MRI versus histological methods for time course monitoring of steatosis amount in a murine model of NAFLD

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KEYWORDS
Magnetic resonance imaging; Animal model; Fatty liver; Histology; Quantification

Abstract
Purpose: Hepatic steatosis is an increasingly frequent disease with potentially severe complications. A simple quantification method is required for pretherapeutic studies to allow steatosis monitoring. This study aimed at evaluating steatosis quantification via a standard 1.5 T MRI machine in a murine model.

Materials and methods: Eleven groups of two rats received a choline methionine deficient diet. MRI was performed at days 0, 2, 4, 5, 6, 7 and 8, and weeks 2, 3, 4 and 5. A phased array surface coil system was used to acquire a GE T1 in- and out-of-phase multi-echo sequence,

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Nonalcoholic fatty liver disease (NAFLD) is increasingly frequent among adults and children. Its prevalence reaches 20–30% in the general population and 75–100% in obese populations [1–3]. Adipose tissue is commonly associated with insulin resistance and it is now widely accepted that fatty liver is an element of metabolic syndrome (MS) [4,5]. Abdominal fat is also a risk factor for a subset of cancers [6]. Furthermore, NAFLD and insulin resistance in hepatocytes share molecular characteristics [7–10]. NAFLD encompasses a wide spectrum of liver lesions, ranging from bland steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and finally cirrhosis with its potentially fatal complications: hepatocellular carcinoma, liver failure, variceal bleeding, and renal failure. Steatosis is present across the entire spectrum of lesions observed in NAFLD. Studies have previously shown that liver steatosis amount is associated with NASH, fibrosis, and metabolic syndrome components in NAFLD [11].

Thus, early management of steatosis is crucial and new therapies are indeed in the pipeline. To evaluate these new treatments in in vivo clinical studies, researchers need murine induced liver steatosis models and a fast, easy, and efficient steatosis quantification technique without mouse sacrifice to permit monitoring.

Several animal models have been developed to obtain steatosis [12]. They work by increas- ing lipids import or synthesis [13,14], affecting methionine metabolism [15], increasing lipid uptake [16–19] and finally by decreasing export of VLDL particles and, to a lesser extend, beta-oxidation with a choline methionine deficient diet (CMDD) [20,21], which is known as a NASH model in rats. However, a protocol to obtain graded steatosis has never been published and obtaining pure steatosis without inflammation has not been described with this model.

Steatosis is usually quantified by standard semi-quantitative histological grading on liver biopsy with visual analysis, evaluating the percentage of hepatocytes containing lipid vacuoles. We have developed another histological method, called area of steatosis (AOS), for the automated and more reliable measurement of lipid vacuole surface on liver specimens [11]. However, obtaining liver histology samples from rats requires their sacrifice and therefore repeated measurements for monitoring are not possible. Moreover, histology suffers from sampling variability [22]. To resolve these difficulties, non-invasive methods for hepatic steatosis quantification have been developed. In humans studies, notwithstanding the utility of ultrasound, MRI is considered to be the most pertinent method [23,24]. Several techniques are applicable [25] including the “3-echoes” method achieved through an “in- and out-of-phase” T1 sequence [26,27]. Most of the studies that validated MRI as a steatosis quantification tool used spectroscopy or histological scores as a reference. To our knowledge, none used total intrahepatic triglycerides (TIT), although it is expected to be the best reference because TIT are indeed what all the techniques aims to quantify. Finally, to broaden the utility of MRI as a steatosis quantification tool, it must be feasible on a standard machine, with neither cardiac nor respiratory synchronization.

The aim of this study was to evaluate hepatic steatosis quantification using a standard 1.5T MRI device, in comparison to classical optical evaluation of steatosis by a pathologist and to AOS, using TIT as reference.

**Methods and materials**

**Animals**

The protocols employed in our laboratory were approved by the French Ministry for Higher Education and Research in conformity with European legislation for research involving animals under the reference number CEEA.2011.1 of the Ethics Committee.

Twenty-two 7 to 8-week-old Sprague Dawley rats (Animalerie hospitalo-universitaire, Angers, France) weighting approximately 280 g were used.

**Experimental model and design**

Steatosis was induced by a specific nutritional regimen based on the choline methionine deficient diet (CMDD; Safe-Diets Inc., Augy France [28]). The rats were housed...
in a polycarbonate cage with a 12-hour light/12-hour dark cycle and controlled temperature (22 ± 2 °C) and humidity (50 ± 10%). They had ad libitum access to food and tap water. The population was divided into eleven experimental groups of 2 rats. Ten groups were subjected to the CMDD during respectively 2, 4, 5, 6, 7 and 8 days and 2, 3, 4 and 5 weeks in order to obtain increasing levels of steatosis. The remaining group was used as a control.

