Applications of magnetic resonance spectroscopy for noninvasive assessment of hepatic steatosis

van Werven, J.R.

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FEASIBILITY OF IN VIVO ANALYSIS OF HEPATIC LIPID COMPOSITION USING 3.0T MR SPECTROSCOPY IN A STEATOTIC RAT MODEL

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

Submitted
ABSTRACT

Purpose
To investigate the feasibility of in vivo assessment of hepatic lipid composition using 3.0T $^1$H-MRS in a steatotic rat model and compare it to histopathological and biochemical assessment.

Materials and Methods
Hepatic steatosis was induced by feeding rats with a methionine/choline-deficient (MCD) diet for 1, 2, 3, 5 or 7 weeks (n=5 per group). At the end of the diet period $^1$H-MRS of the liver was performed and rats were sacrificed for histopathological and biochemical assessment of the liver. From the MR spectra ratios were calculated to estimate hepatic lipid composition.

Results
During MCD diet periods, hepatic steatosis significantly increased on histopathology (p<0.001). The $^1$H-MRS measurements of total hepatic fat content (1.3/(1.3+4.65) ppm) correlated strongly with histological macrovesicular hepatic steatosis (r=0.93, p<0.001) and with the biochemical total hepatic fatty acids (r=0.94, p<0.001). Total unsaturated fatty acids (TUFA, 5.4/(1.3+4.65) ppm) estimated with $^1$H-MRS strongly correlated with the biochemical unsaturated fatty acids (r=0.90, p<0.001). Polyunsaturated fatty acids (PUFA, 2.8/(1.3+4.65) ppm) estimated with $^1$H-MRS strongly correlated with biochemical polyunsaturated fatty acids (r=0.91, p<0.001). The proportion of total unsaturated fatty acids relative to the amount of total fatty acids (rTUFA, 5.4/1.3ppm) measured with $^1$H-MRS strongly correlated with the biochemical amount of unsaturated relative to total hepatic fatty acids (r=0.81, p<0.001). The proportion of polyunsaturated fatty acids relative to the amount of total fatty acids (rPUFA, 2.8/1.3ppm) measured with $^1$H-MRS correlated with the biochemical amount of polyunsaturated fatty acids relative to total fatty acids (r=0.59, p=0.005,) and with the biochemical amount of omega-6 polyunsaturated fatty acids relative to total fatty acids (r=0.73, p<0.001). PUFA at $^1$H-MRS correlated with the histopathologically assessed degree of lobular inflammation in the liver (r=0.51, p=0.023).
Conclusion

3.0T $^1$H-MRS is able to measure poly- and unsaturated hepatic fatty acids and this strongly correlates with biochemical assessment. This study provides evidence that the use of 3.0T $^1$H-MRS is a noninvasive technique to assess hepatic lipid composition.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation. Due to the widespread obesity and metabolic syndrome epidemic [1,2], NAFLD is considered as the most common chronic liver disease in the Western world with an estimated prevalence of 30% [3,4]. Moreover, recent reports suggest that the majority of cryptogenic hepatocellular carcinoma is caused by NAFLD [5]. It is predicted that the rise in NAFLD prevalence could lead to an increased incidence of hepatocellular carcinoma in the next decades [6].

There is increasing evidence that hepatic polyunsaturated fatty acids play an important role in the development of NAFLD. In western societies diets are characterized by an increased intake of omega-6 polyunsaturated fatty acids [7]. An increase in omega-6/omega-3 polyunsaturated fatty acid ratio results in disrupted metabolism of hepatic lipids [8-11]. Recent studies show that the lipid metabolic perturbations in NAFLD and pathophysiology are complex and that NAFLD is associated with numerous changes in lipid composition in the liver [12-14].

Percutaneous liver biopsy is the reference standard for histopathological evaluation of NAFLD, but is accompanied by the risk of complications, inter-observer variability, sampling error and patient discomfort [15-17]. Furthermore, it is only possible to perform an analysis of hepatic lipid composition using biochemical measurements in liver tissue acquired by fine needle liver biopsy.

Proton Magnetic Resonance Spectroscopy (1H-MRS) is a noninvasive diagnostic technique and is considered to be a sensitive and reproducible method to quantify hepatic lipid content [18-21]. It has shown to correlate strongly with histopathological evaluation of liver biopsies in healthy individuals and patients with NAFLD [22-24]. The use of higher magnetic field strengths (≥ 3.0 Tesla) provide higher spectral resolution and may therefore allow to obtain hepatic lipid composition in more detail, e.g., differentiation of (poly)unsaturated fatty acid chains besides total hepatic fat content. A previous study has investigated saturated and unsaturated fatty acid ratios at 1H-MRS in an experimental setting at 4.7 Tesla [25], but without using biochemical confirmation to support the results. Furthermore, the extension to human studies is hampered because the scanner was not a clinical MR scanner.

