral value of fat peak}}{\text{integral value of fat peak + integral value of water peak}}
\]

Fast low angle shot imaging of the entire liver were obtained for both in-phase and out-of-phase transverse dual-echoes. Imaging parameters were as follows: 500/1.47 (out-of-phase) or 1.97 (in-phase); flip angle, 40°; section thickness, 2 mm; matrix, 256×256; field of view, 3.5×3.5cm. A radiologist used these images to position one voxel in the liver parenchyma outside the area of the great vessels for spectroscopy. And the size and location of the ROI matched those of the original 1H MRS voxel on three consecutive in-phase and
out-of-phase images. Signal intensity (SI) was measured at ParaVision4.0 (Bruker BioSpin MRI). The SI for each ROI (3×3mm) was recorded separately, and an average SI was calculated from the three images to gather information for the entire 1H MR spectroscopy voxel volume. We used the following equation:

\[ FC_{\text{IP,OP}} = 100 \times \left[ \frac{(\text{SI}_{\text{IP}} - \text{SI}_{\text{OP}})}{(2 \times \text{SI}_{\text{IP}})} \right] \]  

[2]

where \( \text{SI}_{\text{IP}} \) is SI measured on the in-phase; \( \text{SI}_{\text{OP}} \), SI measured on the out-of-phase image. The in-phase and out-of-phase images were used to study the liver FC.

CSI were also used to study liver FC. Two series of chemical shift selective images, one for fat protons and one for water protons, were derived using rapid acquisition with relaxation enhancement (RARE) sequence. Both series were performed by the 180° refocusing pulse (16, 17). Both the 90° and the 180° pulses were band-selective Gaussian pulses; however, the 90° pulse was applied when the slice-selection gradient was on, and when the 180° pulse is applied, all gradients were off to refocus only in the selected chemical-shift range. The 180° pulse was a 2.3 ms Gaussian pulse, which excited a 700 Hz bandwidth. This was appropriate to discriminate fat and water peaks which, at 7T, have a separation of about 1000 Hz. All images were acquired using the following parameters: 1000/9.9 ms, flip angle, 180°; section thickness, 1mm; matrix, 256×256; field of view, 3.5×3.5cm; and number of excitations, 4. An ROI was drawn at the same site as the voxel used for 1H MRS in the left lobe. The SI in the ROI was recorded for both fat selective images (\( \text{SI}_{\text{fat}} \)) and water selective images (\( \text{SI}_{\text{water}} \)). Liver FC was computed as follows:

\[ FC_{\text{CSI}} = \frac{100 \times \text{SI}_{\text{fat}}}{(\text{SI}_{\text{fat}} + \text{SI}_{\text{water}} \times R)} \]  

[3]

where \( R \) is the ratio of the fat to water proton densities in their pure form: a value \( R = 0.9 \) has been used in literature (25). The water fraction by volume of the sample is thus 1- FC.

**Histopathology**

Each mouse were perfused transcardially with phosphate-buffered saline, followed by freshly prepared 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4); Parts of liver were fixed, dehydrated, embedded and transversely sectioned into 5 μm pieces for hematoxylin and eosin staining. Other parts of liver were frozen in dry ice and then cryostat sectioned at a thickness of 7 um onto poly-l-lysine slides for lipid deposition analysis by Oil
Red O and hematoxylin staining.

All histopathology slides were examined and FC analysis was performed with a semi-automatic vacuole segmentation procedure (HIS-S) developed with the MATLAB software (The MathWorks, Natick, Mass.) as describe by Gaspard et al. (29). The artificial areas such as blood vessels were manually excluded by a pathologist. The percent fat fraction (FC_{HIS-S}) was calculated by following formula:

\[ FC_{HIS-S} = \frac{\text{the area of fat}}{\text{the total tissue area}} \tag{4} \]

**Liver Lipid (LL) Analysis**

Approximately 100 mg of liver (wet weight) was weighed and homogenized in 1 ml of PBS. Lipid was extracted by homogenizing with 2:1 chloroform-methanol (v/v) and separating into three phase by centrifugation, the upper, lower liquid phases and the middle of solid phase. The upper phase was removed as much as possible by siphoning and extracted once more. The lower phase was reserved and dried. Lipid was dissolved in 100 ul 3% Triton X-100. The determination of triglyceride was carried out using enzymatic methods as described by Folch et al. (30).