MRI was performed the evening of the last diet day, after a 12-hour fasting period. On the morning following the MRI, the animals were anesthetized with a mixture of O2 and isoflurane 2.5% for an intracardiac blood sample, then sacrificed by asphyxiation in a CO2-saturated atmosphere. The livers were removed, weighed and sampled for histological assessment. Additional samples were snap frozen in liquid nitrogen for later measurement of TIT.

MRI evaluation

1.5T MRI (General Electrics Excite, Milwaukee, IL) was carried out using a phased array knee coil, with neither cardiac nor respiratory synchronization. The examinations were performed under general anesthesia (O2 and isoflurane 2.5% applied with a handmade gas mask). There was no contention and no respiratory assistance. Rats are in ventral decubitus.

A GE T1 ‘in- and out-of-phase’ multi-echo sequence was performed covering the whole liver, with the following parameters: flip angle = 40°, time of echo = 2.5/4.6/6.7/8.8 ms, DFOV = 12 × 12 cm, matrix of 256×256, thickness = 4 mm, spacing = 4.5 mm, number of slices = 3, acquisition time = 32 s. No cardiac or respiratory gating was needed.

Steatosis quantification was performed on a medical workstation (Synapse, Fuji, Tokyo) by a trained practitioner.

Two operators realized the measures separately. The largest sharp middle liver slice was selected and a free-hand region of interest (ROI) covering the maximum of the liver surface was drawn regardless of vessels except for the inferior vena cava (Fig. 1). The same ROI was used to measure the mean signal value on the 2nd, 3rd and 4th echo. Then steatosis was calculated with the 3-echoes method [29]:

\[
100 \times \frac{[\text{mean}(2\text{nd} + 4\text{th}) - 3\text{rd}]}{[2 \times \text{mean}(2\text{nd} + 4\text{th})]}
\]

All steatosis quantification results are expressed as percentages.

Biochemical analysis

Glucose concentrations in blood serum were measured using a glucometer, and total cholesterol, HDL and LDL cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT) were measured using enzymatic assay kits on a Hitachi 917 automatic analyzer (Roche). Liver triglycerides were determined using the method described by Xu et al. [30]. Briefly, 100 mg of liver tissue was homogenized in 1 ml of ethanol then centrifuged at 15,000 × g for 10 min. TG were measured with a kit (Sigma — Aldrich, St. Louis, MO). Results are expressed as percentages.

![Figure 1](image-url)  MRI steatosis quantification with the 3-echoes method in a rat undergoing a choline methionine deficient diet (CMDD). Rats are in ventral decubitus. Largest sharp middle liver slice, the same region of interest covering the maximum of the liver surface regardless of vessels except for the inferior vena cava, was used to measure the mean signal value on the 2nd, 3rd and 4th echo at respectively 4.6 (a), 6.7 (b) and 8.8 ms (c). Values are expressed in arbitrary unit.
Histological analysis

Liver samples were fixed in 4% buffered formal saline and embedded in paraffin wax. Five-μm thick sections were stained with hematoxylin-eosin-saffron (HES) and 0.1% picrosirius red solution and used for both optical and image analysis.

Optical analysis

Steatosis was calculated as the percentage of hepatocytes containing lipid vacuoles. Microvesicular or macrovesicular steatosis was noted. Inflammation was graded as follows: 0: no inflammation, 1: mild, 2: moderate or severe.

Area of Steatosis

Area of steatosis was measured as previously described [31,32]. Briefly, for image acquisition, we used an Aperio digital slide scanner (Scanscope CS System, Aperio Technologies, Vista CA 92081, USA) image processor that provided high quality 30,000 × 30,000 pixel images at a resolution of 0.5 μm/pixel (magnification ×20). Binary images (white for steatosis and black for the remaining liver tissues) were obtained via an automatic thresholding technique using an algorithm developed in our laboratory. The entire specimen area was analyzed. AOS (%) was calculated as the ratio: area of steatosis vesicles/complete liver surface.

Statistics

Correlations were calculated using Spearman’s coefficient (R), and agreement using intra-class correlation coefficient (ICC). A value of p < 0.05 was considered as statistically significant. Statistical evaluations were performed using SPSS software v18.0 (IBM, Armonk, NY, USA). A Bland Altman analysis was performed between MRI, AOS, Histology and the TIT.