Therefore, the objective of this study was to investigate the feasibility of in vivo assessment of hepatic lipid composition and differentiation between saturated and unsaturated fatty acids using 3.0T 1H-MRS in an experimental steatotic rat model with histopathological assessment and biochemical confirmation provided by gas chromatography.
MATERIALS AND METHODS

Study design
The experiments in this study were approved by the institutional animal ethics committee. Thirty male Wistar rats (Harlan, Zeist, the Netherlands) weighing 250-300 g were acclimatized for one week using standardized laboratory conditions with a 12h-light/dark cycle in a temperature-controlled room. Hepatic steatosis was induced by feeding the rats with a methionine/choline-deficient (MCD) diet (Harlan Teklad, Madison, WI) ad libitum for 1, 2, 3, 5 or 7 weeks (n=5 per group), whereas the control group (n=5) was maintained on standard chow. At the end of the diet period, 1H-MRS of the liver was performed. Following the 1H-MRS procedure the rats were sacrificed and the livers were excised for histopathological determination of hepatic steatosis and biochemical assessment of hepatic lipid composition. The results of the experiments in this study have been used for publication of another scientific paper which only focused on the quantification of total hepatic fat content [26].

A research grant was received from the NutsOhra Foundation (Amsterdam the Netherlands). The NutsOhra Foundation was not involved in designing and conducting this study, did not have access to the data and was not involved in data analysis or preparation of this manuscript.

Histopathological assessment of hepatic steatosis
Livers were excised and liver specimens from multiple lobes were fixed in 10% buffered formalin for 24h, dehydrated and embedded in paraffin. Four µm thick sections were stained with hematoxylin and eosin (H&E). No separate staining for iron was performed and fibrosis was not assessed. Sections were scored by an experienced hepatopathologist ([FJK] with 30 years of experience and blinded for study results) for percentage of macro- and microvesicular steatosis and for inflammation score according to the NASH scoring system defined by Kleiner et al. [27]. Macromesicular steatosis was assessed as the percentage of hepatocytes in the microscopic field containing a lipid vacuole larger than the diameter of the nucleus and displacing the nucleus. Microvesicular steatosis was assessed as the percentage of hepatocytes in the microscopic field containing numerous small vesicles not displacing the nucleus. Total hepatic steatosis was defined as macromesicular plus microvesicular steatosis.
**Hepatic fatty acid extraction and gas chromatography**

Hepatic fatty acids were extracted by a research fellow [HAM] blinded for the study results using a method modified by Srivastava et al. [28]. Frozen liver samples (50mg) were suspended in methanol:chloroform (1mL:2mL) and homogenized with a tissue dispenser. The homogenized tissue was sonicated for 6 min, left overnight, and resonicated for 6 min. Undissolved tissue fragments were pelleted by centrifugation, after which the infranatant was aspirated and filtered. The supernatant was evaporated with nitrogen gas and the lipophilic remnants lyophilized under vacuum at -20ºC and redissolved.

Methylated fatty acids were extracted and quantified using an HP 5890 gas chromatograph (Hewlett-Packhard, Wilmington, DE). Gas chromatography data were processed with Chromeleon software (Dionex, Sunnyvale, CA). Hepatic fatty acids were expressed as mg/g liver tissue.

**3.0 T ¹H-MRS measurements**

¹H-MRS measurements were performed on a clinical 3.0T Philips Intera scanner (Philips Healthcare, Best, The Netherlands) using a receive only surface micro-coil (diameter 5 cm) positioned on the abdomen of the rat in supine position. Rats were anesthetized by an intraperitoneal injection of midazolam (Dormicum, 5 mg/mL) and fentanyl (Hypnorm citrate, 0.315 mg/mL, and fluanisone, 10 mg/mL) in 0.9% saline (1:1:2 ratio, 2.7 mL/kg body weight). Spectra were acquired using a point resolved spectroscopic sequence (PRESS) with TE/TR = 35/2000 msec, 64 signal acquisitions, voxel dimensions 8 mm, 10 mm and 15 mm in the AP, FH and RL directions respectively (1.2 cc). Chemical shift displacements were 3.1, 2.3 and 5.9 mm in the AP, FH and RL directions respectively between the signals at 5.4 and 1.3 ppm. Iterative first order shimming was applied; the average line width of the water peak was 34.7 Hz. Receiver bandwidth was 2000 Hz and the number of data points was 1024.

Spectra both with and without water suppression were obtained; the bandwidth of the water suppression pulse being 100 Hz. ¹H-MRS was performed during free breathing, no respiratory triggering or outer-volume suppression was used. After performing T1-weighted coronal and axial localizer images of the rat abdomen, a voxel was positioned in the rat liver covering multiple hepatic lobes, but avoiding overlap with extra hepatic structures, especially subcutaneous fat (Figure 1).
The chemical shift displacement between the volumes for the peaks at 5.4 and 1.3 ppm was visualized on the localizer images to avoid lipid signal contamination from outside the liver. Voxel size and acquisition time were standardized for all rats.