**Statistical Analysis**

All statistical analyses were performed using SPSS software (SPSS for Windows, version 11.0, 2001; SPSS, Chicago, IL). Numerical data were reported as mean values ± standard deviation (SD). For statistical comparisons, independent-sample \( t \) test, paired-sample \( t \) test and correlation test were applied. A \( P \) value of less than 0.05 was considered to indicate a statistically significant difference.

MR methods determined the volume fractions of lipids in the liver tissue and histological method calculated the percentage of hepatocytes showing visible fat droplets in the microscopic view, but the chemical method measured the TG content of fresh liver tissue. For statistical comparisons, correlation test was applied to assess the relationship between LL and FC measured by three MR and histological methods, both paired-sample \( t \) test and correlation test were applied to assess difference and relationship between MR and histological methods.
RESULTS

Quantitation in Fat/ Water Phantoms

Three MR-based methods of quantitative FC evaluation have been tested in fat/water phantoms (Fig. 1, 2). Results for fat and water quantitation are shown in Table 1. The dual-echo method significantly underestimated the fat concentration compared to the gravimetric reference standard when FC was in range of 10% to 100%. There is a weak correlation between FC calculated with dual-echo method and the known gravimetric reference standard \(r=0.03, P>0.05\). When the actual FC is smaller than 50% (water content higher than FC), fat concentration can be calculated using the formula: \[ FC_{IP,OP} = 100 \times \frac{SI_{IP} - SI_{OP}}{2 \times SI_{IP}} \] but when the actual FC is higher than 50% (water content lower than FC), fat concentration can be calculated using \[ W_{IP,OP} = 100 \times \frac{SI_{IP} - SI_{OP}}{2 \times SI_{IP}}, FC_{IP,OP} = 1 - W_{IP,OP}. \] With the visual analysis of \(^1\text{H} \) MRS fat–water peak size to guide for fat and water dominance on IP-OP images, the corrected IP-OP underestimated fat concentration when FC was in range of 10% to 60%: \((4.5\pm0.0)\%\), \((9.2\pm2.0)\%\), \((16.0\pm1.0)\%\), \((26.2\pm0.1)\%\), \((31.9\pm1.0)\%\) and \((56.1\pm0.2)\%\) respectively. There is a strong correlation \(r=0.972, P<0.01\) between FC calculated with corrected IP-OP \(FC_{IP,OP \text{ correction}}\) and the known gravimetric reference standard.

CSI underestimated fat concentration when FC was in range of 50% to 100%: \((48.0\pm1.0)\%\), \((57.0\pm0.5)\%\), \((67.3\pm0.6)\%\), \((77.3\pm0.6)\%\), \((83.3\pm0.6)\%\) and \((91.0\pm1.0)\%\) respectively \(P<0.05\). MRS overestimated slightly fat concentration when FC was in range of 80%: \((81.6\pm0.6)\% \) \(P<0.05\). CSI and MRS have a high linear correlation with the known gravimetric reference standard (CSI: \(r=0.998\), MRS: \(r=0.999\), \(P<0.01\)), but correlation between corrected IP-OP and the known FC is slightly weakest \(r=0.972, P<0.01\) (Fig. 3).

There are strong correlations between FC calculated with corrected IP-OP and MRS \(r=0.972, P<0.01\), between CSI and MRS \(r=0.999, P<0.01\), and between corrected IP-OP and CSI \(r=0.963, P<0.01\) (Fig. 3).