Results

Non-invasive quantification of steatosis

All the MRI acquisition provided at least one slice with visually good quality and sharpness. The mean signal to noise ratio was 25.7.

TIT, our reference for liver steatosis, was measured at 6.6% in the control rats. Only one rat (#21), which received the CMDD during 5 weeks, presented steatohepatitis (grade 1). MRI quantification was possible for all rats. The agreement between the two observers was excellent: ICC = 0.998 [0.996–0.999].

We first pooled all the results obtained at the various time points to evaluate the correlations between the different methods for steatosis evaluation. MRI and AOS correlated better with TIT than did optical analysis, with respective R of 0.897, 0.889 and 0.753 (all p < 0.001) (Table 1 and Fig. 3). However, agreement with TIT was better for MRI (0.889, p < 0.001) compared to AOS (0.629, p = 0.001) or optical analysis (0.280, p = 0.098). By stepwise multiple linear regression with TIT as the dependent variable and MRI, AOS and optical analysis as independent variables, only MRI was identified as an independent predictor of TIT. Taken together, the results suggested that steatosis amount, as reflected by TIT, was better assessed by MRI.

Table 1 Correlations between methods for quantification of liver steatosis.

<table>
<thead>
<tr>
<th>Method</th>
<th>R</th>
<th>p</th>
<th>ICC</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>TIT vs optical analysis</td>
<td>0.753</td>
<td>&lt;0.001</td>
<td>0.280</td>
<td>0.098</td>
</tr>
<tr>
<td>TIT vs AOS</td>
<td>0.889</td>
<td>&lt;0.001</td>
<td>0.629</td>
<td>0.001</td>
</tr>
<tr>
<td>TIT vs MRI</td>
<td>0.897</td>
<td>&lt;0.001</td>
<td>0.889</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MRI vs optical analysis</td>
<td>0.739</td>
<td>&lt;0.001</td>
<td>0.344</td>
<td>0.054</td>
</tr>
<tr>
<td>MRI vs AOS</td>
<td>0.891</td>
<td>&lt;0.001</td>
<td>0.797</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Optical analysis vs AOS</td>
<td>0.892</td>
<td>&lt;0.001</td>
<td>0.605</td>
<td>0.001</td>
</tr>
</tbody>
</table>

R: Spearman correlation coefficient; ICC: intra-class correlation coefficient; TIT: total intrahepatic triglycerides; AOS: area of steatosis

Monitoring of steatosis time course

According to TIT results, steatosis started to increase progressively at day 7 (11%) of the CMDD diet to reach 33.4% after 5 weeks (Fig. 2). The accuracy of optical analysis for the evaluation of steatosis progression was poor (Fig. 2): steatosis quantification went directly from 0% during the first week to 90% at day 7, then continued giving high values between 80 and 95%. This analysis showed macro vacuoles (except for rat #4 in the 2-day group, which had microvacuoles) and thus a high optical steatosis value (80%) whereas TIT remained at a normal level (9.3%). In comparison, AOS permitted closer monitoring of steatosis (Fig. 2) but the method underestimated steatosis when TIT was < 20% and overestimated it when TIT was above this threshold (Figs. 2 and 3b).

Finally, MRI offered the best performance for monitoring, with a time curve very close to TIT (Fig. 2). MRI evaluated steatosis initially at 8.2% with a slight increase during the first week. Then, MRI quantification started to increase progressively to reach 37% after 5 weeks of the CMDD diet.

Blood markers

Blood cholesterol, LDL and triglycerides levels were significantly correlated with TIT with respective R of −0.799, −0.771 and −0.761 (all p < 0.001). Liver enzymes were also significantly correlated with TIT, with respective R for AST and ALT of 0.458 (p = 0.032) and 0.585 (p = 0.004) (Table 2 and Fig. 4).

Cholesterol, LDL and triglycerides decreased with diet duration whereas liver enzymes increased after week 2. No significant modification of HDL, γGT or glycemia was found.

By stepwise multiple linear regression including MRI and blood markers for the non-invasive quantification of steatosis, independent predictors of TIT were MRI (1st step) and ALT (2nd step). The regression formula of the multivariate analysis was: (0.905*MRI result) − (0.019*ALT [IU/L]) + 1.931. Steatosis estimation using this model was very well correlated with TIT (Fig. 5) with R = 0.910 (p < 0.001) and
Figure 2. Steatosis quantification results with each technique, for every rat, increasing with diet duration. The reference, total intrahepatic triglycerides (TIT), is represented by the solid black line and MRI the dotted line. The dotted gray line is visual optical quantification and the solid gray line is automated area of steatosis (AOS).