Data was processed using jMRUI software [29] by a research fellow [JRW] under direct supervision of an experienced MR physicist ([AJN], 5 years of experience), blinded for study results. Signal resonances (Figure 2) were analyzed and prior knowledge was used for peak localization by using soft constraints. Signal resonances were fitted using Lorentzian line shapes. Phase variation was allowed (40 degrees) around a manually found optimum. Only when lipid moieties of polyunsaturated fatty acid peak were not visible in the unsuppressed spectra in the early MCD diet periods (week 1 and 2), we used the water suppressed spectra to quantify the lipid signal resonances. However, due to the effect of water suppression on the unsaturated fatty acid peak (at 5.4 ppm) next to the water peak (at 4.65 ppm), we did not use this peak for analysis in diet week 1 and 2. From the MR spectra several ratios were calculated to investigate hepatic lipid composition (Table 1):
Figure 2: Examples of the fitting procedure for the 2.8 ppm peaks at week 1, based on the water suppressed spectrum (see inset for unsuppressed spectrum), and for the 5.4 and 2.8 ppm peaks at week 5, based on the unsuppressed spectrum. Residues (upper plots) indicate that in both spectra reliable fits could be obtained.

Table 1: Definitions of $^1$H-MRS ratios

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Fatty acid component</th>
<th>Peak area ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFC</td>
<td>Total hepatic fat content</td>
<td>$-(\text{CH}_2)_n^-$</td>
<td>$1.3/(1.3+4.65)$ ppm</td>
</tr>
<tr>
<td>TUFA</td>
<td>Total unsaturated fatty acids</td>
<td>$-\text{CH}═\text{CH}^-$</td>
<td>$5.4/(1.3+4.65)$ ppm</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
<td>$-\text{CH}═\text{CH}-\text{CH}═\text{CH}^-$</td>
<td>$2.8/(1.3+4.65)$ ppm</td>
</tr>
<tr>
<td>rTUFA</td>
<td>Total unsaturated fatty acids relative to total amount of fatty acids</td>
<td>$-\text{CH}═\text{CH}^-$ / $-(\text{CH}_2)_n^-$</td>
<td>$5.4/1.3$ ppm</td>
</tr>
<tr>
<td>rPUFA</td>
<td>Polyunsaturated fatty acids relative to total amount of fatty acids</td>
<td>$-\text{CH}═\text{CH}-\text{CH}═\text{CH}^-$ / $-(\text{CH}_2)_n^-$</td>
<td>$2.8/1.3$ ppm</td>
</tr>
</tbody>
</table>
1) The total hepatic fat content (HFC) was determined as the ratio between the signal resonance from the methylene \((-\text{CH}_2\text{n-})\) peak at 1.3 ppm and the water peak at 4.65 ppm \((1.3/(1.3+4.65)\) ppm). Calculated hepatic fat content was not corrected for T1 relaxation. Calculated hepatic fat content was corrected for T2 relaxation. T2 measurements were performed in five rats after three weeks of MCD diet by using a multi echo PRESS sequence (TE= 35, 55, 75, 95, 115 and 135 msec). Measured T2 values appeared to be stable in this subset: T2water= 33.2 msec (SD 3.1 msec), T2fat= 61.6 msec (SD 3.0 msec) and were used throughout the entire group for calculating HFC. In addition T2 values for resonances at 5.4 and 2.8 ppm were measured: T25.4=39.8 msec (SD 2.2 msec), T22.8= 57.0 msec (SD 4.7 msec). Since only peak ratios are calculated in the ratios below, no corrections were applied assuming that the variability in T2 has negligible consequences for calculated correlations.

2) In diet week 3, 5 and 7 the ratio of the signal resonance from methine at 5.4 ppm \((-\text{CH}=\text{CH-})\) to water at 4.65 ppm and methylene at 1.3 ppm \((5.4/(1.3+4.65)\) ppm) was used to estimate the amount of Total Unsaturated Fatty Acids (TUFA).

3) The 2.8/(1.3+4.65) ppm signal resonance ratio was used as an indicator to the amount of Poly Unsaturated Fatty Acids (PUFA). The 2.8 ppm signal resonance arises from diallylic protons \((-\text{CH}=\text{CH-CH}_2\text{-CH}=\text{CH-})\) that only exist in polyunsaturated fatty acids.

4) In diet week 3, 5 and 7 the ratio of the methine signal resonance at 5.4 ppm \((-\text{CH}=\text{CH-})\) to methylene \((-\text{CH}_2\text{n-})\) at 1.3 ppm was used to estimate the proportion of total unsaturated fatty acids relative to the amount of total fatty acids \((r\text{TUFA})\).