**In vivo MR imaging and \(^1\text{H} \) MRS of mice**

The liver FCs measured by all three MR methods (Fig. 4, 6), HIS-S (Fig. 5) and LL analysis (Fig. 6) are listed in Table 2. There are significant differences in liver FC between
ob/ob mice and WT mice and between SKO mice and WT mice by using all methods ($P<0.001$), with liver FC of the ob/ob mice and SKO mice all being higher than that of WT mice. Both in vivo T1-weighted imaging and in vitro liver volume calculation prove the liver volume of ob/ob and SKO mice being significantly larger than that of WT mice ($P<0.001$), and there is no significant difference between these two methods ($P=0.798$). Liver index (LI) was calculated as the volume of liver from MR and gravimetric measure divided by the total body weight (LI$_{MR}$, LI$_{GM}$). Both LI$_{MR}$ and LI$_{GM}$ of ob/ob and SKO mice were significantly higher than that of WT mice ($P<0.001$).

Liver FC measured by histological method and the corrected IP-OP is smaller than that by CSI and MRS ($P<0.001$), but no significant difference is observed between FC$_{IP\text{-}OP\text{ correction}}$ and FC$_{HIS\text{-}S}$ ($P=0.556$). Liver FC measured by CSI and MRS are also no significant difference ($P=0.230$) (Table 3). There is a strong correlation between FC calculated with histological method and three MR methods (corrected IP-OP: $r=0.882$, CSI: $r=0.984$, MRS: $r=0.978$ respectively, $P=0.000$); and also a significant linear correlation is observed between LL measured by chemical method and the FC calculated by all three MR methods and HIS-S (corrected IP-OP: $r=0.867$, CSI: $r=0.986$, MR spectroscopy: $r=0.977$, HIS-S: $r=0.960$, $P=0.000$)(Table 4, Fig.6).

A significant linear correlation is observed between liver FC calculated by three MR methods. The FC$_{CSI}$ and FC$_{MRS}$ correlations is slightly stronger (FC$_{CSI}$ vs. FC$_{MRS}$: $r=0.992$, $P<0.001$) than the FC$_{IP\text{-}OP\text{ correction}}$ and FC$_{CSI}$. FC$_{MRS}$ (FC$_{IP\text{-}OP\text{ correction}}$ vs. FC$_{CSI}$: $r=0.891$, FC$_{IP\text{-}OP\text{ correction}}$ vs. FC$_{MRS}$: $r=0.917$, $P=0.000$) (Table 4, Fig.6).
DISCUSSION

There are currently three different types of imaging techniques that allow noninvasive detection of liver FC: 1) ultrasound; 2) computed tomography (CT); and 3) MR methods. However, ultrasound is not considered as a sufficient quantitative tool for fat determination in liver. One major weakness of ultrasound is its operator dependency. CT requires undesired radiation exposure in the examinations of subjects (31). MR methods therefore become the most desirable and useful techniques. A number of MR methods have been proposed for the detection of fatty liver infiltration: 1) Fat-sensitive MR imaging techniques based on signal phase, two-point Dixon and three-point IDEAL, also known as iterative decomposition of water and fat with echo asymmetry and least-squares estimation; 2) Fat-selective MR imaging based on frequency selective excitation, chemical shift selective images, also called spectral fat or water selective imaging; 3) \(^1\)H-MRS (31). \(^1\)H MRS has been by far the most promising and most sensitive noninvasive method to assess liver FC (32).

In our study, liver FCs measured by two-point Dixon IP-OP, CSI and \(^1\)H-MR spectroscopy were compared. Two-point Dixon is a routine clinical method for the semi-quantitative assessment of liver FC. However, this method neglects \(T_2^*\) signal decay and assumes that the signal difference between IP and OP echoes is due to fat-water signal interference only. With OP-then-IP sequential acquisition, the confounding \(T_2^*\) effect is known to cause fat fraction underestimation (33-35), which is also shown in this study.

At 7T MR, the resonance frequencies of methylene and water have a separation of about 1000Hz, the first echo time of in-phase time is 0.5 ms, and of out-of-phase time is 1.0 ms. Our MR machine can only reach the echo time of in-phase time at 1.5 ms and out-of-phase time at 2.0 ms. The corresponding in-phase and out-of-phase image signals were thus lower and the difference between them became smaller (12). Our results agreed with our expectation by proving that two-point Dixon method systematically resulted in an underestimation of FC in both phantom and \emph{in vivo} evaluations, and the degree of underestimation increased along with increasing FC.

