Applications of magnetic resonance spectroscopy for noninvasive assessment of hepatic steatosis

van Werven, J.R.

Citation for published version (APA):
NONINVASIVE QUANTIFICATION OF HEPATIC STEATOSIS IN RATS USING 3.0T $^1$H-MAGNETIC RESONANCE SPECTROSCOPY

Hendrik A. Marsman
Jochem R. van Werven
Aart J. Nederveen
Fiebo J.W. ten Kate
Michal Heger
Jaap Stoker
Thomas M. van Gulik

ABSTRACT

Purpose:
To assess the accuracy of noninvasive 3.0 T $^1$H-MRS in an experimental steatosis model for the discrimination of clinically relevant macrovesicular steatosis degrees and to evaluate three different $^1$H-MR spectrum-based fat quantification methods.

Materials and methods:
Steatosis was induced in rats by a methionine-choline deficient diet during 0-5 weeks. $^1$H-MRS measurements of hepatic fat content were compared with histopathological and biochemical steatosis degree. In $^1$H-MR spectra, areas under the curve (AUC) of fat (1.3ppm), water (4.7ppm), total fat (0.5-5.3 ppm), and total spectrum peaks (0.5-5.3ppm) were determined and hepatic fat content calculated as follows; $\frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}}$, $\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{fat}} + (\text{AUC}_{\text{water}}/0.7)}$, and $\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}$.

Results:
A significant correlation was found between $^1$H-MRS and macrovesicular steatosis ($r=0.932, p<0.0001$) and between $^1$H-MRS and total fatty acids ($r=0.935, p<0.0001$). $^1$H-MRS accurately distinguished mild from moderate and moderate from severe steatosis. Calculations using $\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}$ ratio in severe steatotic livers resulted in higher hepatic fat percentages as compared to the other methods due to a decrease in hepatic water content.

Conclusion:
$^1$H-MRS quantification of hepatic fat content showed high correlations with histological and biochemical steatosis determination in an experimental steatosis rat model, and accurately discriminated between clinically relevant steatosis degrees. These results encourage further application of $^1$H-MRS in patients for accurate steatosis assessment.
INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by increased lipid accumulation in the liver and is strongly associated with the metabolic syndrome (obesity, diabetes, and dyslipidemia) [1]. In the Western world NAFLD is currently considered the most common chronic liver disease, affecting up to 30% of the general population [1] and 90% of morbidly obese patients undergoing bariatric surgery [2]. The disease spectrum of NAFLD ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), which can further progress into fibrosis, cirrhosis, and ultimately into end-stage liver disease, posing a significant burden on liver transplantation waiting lists. Moreover, clinical studies identified severe macrovesicular steatosis (>66%) as an exclusion criterion for patients requiring major liver resection because of the concomitant high risk of postoperative complications [3,4]. In living donor liver transplantation (LDLT) procedures steatosis is considered even more deleterious, posing a risk for both the donor and the recipient. Consequently, moderate (33-66%) macrovesicular steatosis constitutes a preclusion criterion for LDLT [5,6]. It is therefore recommended to routinely perform a liver biopsy for steatosis assessment, in LDLT donors in particular [5-7]. However, Brown et al. [8] reported that of 42 U.S. centers performing LDLTs, only 14% routinely perform a liver biopsy, 60% in select cases, and 26% never perform liver biopsies. Although reasons to refrain from implementing this procedure were not investigated, it is likely that the invasive nature and the associated risks [9] for the living donor have led to abandonment of this procedure in several centers. Furthermore, histological assessment of a needle biopsy specimen is potentially inaccurate since heterogenic manifestation of hepatic steatosis can lead to underscoring of the degree of steatosis or to false-positive results [10].