5) The 2.8/1.3 ppm signal resonance ratio was used to assess the proportion of polyunsaturated fatty acids relative to the amount of total fatty acids \((r\text{PUFA})\). Throughout this paper the abbreviations HFC, TUFA, PUFA, rTUFA and rPUFA are solely used to indicate the outcome of the \(^1\text{H}-\text{MRS} \) measurements. The magnitude of the signal resonance peaks increases when the amount of hepatic fatty acids increases. In this study no contribution to the signal resonances other than lipid was assumed, since lipid resonances are predominant in MR spectra from steatotic livers. It should be noted that the ratios presented above do not probe volume ratios between lipids directly but only contain ratios of amount of resonating protons at different locations in the fatty acid chains. Total scanning time including anesthesia, positioning of the rats and acquisition of localizers amounted to 30 minutes.
Statistical analysis

Differences between groups were assessed with a one-way ANOVA using post hoc Bonferroni correction. For Bonferroni corrections the level of significance was corrected for the number of comparisons (p-value: 0.05/5 = 0.01). Correlations between $^1$H-MRS and histopathological and biochemical measurements were assessed using Pearson’s correlation coefficient. Normal data distribution was tested using a Shapiro-Wilk test. For all other statistical analysis a p-value <0.05 was considered significant. We did not evaluate reproducibility, intra- and inter-observer variability. For analyses SPSS version 16.0 was used (SPSS Inc. Chicago Ill).

RESULTS

Histopathologic assessment of hepatic steatosis

Table 2 shows that there was no macrovesicular hepatic steatosis on histopathological assessment of the control livers. After two weeks of MCD diet macrovesicular hepatic steatosis was 38±9% and predominantly located in the centrilobular zone. Compared to the control livers, this significantly (p<0.001) increased to 82±9% after seven weeks of MCD diet and was associated with panlobular hepatic steatosis. Microvesicular hepatic steatosis in the control livers was 2±1%. This significantly (p=0.009) increased to 23±6% after two weeks of MCD diet. After seven weeks of MCD diet microvesicular steatosis was decreased to 5±6% (not significant). Histopathologic assessment of total hepatic steatosis significantly (p<0.001) increased to 87±8% total hepatic steatosis after seven weeks of MCD diet. Importantly, compared to the controls, an increased duration of the MCD diet at seven weeks was associated with significant (p=0.009) increased lobular inflammation (Table 3).
Table 2: Histopathology assessment

<table>
<thead>
<tr>
<th></th>
<th>Baseline Controls (n=5)</th>
<th>MCD 1 week (n=5)</th>
<th>MCD 2 weeks (n=5)</th>
<th>MCD 3 weeks (n=5)</th>
<th>MCD 5 weeks (n=5)</th>
<th>MCD 7 weeks (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroposecular steatosis (%)</td>
<td>0±0</td>
<td>6±7</td>
<td>38±9</td>
<td>58±11</td>
<td>84±8</td>
<td>82±9*</td>
</tr>
<tr>
<td>Microvesicular steatosis (%)</td>
<td>2±1</td>
<td>16±2</td>
<td>23±6</td>
<td>16±5</td>
<td>4±9</td>
<td>5±6*</td>
</tr>
<tr>
<td>Total hepatic steatosis (%)</td>
<td>2±1</td>
<td>23±2</td>
<td>63±2</td>
<td>76±2</td>
<td>88±6</td>
<td>87±8*</td>
</tr>
</tbody>
</table>

Mean ± standard deviation
*, significant difference (p<0.001) controls, week 1, 2 and 3
¥, significant difference (p<0.001) controls and week 1
#, significant difference (p<0.001) week 2

Table 3: Hepatic inflammation score in the rat liver during MCD diet

<table>
<thead>
<tr>
<th>MCD diet (in weeks)</th>
<th>Inflammation score per rat (N=5 per MCD diet group)</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 0 0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0 0 1 1 0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1 1 0 0 1</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>1 1 2 1 0</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1 1 1 1 2</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>1 1 1 1 2</td>
<td>7*</td>
</tr>
</tbody>
</table>

*, significant increase (p=0.009) in inflammation score (Kruskal-Wallis analysis)
Gas chromatography

Total hepatic fat content assessed with gas chromatography significantly (p<0.001) increased from 11.87±2.28 mg/g in the control livers to 110.11±37.63 mg/g after seven weeks of MCD diet (Table 4). According to gas chromatography total-, mono- and poly- unsaturated fatty acids significantly increased during the MCD diet periods (p<0.001) (Table 4). The contribution of polyunsaturated fatty acids to this increase was predominant. Furthermore, the increase in polyunsaturated fatty acids was mainly caused by an increase in omega-6 polyunsaturated fatty acids and therefore the omega-6 / omega-3 ratio was significantly increased (p<0.001) after 7 weeks of MCD diet (Figure 3). Furthermore gas chromatography showed that linoleic acid (C18:2(n-6), C<sub>18</sub>H<sub>32</sub>O<sub>2</sub>) was the most predominant omega-6 polyunsaturated fatty acid (Figure 4). Gas chromatography showed that both the total and mono-unsaturated fatty acids relative to total amount of hepatic fat were significantly increased (p<0.001) after seven weeks of MCD diet. However, the polyunsaturated fatty acids relative to total amount of hepatic fat were not significantly increased (Table 4).