In an in-phase image, the fat and water signals within a voxel are additive; in an opposed-phase image, they are subtracted from each other. The decrease in signal intensity from the in-phase to an opposed-phase image indicates the presence of both water and fat.
within a voxel. When the actual FC is higher than 50% (water content lower than FC), fat concentration were corrected through above formulate. But IP-OP method also presented greater underestimation compared to other methods due to the higher FC in the liver of ob/ob and SKO mice.

CSI includes two series, selective fat-protons imaging and selective water-protons imaging. Our CSI method was derived partly from the method described by Sbandrea et al. (16), who evaluated the accuracy of CSI on brown adipose tissue in rats using a 4.7T MR scanner. In phantoms, we proved using a 7T MR system that CSI was accurate in lipid quantification when FC was lower than 50%, and approximately 9% underestimation of fat occurred from 50% to 100% of FC. Since centers of excitation frequency are methylene and water proton, the degree of FC underestimation increased in higher FC. Liver FC usually is lower than 50%, CSI is thus able to not only accurately measure the FC, but also evaluate its distribution.

Besides liver histological and chemical analysis, as current standard for diagnosis and grading of hepatic FC, 1H MRS also allows non-invasive studies in the molecular composition of tissues in vivo. 1H MRS recorded from liver tissue usually shows two dominant signal portions, namely the water signal (positioned at 4.7 ppm) and the signal from methylene protons of fatty acids (positioned at 1.3 ppm). In 1H MRS, peak area is proportional to concentration of the metabolite containing the relevant nuclei which is also influenced by T1 and T2 relaxation times (36). To minimize T1 effects, one sequence with a long repetition time (10 s) and one sequence with 1.8 s of repetition time were used by Strobel et al (26). A correction factor was also used on the difference of the two spectra. In order to correct T2 relaxation, a sequence with a series of different echo times was used to calculate the spin-spin relaxation time (T2). We also used a series of different echo time to correct the spectral data. In phantom experiments, we proved that there was more accurate that quantifying FC with MRS method than with CSI when FC was in range of 50% to 100%. MRS allows quantifying FC in different tissues, and CSI allows identifying the fat distribution. Therefore, CSI and MRS should be used together in order to evaluate liver FC comprehensively.

Interestingly, the percentage values of liver FC from MRS and CSI were slight different
compared to HIS-S examination. Gaspard et al. suggested that this might be due to the fact that MR techniques determined the volume fractions of lipids in the liver tissue rather than the percentage of hepatocytes showing visible fat droplets in the microscopic view (29). In addition, this discrepancy might also be explained by the difficulty of quantifying microvesicular fat. In fact, automated software estimated the area occupied by fat droplets with a minimum diameter of 5 μm. Fat existing in droplets of diameters less than 5 μm was therefore not included. Since the histological image was obtained by light transmitting through the slice, droplets failed to pass through the slice completely would not be quantified.

Similarly, the results obtained by automated calculation software from histological slides and by isolation and purification of LL were not comparable to each other because the automated software measured the specific surface area of fat droplets in the microscopic field, whereas LL analysis measured the quantity of lipid. However, the data of LL chemical analysis and CSI and 1H MRS correlated very well. This may be because volume fractions of lipid in liver tissue were measured in MR methods and the TG content of fresh liver tissue was detected in chemical method, rather than surface area of fat droplets in the HIS-S method.

In addition to the above mentioned MR methods, traditional T1-weighted imaging method was also used to observe the anatomic details of liver, calculate liver volume, and identify the distribution of fat in subcutaneous and internal organs (data not shown). Ob/ob mice had significantly larger liver than other groups, indicating that severity of the fatty liver positively correlate with the liver volume.