Noninvasive modalities such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) have been employed for the assessment of hepatic steatosis [11-13]. However, these modalities do not specifically measure hepatic fat content, are semi-quantitative, and lack high sensitivity and specificity [13]. $^1$H-magnetic resonance spectroscopy ($^1$H-MRS) has been studied in the experimental and clinical setting as an alternative for the noninvasive detection of hepatic steatosis [14-19]. $^1$H-MRS measures the resonance signals derived from protons in triglycerides, which can be quantified and used as a noninvasive measure for the degree of steatosis. No studies have yet evaluated $^1$H-MRS for the discrimination of clinically relevant degrees of macrovesicular steatosis (i.e., mild, moderate, and severe macrovesicular steatosis).
Furthermore, different methods have been employed for the quantification of the percentage hepatic fat content derived from \(^1\)H-MR spectra. The aim of this study was therefore to investigate the quantitative and discriminative capacity of \(^1\)H-MRS using a clinical 3.0 T MRI scanner in an experimental rat steatosis model and to compare different quantification methods.

**MATERIALS AND METHODS**

**Study design**
The experiments were performed in accordance with the guidelines and approval of the animal ethics committee of the University of Amsterdam. Male Wistar rats (Harlan, Zeist, the Netherlands) weighing 250-300 g were acclimatized for one week under standardized laboratory conditions in a temperature-controlled room with a 12-hour light/dark cycle. Steatosis was induced by feeding a methionine/choline-deficient (MCD) diet (Harlan Teklad, Madison, WI) ad libitum for 1, 2, 3, or 5 weeks (n=5/group) as described earlier [20]. The control group (n=5) was maintained on standard chow. At the end of the diet period the degree of steatosis was quantified by \(^1\)H-MRS. Additionally, blood was drawn before animal sacrifice for the assessment of liver enzymes and the livers were excised for histological determination of the macrovesicular steatosis grade, biochemical analysis of fat content, and determination of water content.

**\(^1\)H-MRS**
\(^1\)H-MRS measurements were performed on a clinical 3.0 T Philips Intera scanner (Philips Healthcare, Best, The Netherlands). Animals were anesthetized by intraperitoneal injection of dormicum (midazolam, 5 mg/mL) and hypnorm (fentanyl citrate, 0.315 mg/mL, and fluanisone, 10 mg/mL) in 0.9% saline (1:1:2 ratio, 2.7 mL/kg body weight). A 5-cm diameter experimental \(^1\)H-MRS micro-coil (Philips Research Laboratories, Hamburg, Germany) was positioned on the abdomen (Figure 1A) and scout images were acquired to localize the liver and surrounding structures. A voxel of 8 × 10 × 15 mm (1200 mm\(^2\)) was delineated in the liver, avoiding inclusion of extra-hepatic structures (Figure 1B, C). The voxel size and acquisition time were standardized for all animals. Spectra were acquired using first order iterative shimming, a PRESS sequence with TE/TR = 35/2000 msec and 64 signal acquisitions. Calculated peak areas of water and fat were corrected for T2 relaxations (T2 water = 34 msec, T2 fat = 68 msec) [21].
The total fat peak area under the curve (AUC$_{\text{total fat peaks}}$, 0.5-3.0 ppm) and the total spectral peak areas (AUC$_{\text{total peaks}}$, 0.5-5.3 ppm) were determined using the AMARES algorithm with jMRUI software [22]. The percentage hepatic fat content was determined from the ratio [AUC$_{\text{total fat peaks}}$ / AUC$_{\text{total peaks}}$] as described by Longo et al. [17].

**Figure 1:** Under light anesthesia, rats were placed in supine position in a clinical 3.0 T MR scanner. An experimental amplifier coil was positioned on the abdomen covering the hepatic region for optimal signal acquisition. Scout MR images were obtained from an intrahepatically positioned voxel (1200 mm$^3$) viewed transversally and coronally. Right image; Voxel position in the rat liver at a coronal and axial slice. The two boxes represent the chemical shift displacement.

**Histological assessment of steatosis**

Excised livers were fixed in 10% buffered formalin for 24 hours. Four-μm thick sections were stained with hematoxylin and eosin (H&E) and Sirius red for fibrosis detection. Sections were scored by a hepatopathologist in a blinded fashion for percentage of hepatocytes containing macrovesicular steatosis and for inflammation according to the NASH scoring system [23]. Briefly, no lobular inflammatory foci, <2, 2-4, and >4 foci were scored as 0, 1, 2 and 3, respectively.