Figure 3. Correlation graphs between total intrahepatic triglycerides (TIT) and MRI ICC = 0.889; p < 0.001 (a), area of steatosis (AOS) ICC = 0.629; p = 0.001 (b), and visual histology ICC = 0.280; p = 0.098 (c). Bland Altman analysis between MRI and TIT (d).
Table 2  Correlations between total intrahepatic triglycerides and blood markers.

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>−0.247</td>
<td>0.268</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>−0.799</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL</td>
<td>−0.067</td>
<td>0.766</td>
</tr>
<tr>
<td>LDL</td>
<td>−0.771</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>−0.761</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST</td>
<td>0.458</td>
<td>0.032</td>
</tr>
<tr>
<td>ALT</td>
<td>0.585</td>
<td>0.004</td>
</tr>
</tbody>
</table>

R: Spearman correlation coefficient.

Figure 4. Correlation between total intrahepatic triglycerides (TIT) and ALT $R = 0.585; p = 0.004$.

ICC = 0.952 ($p < 0.001$) and permitted very close monitoring of steatosis evolution over time (Fig. 2).

Discussion

Steatosis indeed occurs in over nutrition but it is mainly due to an imbalance between dietary practices and today’s increasingly sedentary lifestyle and has thus become a major health problem worldwide [33]. The consequences of the disease, including roles in insulin resistance and the possible evolution to steatohepatitis and fibrosis, are well known now, but the mechanisms behind them remain incompletely understood.

Murine models are thus needed to gain a better understanding of the pathology and its diverse elements, and to make therapeutic studies possible.

The choline methionine deficient diet (CMDD) is a NASH model that results in high values of steatosis and hepatitis. In this study we demonstrated that CMDD can be used to create increasing degrees of steatosis free from inflammation in a relatively short period.

Via this gamut of steatosis thus created, we were able to evaluate different steatosis quantification techniques at different levels of steatosis in good conditions.

The classical visual evaluation of steatosis measures the percentage of hepatocytes containing lipid vacuoles. However, vacuoles appear when steatosis starts to increase, and in our study, the result furnished by the test leaped directly from 0 to nearly 100%. This “all or nothing” result, with no ability to illustrate progression, is thus clearly unsuited for the precise measurement of steatosis, and even more so for the demonstration of small variations regarding a therapeutic effect. This can explain the variability of the results in the D7 group with histology: as steatosis starts to raise, lipids vacuoles appears but are neglected by the anatomopathologist because there are too small until they reach simultaneously a sufficient size. On the opposite, the same difficulty can also explain the variability in the D2 group as the rat with high results of steatosis had microvacuoles in 80% of hepatocytes whereas they did not contain much lipids or represent a significant surface with AOS.

AOS was well correlated with TIT in our study, and may well be the best histological technique for quantifying steatosis. However we did observe that AOS tended to overestimate steatosis. This observation may be explained by the presence of vacuoles containing more than just lipids or by the modification of vacuole size during histological preparation.

Finally, among the different techniques tested in our study, MRI provided the best correlation with TIT. Its correlation was furthermore highly statistically significant despite the small number of subjects. MRI also offers the additional advantage of being non-invasive.

Our results confirmed the poor reliability of biological markers for evaluating steatosis. Biological markers could,
in principle, be used in conjunction with MRI, but it is our opinion that any possible improvement in results would not be worth the price and complexity of the test.

The use of a standard 1.5 T MRI obliges a compromise. To limit T1 pollution in quantification, authors have recommended using a small flip angle, usually 20° [34,35]. However in our study, due to the small size of the rats, we needed to use a 40° flip angle to increase the signal. The use of a 3 T MRI device or a dedicated surface coil would permit a smaller flip angle.

However, our goal in the present work was to present a convenient way to quantify steatosis in important cohorts of rats with non-specific equipment. In the technique described here, examination times are short, a standard 1.5 T machine is sufficient and there is no need for respiratory synchronization. We also assumed several technical imperfections to avoid dealing with the multiple chemical profiles of fat. Similarly, we did not test more precise techniques, such as the correction of confounding factors or synchrotron Fourier transform infrared microspectroscopy, which are much more difficult to implement and to handle [36,37].

Conclusion

In conclusion, liver steatosis in rat models can be easily and precisely quantified with a standard 1.5 T MRI device. MRI not only eliminates the need for animal sacrifice, thus enabling monitoring, it also correlates better with total liver triglycerides than histological quantification methods.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

References