This study provided some information about fatty liver that may be useful when ob/ob mice are used as an animal model of disease. The liver is the organ most often involved in fat accumulation. In its parenchyma, the fat depot appears to be homogeneous, and it is quantified by in vivo localized spectroscopy, MR imaging and by anatomical histological and chemical surveys. Considering that ob/ob mice are a model for hepatic steatosis and SKO mice are frequently with fat accumulation in liver, 1H MR spectroscopy and imaging seem to be promising tools to follow the time course of fat accumulation.

The limitations of our study must be acknowledged. Firstly, the number of mice used in the experiments was fairly small. Secondly, the amount of fat measured by HIS-S and liver lipid analysis might not be representative of the amount of fat throughout the entire liver (9,
Thirdly, the FC measured using our MR based methods represents the signal derived from protons contained in fatty acid molecules, as opposed to the weight of lipid per unit of liver volume.

CONCLUSION

T₁WI can be used to measure liver volume and observe fat distribution; IP-OP method gives a FC that is significantly lower than the actual value, especially in higher fat concentrations; CSI and ¹H MRS are accurate in quantifying fat in both phantoms and liver in vivo. Given their excellent correlation and concordance with LL analysis, CSI and MRS may replace liver fat histological and chemical analysis in longitudinal studies.
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REFERENCES


CAPTIONS FOR ILLUSTRATION

**Figure 1:** Quantitative MR imaging of diluted fat/water phantoms. **A:** chemical shift imaging (selective fat protons imaging and selective water protons imaging, CSI-fat and CSI-water); **B:** Dixon dual-echo MR imaging (in-phase and out-of-phase).

**Figure 2:** $^1$H-MRS of fat/water phantoms (A) and results of fat content calculated by using IP-OP, corrected IP-OP, CSI, and MRS (B).

**Figure 3:** Graph shows correlation between known FC and FC measured by CSI ($r=0.998$, $P<0.01$, A), and MRS ($r=0.999$, $P<0.01$, B) in phantom; and relationship between FC$_{\text{CSI}}$ and FC$_{\text{MRS}}$ ($r=0.998$, $P<0.01$, C).

**Figure 4:** *In vivo* Dixon dual-echo IP-OP MR imaging, CSI and $^1$H MRS in mice liver.

**Figure 5:** Histology of liver (scale bar, 20 μm). **A:** H&E staining ($\times400$); **B:** Oil Red O staining ($\times400$).

**Figure 6:** *In vivo* and *in vitro* measurements of liver FC and LL in mice. **A, B:** liver FC measured by IP-OP, CSI and $^1$H MRS *in vivo* and by HIS-S *in vitro* from ob/ob and SKO mice, respectively; **C:** LL calculated by chemical method. Graph shows correlation between Liver FC by chemical method and FC measured by CSI ($r=0.986$, $P<0.001$, D), and MRS ($r=0.977$, $P<0.001$, E); and relationship between CSI and MRS ($r=0.992$, $P<0.001$, F).
Table 1: Known gravimetric FC and measured FC by MR imaging and MR spectroscopy in phantoms.