**Hepatic fatty acid quantification by gas chromatography (GC)**

Hepatic lipids were extracted using a method modified from Srivastava et al. [24]. Frozen liver samples were homogenized and sonicated in a methanol:chloroform (1:3) solution, followed by centrifugation. The supernatant was evaporated using nitrogen gas and the lipid pellets were lyophilized and resuspended in 2% Triton X-100. For GC, methylated fatty acids were extracted using methanolic HCl (Sigma-Aldrich, St. Louis, MO) and quantified by capillary GC using an HP 5890 gas chromatograph (Hewlett-Packard, Wilmington, DE). Total hepatic fatty acids were expressed as mg/g liver tissue.
Comparison of $^1$H-MRS fat quantification methods

The computational method to derive hepatic fat content from $^1$H-MR spectra varies among research groups. Typically, at $\geq 1.5$T, six resonance signals are visible in addition to water (4.7 ppm), namely 0.8-1.0 ppm (-CH$_3$, methyl), 1.2-1.6 ppm (-CH$_2$, methylene), 2.0-2.1 ppm (-CH$_2$-CH=CH-, allylic), 2.2-2.4 ppm (COO-CH$_2$-CH$_2$-, α-methylene to carboxyl), 2.8-3.1 ppm (=CH-CH$_2$-CH=, diallylic), and 5.3 ppm (-CH=CH-, methene).

Longo et al. [17] described a method in which total fat (0.5-3.0 ppm) is divided by total peak area (0.5-5.5 ppm); $[\text{AUC}_{\text{total fat peaks}} / \text{AUC}_{\text{total peaks}}]$. Other groups [16,25] have used only the methylene signal (1.2-1.4 ppm) normalized to the cumulative methylene and water signal (4.7 ppm) peak, using a correction factor of 0.7 for water; $[\text{AUC}_{\text{fat}} / (\text{AUC}_{\text{fat}} + (\text{AUC}_{\text{water}} / 0.7))]$. Another method is to normalize the integrated methylene peak by the integrated water peak; $[\text{AUC}_{\text{fat}} / \text{AUC}_{\text{water}}]$ [12,26]. These methods, $[\text{AUC}_{\text{total fat peaks}} / \text{AUC}_{\text{total peaks}}]$, $[\text{AUC}_{\text{fat}} / (\text{AUC}_{\text{fat}} + (\text{AUC}_{\text{water}} / 0.7))]$, and $[\text{AUC}_{\text{fat}} / \text{AUC}_{\text{water}}]$ were employed for hepatic fat determination from the spectra acquired by $^1$H-MRS.

Determination of hepatic water content

Since the water fraction (4.7 ppm) in the $^1$H-MR spectrum comprises an important constituent in the fat quantification methods described in the previous section, the dry/wet weight ratios were determined in samples from the same livers used for histological and biochemical fat determination. After animal sacrifice, the liver samples were weighed directly before and after 4 weeks of incubation at 60°C. Water content was calculated by: $[1-(\text{dry weight} / \text{wet weight})] \times 100\%$.

Hepatocellular damage

Plasma was analyzed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by routine clinical chemistry. The AST:ALT ratio was calculated as a blood plasma marker for steatosis-induced hepatocellular injury [27].

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences software (SPSS, Chicago, Ill). Histological, $^1$H-MRS, and biochemical measurements of hepatic fat are expressed as mean ± SEM throughout the manuscript. The Pearson correlation coefficient $r$ was used to determine the correlation between ordinal variables.
The Gaussian distribution of the data sets was tested with a Shapiro-Wilk test. Differences between $^1$H-MRS data in the categorical steatosis groups were assessed with a one-way ANOVA using post-hoc Bonferroni correction. P-values of $<$0.05 were considered significant.