Figure 3: Box plot representing omega-6/ omega-3 polyunsaturated fatty acid ratio in the rat liver during MCD diet in weeks.
Figure 4: Bar chart representing the significant increase in omega-6 polyunsaturated fatty acids in the rat liver, especially linoleic acid (C18:2(n-6)), during MCD diet in weeks.
### Table 4: Gas chromatography assessment

<table>
<thead>
<tr>
<th></th>
<th>Baseline Controls (n=5)</th>
<th>MCD 1 week (n=5)</th>
<th>MCD 2 weeks (n=5)</th>
<th>MCD 3 weeks (n=5)</th>
<th>MCD 5 weeks (n=5)</th>
<th>MCD 7 weeks (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total fatty acids (mg/g)</strong></td>
<td>11.87±2.28</td>
<td>24.66±9.07</td>
<td>45.04±0.09</td>
<td>67.19±3.15</td>
<td>120.29±13.56</td>
<td>110.11±37.63</td>
</tr>
<tr>
<td><strong>Total saturated fatty acids (mg/g)</strong></td>
<td>4.34±0.82</td>
<td>7.47±2.76</td>
<td>12.78±2.57</td>
<td>19.23±8.95</td>
<td>28.71±4.32</td>
<td>24.69±1.03§</td>
</tr>
<tr>
<td><strong>Total unsaturated fatty acids (mg/g)</strong></td>
<td>7.53±1.47</td>
<td>17.19±6.32</td>
<td>32.26±6.69</td>
<td>47.97±2.26</td>
<td>91.58±10.07</td>
<td>85.43±2.75*</td>
</tr>
<tr>
<td><strong>Mono-unsaturated fatty acids (mg/g)</strong></td>
<td>2.48±1.91</td>
<td>4.28±1.50</td>
<td>8.70±1.81</td>
<td>14.48±7.86</td>
<td>27.09±3.28</td>
<td>23.72±9.42</td>
</tr>
<tr>
<td><strong>Polyunsaturated fatty acids (mg/g)</strong></td>
<td>5.60±1.17</td>
<td>12.91±4.94</td>
<td>23.56±4.93</td>
<td>33.49±14.80</td>
<td>68.49±15.39</td>
<td>61.71±18.25*</td>
</tr>
<tr>
<td><strong>Omega-3 polyunsaturated fatty acids (mg/g)</strong></td>
<td>0.99±0.22</td>
<td>1.28±0.48</td>
<td>1.76±0.24</td>
<td>1.37±0.31</td>
<td>2.07±0.17</td>
<td>2.01±0.57¥</td>
</tr>
<tr>
<td><strong>Omega-6 polyunsaturated fatty acids (mg/g)</strong></td>
<td>4.54±0.92</td>
<td>11.53±4.43</td>
<td>21.78±4.72</td>
<td>31.99±14.80</td>
<td>62.18±7.25</td>
<td>59.56±17.71*</td>
</tr>
<tr>
<td><strong>Omega-6/3 polyunsaturated fatty acid ratio</strong></td>
<td>4.62±0.17</td>
<td>9.09±0.93</td>
<td>12.29±1.46</td>
<td>22.43±6.33</td>
<td>30.12±2.68</td>
<td>29.74±5.94</td>
</tr>
<tr>
<td><strong>Linoleic acid (mg/g)</strong></td>
<td>1.93±0.42</td>
<td>6.54±2.69</td>
<td>12.61±3.12</td>
<td>20.94±10.94</td>
<td>42.41±6.03</td>
<td>37.21±13.20*</td>
</tr>
<tr>
<td><strong>Total unsaturated fatty acids relative to total amount of fatty acids</strong></td>
<td>0.63±0.01</td>
<td>0.70±0.01</td>
<td>0.72±0.02</td>
<td>0.71±0.01</td>
<td>0.76±0.02</td>
<td>0.78±0.02¥</td>
</tr>
<tr>
<td><strong>Mono-unsaturated fatty acids relative to total amount of fatty acids</strong></td>
<td>0.16±0.02</td>
<td>0.18±0.02</td>
<td>0.19±0.01</td>
<td>0.20±0.03</td>
<td>0.23±0.01</td>
<td>0.21±0.02¥</td>
</tr>
<tr>
<td><strong>Polyunsaturated fatty acids relative to total amount of fatty acids</strong></td>
<td>0.47±0.03</td>
<td>0.52±0.03</td>
<td>0.52±0.02</td>
<td>0.51±0.03</td>
<td>0.57±0.08</td>
<td>0.57±0.03</td>
</tr>
</tbody>
</table>