<table>
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<tr>
<th>Known FC (%)</th>
<th>FC&lt;sub&gt;IP-OP&lt;/sub&gt; (%)</th>
<th>P</th>
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<th>FC&lt;sub&gt;CST&lt;/sub&gt; (%)</th>
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<td>9.6±2.0</td>
<td>0.085</td>
<td>20.0±1.0</td>
<td>1.000</td>
<td>19.7±0.7</td>
<td>0.585</td>
</tr>
<tr>
<td>30</td>
<td>16.0±1.0</td>
<td>0.033*</td>
<td>16.0±1.0</td>
<td>0.033*</td>
<td>29.0±1.0</td>
<td>0.184</td>
<td>29.4±0.3</td>
<td>0.363</td>
</tr>
<tr>
<td>40</td>
<td>26.2±0.1</td>
<td>0.004*</td>
<td>26.2±0.1</td>
<td>0.004*</td>
<td>39.0±1.0</td>
<td>0.225</td>
<td>40.7±1.2</td>
<td>0.452</td>
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<tr>
<td>50</td>
<td>31.9±1.0</td>
<td>0.029*</td>
<td>31.9±1.0</td>
<td>0.029*</td>
<td>48.0±1.0</td>
<td>0.038*</td>
<td>50.6±1.7</td>
<td>0.638</td>
</tr>
<tr>
<td>60</td>
<td>43.9±0.2</td>
<td>0.007*</td>
<td>56.1±0.2</td>
<td>0.016*</td>
<td>57.0±0.5</td>
<td>0.015*</td>
<td>60.8±1.2</td>
<td>0.363</td>
</tr>
<tr>
<td>70</td>
<td>37.4±1.8</td>
<td>0.025*</td>
<td>62.6±1.8</td>
<td>0.073</td>
<td>67.3±0.6</td>
<td>0.015*</td>
<td>71.2±0.9</td>
<td>0.160</td>
</tr>
<tr>
<td>80</td>
<td>22.0±0.3</td>
<td>0.002*</td>
<td>77.0±0.3</td>
<td>0.063</td>
<td>77.3±0.6</td>
<td>0.015*</td>
<td>81.6±0.6</td>
<td>0.045*</td>
</tr>
<tr>
<td>90</td>
<td>2.6±1.0</td>
<td>0.006*</td>
<td>97.4±1.0</td>
<td>0.072</td>
<td>83.3±0.6</td>
<td>0.002*</td>
<td>91.6±1.6</td>
<td>0.215</td>
</tr>
<tr>
<td>100</td>
<td>0.4±0</td>
<td>0.000*</td>
<td>99.6±0</td>
<td>0.258</td>
<td>91.0±1.0</td>
<td>0.008*</td>
<td>1</td>
<td>1.000</td>
</tr>
</tbody>
</table>

All values are means ± SD. Statistical analysis was done with independent-samples t test. n represents number of tested phantoms, n = 3. *P<0.05.
Table 2: Liver FC measured by IP-OP, CSI, MR spectroscopy, HIS-S and LL, and volume of liver and LI obtained by MR $T_1$-weighted imaging and volumetric methods in *ob/ob* and WT mice

<table>
<thead>
<tr>
<th></th>
<th><em>ob/ob</em></th>
<th>WT</th>
<th><em>P</em></th>
<th>SKO</th>
<th>WT</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>FC_{IP-OP} (%)</td>
<td>23.62±5.18</td>
<td>1.50±1.90</td>
<td>0.000*</td>
<td>2.90±0.30</td>
<td>1.70±1.40</td>
<td>0.000*</td>
</tr>
<tr>
<td>FC_{CSI} (%)</td>
<td>54.44±5.02</td>
<td>4.90±1.10</td>
<td>0.000*</td>
<td>17.66±4.45</td>
<td>3.04±0.80</td>
<td>0.000*</td>
</tr>
<tr>
<td>FC_{MRS} (%)</td>
<td>55.83±7.01</td>
<td>3.30±0.99</td>
<td>0.000*</td>
<td>14.78±4.10</td>
<td>1.31±0.88</td>
<td>0.000*</td>
</tr>
<tr>
<td>FC_{HIS-S} (%)</td>
<td>23.03±2.59</td>
<td>0.53±0.19</td>
<td>0.000*</td>
<td>7.81±0.89</td>
<td>0.49±0.18</td>
<td>0.000*</td>
</tr>
<tr>
<td>LL (mg/g)</td>
<td>55.30±4.3</td>
<td>5.40±0.78</td>
<td>0.000*</td>
<td>18.50±6.42</td>
<td>4.81±0.93</td>
<td>0.000*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>47.50±1.54</td>
<td>26.00±0.71</td>
<td>0.000*</td>
<td>21.80±1.61</td>
<td>22.60±1.25</td>
<td>0.000*</td>
</tr>
<tr>
<td>Volume_{MR} (mm$^3$)</td>
<td>4260±547</td>
<td>1503±199</td>
<td>0.000*</td>
<td>2100±221</td>
<td>922±183</td>
<td>0.000*</td>
</tr>
<tr>
<td>Volume_{GM} (mm$^3$)</td>
<td>4397±416</td>
<td>1410±205</td>
<td>0.000*</td>
<td>1720±328</td>
<td>1109±123</td>
<td>0.000*</td>
</tr>
<tr>
<td>LI_{MR}</td>
<td>89.54±9.22</td>
<td>56.77±6.85</td>
<td>0.000*</td>
<td>96.77±13.40</td>
<td>40.79±7.79</td>
<td>0.000*</td>
</tr>
<tr>
<td>LI_{GM}</td>
<td>92.45±6.10</td>
<td>54.25±7.68</td>
<td>0.000*</td>
<td>78.95±6.19</td>
<td>49.04±2.94</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