**RESULTS**

**Steatosis induction by the MCD diet**

Histological evaluation of control livers revealed no steatosis, whereas a 1-week MCD diet induced no-to-mild steatosis of $6 \pm 7\%$. Mild to moderate steatosis ($38 \pm 9\%$), located mainly in the centrilobular zone, was observed following a 2-week MCD diet. A 3- and 5-week MCD diet was associated with panlobular moderate to severe steatosis, amounting to $58 \pm 11\%$ and $84 \pm 8\%$, respectively. Histologically mild (0-33%), moderate (33-66%), and severe (66-100%) macrovesicular steatosis degrees induced by the MCD diet are depicted in Figure 2. GC determination of total hepatic fatty acids yielded $11.9 \pm 1.9 \text{ mg/g}$ in control, $24.7 \pm 7.3 \text{ mg/g}$ after 1 week, $45.0 \pm 7.1 \text{ mg/g}$ after 2 weeks, $67.2 \pm 26.9 \text{ mg/g}$ after 3 weeks, and $120.3 \pm 11.4 \text{ mg/g}$ after 5 weeks of the MCD diet. An increased duration of the MCD diet was further associated with an augmented number of lobular inflammatory foci and a significantly decreased plasma AST:ALT ratio (results not shown). Sirius red staining revealed no fibrosis in any of the groups.

*Figure 2: Histological micrographs (H&E, 4x magnification) of mild (0-33%), moderate (33-66%), and severe (66-100%) steatosis induced by the MCD diet after 2, 3, and 5 weeks, respectively.*
\(^1\text{H-MRS} \text{ fat quantification and comparison to histology and GC}\)

Representative \(^1\text{H-MR}\) spectra of varying degrees of hepatic fat content in the experimental groups are shown in Figure 3. Minimal fat content was detected by \(^1\text{H-MRS} (\text{[AUC}_{\text{total fat peaks}} / \text{AUC}_{\text{total peaks}}])\) in the control group, i.e., 1.6 ± 0.5%, which increased after a 1-week MCD diet to 6.3 ± 1.5%. After 2 weeks of MCD diet the hepatic fat content had reached a level of 15.6 ± 5.7%, which gradually increased to 25.2 ± 7.8% and 49.2 ± 2.7% at 3 and 5 weeks, respectively. The \(^1\text{H-MRS}\) measurements of hepatic fat content showed a strong positive correlation with the degree of histological macrovesicular steatosis (\(r =0.932, p<0.0001\), Figure 4A). Furthermore, the positive correlation between total hepatic fat content as determined by GC and \(^1\text{H-MRS}\) was equally strong (\(r =0.935, P<0.0001\), Figure 4B).

Figure 3: Representative \(^1\text{H-MRS}\) spectra of rat livers after steatosis induction with the methionine-choline deficient (MCD) diet during 0-5 weeks. The fat and water signals reside at 1.3 and 4.7 ppm, respectively. In the control livers (week 0) a minimal fat peak is seen, which increases with the duration of the MCD diet. Conversely, the water peak exhibits a gradual decrease in signal amplitude as a function of MCD diet time.
Discriminative power of $^1$H-MRS in relation to steatosis degrees

We investigated if $^1$H-MRS is capable of discriminating between clinically relevant histological steatosis degrees by categorizing the measurements according to the following percentages of macrovesicular steatosis: 0-33% (mild), 33-66% (moderate), and 66-100% (severe). Accordingly, 13 rats had mild, six had moderate, and six had severe macrovesicular steatosis. As shown in Figure 5, $^1$H-MRS measurements of mild steatosis (6.4 ± 4%) were significantly lower ($p < 0.001$) than $^1$H-MRS measurements of livers with moderate steatosis (23 ± 4%). Similarly, $^1$H-MRS measurements of moderate steatosis were significantly lower ($p < 0.001$) than of severe steatosis (47 ± 4%). These data show that $^1$H-MRS can distinguish mild from moderate and moderate from severe macrovesicular steatosis.