Mean ± standard deviation

#, significant difference (p<0.001) controls, week 1, 2 and 3

*, significant difference (p<0.001) controls, week 1 and 2

§, significant difference (p<0.001) controls, week 1

¥, significant difference (p<0.001) controls
**1H-MRS hepatic lipid composition: Comparison to histology and gas chromatography**

1) Minimal HFC (2±1%) was detected by 1H-MRS in the control group, which increased after one week MCD diet to 6±2%. After two weeks of MCD diet the HFC was 16±6%, after three weeks 25±8%, after five weeks 49±3% and after seven weeks 40±4% (Table 5). The 1H-MRS measurements of HFC were strongly correlated with histological macrovesicular hepatic steatosis (r=0.93, p<0.001) and the total hepatic fatty acids determined by gas chromatography (r=0.94, p<0.001) (Table 6). The 1H-MRS measurements of HFC were also correlated with total histological hepatic steatosis (r=0.84, p<0.001), but not to histological microvesicular hepatic steatosis.

2) The 1H-MRS resonances from the TUFA increased (5.4/(1.3+4.65) ppm, Table 5) during the diet period. At five weeks MCD diet this increase in resonances was statistically significant (p<0.001). The TUFA at 1H-MRS strongly correlated with the unsaturated fatty acids assessed with gas chromatography (r=0.90, p<0.001) (Figure 5A, Table 6).

3) The PUFA at 1H-MRS (2.8/(1.3+4.65) ppm) were significantly increased after five weeks of MCD diet (p<0.001, Table 5). This significant increase leveled out at seven weeks of MCD diet. The PUFA assessed with 1H-MRS strongly correlated with polyunsaturated fatty acids assessed with gas chromatography (r=0.91, p<0.001) (Figure 5B, Table 6).

4) rTUFA (5.4/1.3ppm) at 1H-MRS strongly correlated with the amount of unsaturated relative to total hepatic fatty acids assessed by gas chromatography (r=0.81, p<0.001, Figure 5C, Table 6). This means that changes in unsaturated fatty acids can be measured irrespectively from the total amount of hepatic fat with 1H-MRS.

5) rPUFA significantly correlated with the amount of omega-6 polyunsaturated fatty acids relative to total fatty acids (r=0.73,p<0.001) (Figure 5D, Table 6). Moreover, rPUFA (2.8/1.3ppm) at 1H-MRS was correlated with the amount of polyunsaturated relative to total fatty acids (r=0.59, p=0.005, Figure 5E, Table 6) assessed with gas chromatography, but did not correlate with the amount of mono-unsaturated fatty acid relative to total fat ratio (r=-0.07, p=0.763), meaning that 1H-MRS is able to measure changes in polyunsaturated fatty acids separate from the total amount of hepatic fat.
Finally we investigated the degree of lobular inflammation assessed by histopathology in relation to gas chromatography and $^1$H-MRS. The degree of lobular inflammation positively correlated with the amount of omega-6 polyunsaturated fatty acids ($r=0.69$, $p<0.001$, Table 6) assessed by gas chromatography. Moreover, PUFA at $^1$H-MRS were correlated with the histopathologically assessed degree of lobular inflammation ($r=0.57$, $p=0.001$, Table 6) in the liver.

Table 5: $^1$H-MRS ratios during MCD diet periods

<table>
<thead>
<tr>
<th></th>
<th>Baseline Controls (n=5)</th>
<th>MCD 1 week (n=5)</th>
<th>MCD 2 weeks (n=5)</th>
<th>MCD 3 weeks (n=5)</th>
<th>MCD 5 weeks (n=5)</th>
<th>MCD 7 weeks (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>unsuppressed</td>
<td>suppressed</td>
<td>suppressed</td>
<td>unsuppressed</td>
<td>unsuppressed</td>
<td>unsuppressed</td>
</tr>
<tr>
<td>HFC</td>
<td>0.02±0.01</td>
<td>0.060±0.020</td>
<td>0.160±0.060</td>
<td>0.250±0.080</td>
<td>0.490±0.030</td>
<td>0.400±0.040*</td>
</tr>
<tr>
<td>TUFA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.010±0.005</td>
<td>0.046±0.006</td>
<td>0.042±0.0009§</td>
</tr>
<tr>
<td>PUFA</td>
<td>ND</td>
<td>0.003±0.003</td>
<td>0.007±0.007</td>
<td>0.012±0.007</td>
<td>0.039±0.003</td>
<td>0.041±0.0006*</td>
</tr>
<tr>
<td>rTUFA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.097±0.023</td>
<td>0.137±0.017</td>
<td>0.150±0.008£</td>
</tr>
<tr>
<td>rPUFA</td>
<td>ND</td>
<td>0.110±0.025</td>
<td>0.116±0.018</td>
<td>0.112±0.001</td>
<td>0.116±0.014</td>
<td>0.149±0.015¥</td>
</tr>
</tbody>
</table>