All values are means ± SD. Statistical analysis was done by independent-samples $t$ test. *P*<0.05. Volume_{MR} (Volume from MR measure), Volume_{GM} (Volume from gravimetric measure), LI_{MR} (Volume from MR measure/Body weight), LI_{GM} (Volume from gravimetric measure/Body weight).
Table 3: Differences between MR and histological HIS-S were measured for mice.

<table>
<thead>
<tr>
<th>Parameter 1</th>
<th>Parameter 2</th>
<th>$t$, $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{\text{CHI-S}}$</td>
<td>$F_{\text{IP-OP}}$</td>
<td>-0.600, 0.556</td>
</tr>
<tr>
<td></td>
<td>$F_{\text{CSI}}$</td>
<td>4.394, 0.000*</td>
</tr>
<tr>
<td></td>
<td>$F_{\text{MRS}}$</td>
<td>3.548, 0.002*</td>
</tr>
<tr>
<td>$F_{\text{MRS}}$</td>
<td>$F_{\text{IP-OP}}$</td>
<td>-3.695, 0.002*</td>
</tr>
<tr>
<td></td>
<td>$F_{\text{CSI}}$</td>
<td>2.494, 0.230</td>
</tr>
<tr>
<td>$F_{\text{CSI}}$</td>
<td>$F_{\text{IP-OP}}$</td>
<td>4.432, 0.000*</td>
</tr>
</tbody>
</table>

Statistical analysis was done with paired-samples $t$ test. $^*P < 0.05.$
Table 4: Correlations between MR, histological HIS-S and chemical LL analysis were measured for mice.

<table>
<thead>
<tr>
<th>Parameter 1</th>
<th>Parameter 2</th>
<th>r, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC_{HIS-S}</td>
<td>FC_{IP-OP}</td>
<td>0.882, 0.000*</td>
</tr>
<tr>
<td>FC_{CSI}</td>
<td>FC_{CSI}</td>
<td>0.984, 0.000*</td>
</tr>
<tr>
<td>FC_{MRS}</td>
<td>FC_{CSI}</td>
<td>0.978, 0.000*</td>
</tr>
<tr>
<td>LL</td>
<td>FC_{IP-OP}</td>
<td>0.867, 0.000*</td>
</tr>
<tr>
<td></td>
<td>FC_{CSI}</td>
<td>0.986, 0.000*</td>
</tr>
<tr>
<td></td>
<td>FC_{MRS}</td>
<td>0.977, 0.000*</td>
</tr>
<tr>
<td></td>
<td>FC_{HIS-S}</td>
<td>0.960, 0.000*</td>
</tr>
<tr>
<td>FC_{MRS}</td>
<td>FC_{IP-OP}</td>
<td>0.917, 0.000*</td>
</tr>
<tr>
<td></td>
<td>FC_{CSI}</td>
<td>0.992, 0.000*</td>
</tr>
<tr>
<td>FC_{CSI}</td>
<td>FC_{IP-OP}</td>
<td>0.891, 0.000*</td>
</tr>
</tbody>
</table>

Statistical analysis was done with correlation test. *P < 0.05.
Figure 1
Figure 2

A

$^1$H-MRS in phantsoms

B

Calculated Fat Content [%] vs Known Fat Content [%]

- Known Fat Content
- MRS data
- CSI data
- IP-OP-data
- IP-OP-correction

0 10 20 30 40 50 60 70 80 90 100

0 20 40 60 80 100
Figure 3
Figure 4
Figure 5
Figure 6