Figure 4: $^1$H-MRS measurements of hepatic fat content were strongly correlated to (A) histological assessment of macrovesicular steatosis ($P < 0.001$) and (B) fatty acid determination by gas chromatography ($P < 0.001$).
Comparison between different quantification methods

An analysis of the three most frequently applied methods for the quantification of hepatic fat from $^1$H-MR spectra, namely $\frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}}$, $\frac{\text{AUC}_{\text{fat}}}{(\text{AUC}_{\text{fat}} + \frac{\text{AUC}_{\text{water}}}{0.7})}$ and $\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}$ was performed. Data were categorized according to the degrees of clinically relevant macrovesicular steatosis (i.e. mild, moderate, and severe). In rat livers with mild and moderate steatosis there were no differences in the percentages of hepatic fat between the calculation methods (Figure 6). Fat quantification in severely steatotic livers by $\frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}}$ and $\frac{\text{AUC}_{\text{fat}}}{(\text{AUC}_{\text{fat}} + \frac{\text{AUC}_{\text{water}}}{0.7})}$ resulted in similar values (47 ± 4% and 43 ± 5%, respectively), whereas quantification by $\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}$ resulted in a significantly higher hepatic fat content (78 ± 16%). This discrepancy can be observed in the $^1$H-MR spectra shown in Figure 3, in which the water signals (4.7 ppm) decreased in rat livers after 3 and 5 weeks on the MCD diet. Inasmuch as the latter formula normalizes the total fat content to the AUC of the water peak, the reduced hepatic water content in conjunction with the increased fat content will lead to a higher total hepatic fat percentage, as compared to the other formulas.

Figure 5: $^1$H-MRS can accurately discriminate between clinically relevant histological degrees of steatosis, i.e., mild (0-33%), moderate (33-66%), and severe (66-100%) macrovesicular steatosis. Significant differences were found between $^1$H-MRS measurements of mild versus moderate steatosis (#, $P < 0.001$) and moderate versus severe steatosis (##, $P < 0.001$).

Chapter 6: 3.0T $^1$H-MRS to assess hepatic steatosis in rats
**Chapter 6: 3.0T $^1$H-MRS to assess hepatic steatosis in rats**

**Figure 6**: Comparison between three commonly used methods for quantifying hepatic fat content from $^1$H-MR spectra. In mild and moderate steatosis, no notable differences between the methods were observed, whereas $\text{[AUC}_{\text{fat}}/\text{AUC}_{\text{water}}]$ yielded a significantly higher fat percentage in severe steatosis compared to $\text{[AUC}_{\text{fat}} / \text{AUC}_{\text{fat}} + (\text{AUC}_{\text{water}} / 0.7)]$ and $\text{[AUC}_{\text{fat}} / \text{AUC}_{\text{water}}]} \ (P < 0.01, *)$

**Decreased water content in livers with severe steatosis**

To further elucidate the abovementioned phenomenon, the AUC of the water peak was analyzed and juxtaposed to the respective water fraction as determined by the dry/wet weight ratio. The use of the $\text{[AUC}_{\text{fat}} / \text{AUC}_{\text{water}}]$ equation for the determination of hepatic fat is based upon the assumption that the water fraction in the liver remains stable with increasing degrees of steatosis and therefore can be used as a reference signal [12,26]. In severe steatosis, however, $^1$H-MR spectra exhibited a reduced water signal (AUC at 4.7 ppm) relative to livers with mild and moderate steatosis (Figure 7A). This observation was corroborated by the dry/wet weight ratios found in livers with increasing degrees of steatosis (Figure 7B). Compared to mild steatosis, dry/wet weight ratios in livers with moderate and severe steatosis were significantly lower. These data demonstrate that the water signal in severe fatty livers is strongly influenced by the amount of fat content. Therefore, the fat quantification method using the $\text{[AUC}_{\text{fat}} / \text{AUC}_{\text{water}}]}$ equation substantially skews the hepatic fat content to higher degrees of steatosis. The other two methods yielded similar hepatic fat percentages, independent of the steatosis degree.
Chapter 6: 3.0T $^1$H-MRS to assess hepatic steatosis in rats

The method originally described by Longo et al., \( \frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}} \), incorporates all the fat peaks (0.5-3.0 ppm) and reflects total hepatic fat content more accurately. This total fat compartment is then corrected for by the total peak area (0.5-5.5 ppm) and not only the water content. In order to maintain conformity in data derived from $^1$H-MRS measurements, we therefore recommend the \( \frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}} \) method from Longo et al. for the calculation of hepatic fat content.