Mean ± standard deviation
ND, not determined
* significant difference ($p<0.001$) controls, week 1, 2 and 3
#, significant difference ($p<0.001$) week 1, 2 and 3
¥, significant difference ($p<0.001$) week 1 and 3
§, significant difference ($p<0.001$) controls and week 3
£, significant difference ($p<0.001$) week 3
Table 6: Correlations

<table>
<thead>
<tr>
<th>Parameters:</th>
<th>Pearson correlation coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1H-MRS, histopathology (HP) or gas chromatography (GC)</strong></td>
<td></td>
</tr>
<tr>
<td>HFC (1H-MRS) vs. histopathologic steatosis (HP)</td>
<td>( r = 0.93, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>HFC (1H-MRS) vs. total fatty acids (GC)</td>
<td>( r = 0.94, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>TUFA (1H-MRS) vs. total unsaturated fatty acids (GC)</td>
<td>( r = 0.90, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>PUFA (1H-MRS) vs. polyunsaturated fatty acids (GC)</td>
<td>( r = 0.91, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>PUFA (1H-MRS) vs. linoleic acid (GC)</td>
<td>( r = 0.90, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>PUFA (1H-MRS) vs. lobular inflammation (HP)</td>
<td>( r = 0.57, \ p = 0.001 )</td>
</tr>
<tr>
<td>rTUFA (1H-MRS) vs. total unsaturated/ total fatty acid ratio (GC)</td>
<td>( r = 0.81, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>rPUFA (1H-MRS) vs. polyunsaturated/ total fatty acid ratio (GC)</td>
<td>( r = 0.59, \ p = 0.005 )</td>
</tr>
<tr>
<td>rPUFA (1H-MRS) vs. omega-6 polyunsaturated fatty acids/ total fatty acid ratio (GC)</td>
<td>( r = 0.73, \ p &lt; 0.001 )</td>
</tr>
<tr>
<td>Omega-6 polyunsaturated fatty acids (GC) vs. lobular inflammation (HP)</td>
<td>( r = 0.69, \ p &lt; 0.001 )</td>
</tr>
</tbody>
</table>
Chapter 7: 3.0T $^1$H-MRS to assess hepatic lipid composition in rats

(A) T-FA (H-MRS) vs. Unsaturated fatty acids (mg/g) with $r = 0.896$

(B) P-FA (H-MRS) vs. Polyunsaturated fatty acids (mg/g) with $r = 0.921$

(C) T-FA (H-MRS) vs. Ratio unsaturated/total fat at gas chromatography with $r = 0.899$
Figure 5:
Scatter plots of the correlations between hepatic (poly) unsaturated fatty acids assessed by $^1$H-MRS versus gas chromatography.
A) Correlation between TUFA ($^1$H-MRS) at the $y$-axis versus total unsaturated fatty acids assessed with gas chromatography at the $x$-axis.
B) Correlation between PUFA ($^1$H-MRS) at the $y$-axis versus polyunsaturated fatty acids assessed with gas chromatography at the $x$-axis.
C) Correlation between rTUFA ($^1$H-MRS) at the $y$-axis versus the total unsaturated fatty acids relative to the total amount of fatty acids assessed with gas chromatography at the $x$-axis.
D) Correlation between rPUFA ($^1$H-MRS) at the $y$-axis versus omega-6 polyunsaturated fatty acids relative to the total amount of fatty acids assessed with gas chromatography at the $x$-axis.
E) Correlation between rPUFA ($^1$H-MRS) at the $y$-axis versus polyunsaturated fatty acids relative to the total amount of fatty acids assessed with gas chromatography at the $x$-axis.
Chapter 7: 3.0T $^1$H-MRS to assess hepatic lipid composition in rats

**DISCUSSION**

This study shows that 3.0T $^1$H-MRS is able to noninvasively detect total unsaturated and polyunsaturated fatty acids independent of the total hepatic fat content and this is strongly correlated with biochemical data. Furthermore, PUFA detected by $^1$H-MRS are associated with lobular inflammation of the liver.