![Figure 7](image)

**Figure 7:** (A) decrease in water content (4.7 ppm) was measured by $^1$H-MRS in livers with severe macrovesicular steatosis compared to livers with moderate steatosis (*, $P < 0.05$) and mild steatosis (#, $P < 0.001$) (A). Also shown are the integrated areas under the curve of the fat signal (1.2-1.4 ppm, dotted line) as a function of the steatosis grade. The wet/dry weight ratios were significantly lower in severely steatotic livers compared to livers with moderate (#, $P < 0.01$) and mild steatosis (*, $P < 0.001$) (B).
DISCUSSION

The aim of this study was to investigate the accuracy of \(^1\)H-MRS measurements in a hepatic steatosis model in rats. Using a clinical 3.0 T MR scanner we have demonstrated that \(^1\)H-MRS is capable of discriminating between clinically relevant degrees of steatosis (i.e., mild, moderate, and severe). We have also shown that the development of steatosis in rat livers occurred at the expense of hepatic water content. Consequently, the most frequently employed \(^1\)H-MRS methods to calculate hepatic fat content were re-evaluated in light of these findings to ascertain which of these calculation methods is most suitable. The first reports on the use of \(^1\)H-MRS for the detection of fat content in (human) tissues were mainly experimental and were not validated by histological or biochemical fat determination [14,15]. Szczepaniak et al. [18] validated \(^1\)H-MRS in an experimental dog steatosis model using intravenous norepinephrine infusion and in a rabbit steatosis model using an intravenous inhibitor of hepatic mitochondrial fatty acid oxidation. Unfortunately, these models were not associated with the induction of a clinically relevant macrovesicular steatosis, and therefore a quantitative comparison of \(^1\)H-MRS with the histological degree of steatosis could not be made. Nevertheless, a close correlation between \(^1\)H-MRS and hepatic triglyceride content was found (r =0.93).

With respect to clinical studies, Longo et al. [17] validated \(^1\)H-MRS measurements with histomorphometric analysis of liver biopsies in 29 patients suspected of having NAFLD, yielding a good correlation between \(^1\)H-MRS and histomorphometrically derived fat content (r =0.70). In this study we corroborated the strong correlation between \(^1\)H-MRS fat peaks and actual fat content as determined by GC (r =0.94) and histological analysis (r =0.93). A limitation of \(^1\)H-MRS is the inability to differentiate between macro- and microvesicular steatosis. In Figure 4A relative high hepatic \(^1\)H-MRS fat percentages in rat livers with 0% macrovesicular steatosis groups were detected. This can be explained by a microvesicular steatosis component, which sometimes precedes the formation of macrovesicular steatosis after 1 or 2 weeks MCD diet in rats. However, the macrovesicular component is the most relevant in patients undergoing liver resection or transplantation and encountered most frequently, and was therefore compared with \(^1\)H-MRS measurements.

Another significant finding was the strong discriminative power of \(^1\)H-MRS between the clinically relevant degrees of macrovesicular steatosis, which is particularly important in relation to LDLT procedures since moderate and severe macrovesicular steatosis are considered exclusion criteria for donation [5,6].
It should be noted, however, that the $^1$H-MRS measurements in our study were performed under highly standardized experimental conditions. $^1$H-MR spectra of relatively high quality were acquired with an experimental surface coil on anesthetized animals with minimal motion artifacts. In the clinical setting different MR hardware is used, which can result in suboptimal signal detection, particularly in the presence of excessive abdominal fat. Furthermore, the relative large voxel size used in rodent studies, covering the total liver, is not hampered by a possible heterogeneity of the steatosis distribution. In the clinical application, and especially in longitudinal studies, however, voxel placement should be performed in the exact same position in liver to overcome this problem. Consequently, the accuracy of $^1$H-MRS in distinguishing clinically relevant degrees of steatosis has to be properly validated by histopathological studies in patients with NAFLD.