Corbin et al. studied hepatic fat content and composition of hepatic triglycerides in two different mice models [25]. Using $^1$H-MRS they showed a significant increase in saturated and unsaturated fatty acids during prolonged durations of the experiments. Minor differences with our study are the use of two different mice models, the duration of the MCD diet, the use of a smaller voxel and respiratory triggering for $^1$H-MRS acquisitions. Major differences with our study are the use of a high field 4.7T MR magnet and different calculated $^1$H-MRS ratios to determine hepatic lipid composition (Corbin et al. used the 0.9 ppm resonance peak as an internal chemical shift reference). The most important difference with Corbin et al. is that in our study $^1$H-MRS results for the assessment of hepatic lipid composition were compared and confirmed to accurate biochemical hepatic fat analysis. Our study shows that the assessment of (poly)unsaturated fatty acids at $^1$H-MRS is also feasible on a clinical 3.0T MR scanner and that results are in agreement with biochemical data. Previous research showed that hepatic unsaturated lipids can be assessed with $^1$H-MRS at 3.0T and that they correlated with clinical and metabolic parameters associated with NAFLD [30]. Hepatic unsaturated lipids measured with $^1$H-MRS were increased in patients with diabetes type 2 (DM2). Unfortunately, in the latter study no liver biopsies were performed for histopathological or biochemical assessment. The comparison between the assessment of hepatic lipid composition with $^1$H-MRS and biochemical confirmation provides more insight in noninvasive assessment of the different $^1$H-MRS signal resonances in the liver.

3.0T $^1$H-MRS is able to measure unsaturated fatty acids (TUFA; total unsaturated fatty acids (5.4/(1.3+4.65) ppm) and PUFA; polyunsaturated fatty acids (2.8/(1.3+4.65) ppm) and total hepatic fat content (1.3/ (1.3+4.65) ppm). Furthermore, an increase in unsaturated fatty acids can be measured independently of the total amount of hepatic fat content using $^1$H-MRS (TUFA). This is confirmed by gas chromatography and supports the result of an earlier study demonstrating that compared to non diabetic patients, $^1$H-MRS assessed hepatic unsaturated fatty acids are increased in patients with DM2 independently from the total amount of hepatic fat [30].

Our study shows that $^1$H-MRS is able to measure increased PUFA in the steatotic rat liver, confirmed by gas chromatography.
Omega-6 polyunsaturated fatty acids increase more than omega-3 polyunsaturated fatty acids, resulting in a significantly increased omega-6/omega-3 ratio. Omega-6 polyunsaturated fatty acids are pro-inflammatory and could play a role in the development of NAFLD towards more severe non-alcoholic steatohepatitis (NASH) [31]. Hepatic polyunsaturated fatty acids could be a biomarker in this process because NASH induced in animal models show increased polyunsaturated fatty acids [25]. Furthermore, in recent clinical trials supplementation of omega-3 polyunsaturated fatty acids significantly improved metabolic and inflammatory markers in patients with NASH by improving omega-6/omega-3 polyunsaturated fatty acid ratio [9,32].

Our study has some limitations. First; as this was an exploratory pilot study no formal sample size calculation was performed. We chose a sample size that was considered appropriate to address the study aims. We did however use a control group. Since this was not a main objective of this study, reproducibility, inter- and intra observer variability was not assessed for the techniques used. However, reproducibility of $^1$H-MRS to measure hepatic fat content in humans has shown to be very reproducible [19].

$^1$H-MRS measurements were performed on a clinical 3.0T MR scanner. The assessment of resonances of several hepatic fatty acids is hampered at 3.0 Tesla by a lower spectral resolution, e.g. detection of the 0.9 methyl peak is impossible when the total amount of hepatic fat is low and separation of 2.1 and 2.3 peaks is not reliable at 3.0T because the latter two signal resonance peaks can easily be interpreted as one peak (Figure 2). $^1$H-MRS was performed during free breathing of the rats. This is a potential limitation because the volume interrogated by $^1$H-MRS could be blurred in the longitudinal direction by respiratory excursions in the rats. This could lead to an increased line width. Furthermore we used a point resolved spectroscopic sequence (PRESS) which can be confounded by J-coupling effects [33]. We corrected for T2 relaxation by using fixed T2-values at three weeks of MCD diet (five rats). This ignores the variability in T2 relaxation values between rats from other MCD diet periods. We only applied corrections for T2 relaxation for water at 4.65 ppm and fat at 1.3 ppm, not for secondary peak resonances. We did not correct for T1 relaxation. A limitation for $^1$H-MRS in general is that the differentiation between omega-3 and omega-6 polyunsaturated fatty acids is not possible.

In this study the peaks related to polyunsaturated fatty acid quantification at 2.8 ppm (PUFA and rPUFA) were quantified differently in weeks 1 and 2 compared to weeks 3, 5 and 7 (see Figure 2): for week 1 and 2 we used the water suppressed spectra and for week 3, 5 and 7 we used water unsuppressed spectra.

Separate staining for iron or fibrosis was not performed. This could confound the results.
of our study since elevated iron or fibrosis may be present in rats with steatotic livers. In conclusion, 3.0T $^1$H-MRS is able to noninvasively measure total unsaturated fatty acids and polyunsaturated fatty acids irrespectively of the total hepatic fat content (rTUFA and rPUFA). This assessment strongly correlates with biochemical data from gas chromatography. This experimental rat study provides evidence for the use of 3.0T $^1$H-MRS as a noninvasive diagnostic tool to assess hepatic lipid composition. This could be used in future research on hepatic steatosis.
REFERENCES