In the previously mentioned clinical study by Longo et al. [17] a proposal for the $(\text{AUC}_{\text{total fat peaks}} / \text{AUC}_{\text{total peaks}})$ method for calculating hepatic fat content from $^1$H-MR spectra was advocated. The same method was applied in a large study by Szczepaniak et al. [19], evaluating the prevalence of hepatic steatosis in over 2300 participants of the Dallas Heart Study population. The maximum hepatic fat content measured by Longo et al. was little over 30%, and Szczepaniak et al. found a maximum of around 40%, with a peak of approximately 48% in one patient. These statistics imply a relatively small intrastudy variability with respect to the higher steatosis grades. Another definition of hepatic fat percentage by $^1$H-MRS is the ratio of the fat peak (1.3 ppm) to the water peak (4.7 ppm), i.e., $\left[\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}\right]$ [26]. Using this definition, Thomas et al. [26] reported on the relationship between body adiposity and steatosis in 11 NASH patients and measured hepatic fat percentages of up to 75%, which is disproportionally higher than the maximum percentages reported by Longo et al. and Szczepaniak et al. The same discrepancy was also found in our experimental data when comparing the three most frequently used methodologies for hepatic fat calculation, $\left[\frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}}, \frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{total peaks}}}, \frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \right]$ and $\left[\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \right]$. We found that in cases of severe steatosis, $\left[\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \right]$ resulted in a significantly higher hepatic fat percentage ($78 \pm 16\%$) compared to $\left[\frac{\text{AUC}_{\text{total fat peaks}}}{\text{AUC}_{\text{total peaks}}}, \right]$ ($47 \pm 4\%$) and $\left[\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \right]$ ($43 \pm 5\%$). The reason for the higher hepatic fat content when using $\left[\frac{\text{AUC}_{\text{fat}}}{\text{AUC}_{\text{water}}}, \right]$, is the decreased water content with increasing degrees of steatosis.
Consequently, $^1$H-MRS quantification of hepatic fat should be standardized to facilitate a reliable assessment of high risk patients for extended liver resections and donors for LDLT procedures as well as to enable a valid comparison of data generated by interventional studies in patients with NAFLD.

Since the assumption that water content is constant and can be used a reference standard in the $[\text{AUC}_{\text{fat}} / \text{AUC}_{\text{water}}]$ formula does not hold true, our recommendation is to abolish this method. The other two methods, i.e., $[\text{AUC}_{\text{total fat peaks}} / \text{AUC}_{\text{total peaks}}]$ and $[\text{AUC}_{\text{fat}} / (\text{AUC}_{\text{fat}} + (\text{AUC}_{\text{water}} / 0.7))]$, resulted in similar values of hepatic fat percentage. However, we recommend the method originally described by Longo et al., $[\text{AUC}_{\text{total fat peaks}} / \text{AUC}_{\text{total peaks}}]$, inasmuch as this method incorporates all the fat peaks in $^1$H-MR spectra instead of only the saturated fatty acid peak at 1.3 ppm. This method takes into account possible variations in unsaturated fatty acids in patients with steatosis, whereas $[\text{AUC}_{\text{fat}} / (\text{AUC}_{\text{fat}} + (\text{AUC}_{\text{water}} / 0.7))]$ does not.

In conclusion, quantification of experimental hepatic steatosis using $^1$H-MRS accurately discriminates between clinically relevant degrees of steatosis. The results of this study justify further clinical trials applying $^1$H-MRS for noninvasive measurement of steatosis in patients undergoing extensive liver resection or in LDLT procedures.
REFERENCES