Diffuse Reflectance Spectroscopy: Towards Real-Time Quantification of Steatosis in Liver

Transplant International 2015; 28: 465-474

Daniel J. Evers
Andrie C. Westerkamp
Jarich W. Spliethoff
Vishnu V. Pully
Daphne Hompes
Benno H.W. Hendriks
Warner Prevoo
Marie-Louise F. van Velthuysen
Robert J. Porte
Theo J. M. Ruers
Abstract

**Background:** Assessment of fatty liver grafts during orthotopic liver transplantation is a challenge due to the lack of real-time analysis options. Diffuse reflectance spectroscopy (DRS) could be a new diagnostic tool to quickly assess the degree of steatosis.

**Methods:** Eight hundred seventy-eight optical measurements were performed *in vivo* in 17 patients in liver tissue during surgery and *ex vivo* on liver resection specimens from 41 patients. The degree of liver steatosis per measurement location was quantified from the collected optical spectra and compared to the histology analysis, which was performed by three independent pathologists.

**Results:** Twenty-two patients were diagnosed with less than 5% steatosis, fifteen patients had mild steatosis (5-33%) and four patients displayed moderate (33-66%) steatosis in their liver biopsies. Severe steatosis (> 66%) was not identified. Intra-class correlation between the pathologists analysis was 0.949. A correlation of 0.854 was found between histological assessment and DRS analyses of liver steatosis *ex vivo*. For the same liver tissue, a correlation of 0.925 was demonstrated between *in vivo* and *ex vivo* DRS analysis for steatosis quantification.

**Conclusion:** DRS can quantify steatosis in liver tissue both *in vivo* and *ex vivo* with good agreement compared to histopathological analysis. DRS analysis can be performed real-time and may therefore be useful for fast and objective assessment of liver steatosis during organ procurement procedures.
Introduction

Liver steatosis is one of the most important risk factors for primary non-function or early graft failure after orthotopic liver transplantation (OLT). Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the world and may ultimately lead to severe non-alcoholic steatohepatitis (NASH) or cirrhosis. Estimations of NAFLD prevalence vary between 20 and 30% in unselected populations from developed countries (1). Steatosis is generally characterized quantitatively and qualitatively. Steatosis is traditionally quantified as none (< 5%), mild (5% to 33%), moderate (33% to 66%), or severe (> 66%) dependent on the percentage of hepatocytes containing fat vacuoles (2-6). Within the degree of fat accumulation in the hepatocytes, the histological evaluation of steatosis can be qualified in two major patterns: microvesicular and macrovesicular steatosis. Microvesicular steatosis solely, has been shown to have no negative impact on outcome (7-9). Yet, several studies have shown that moderate and severe macrovesicular steatosis of liver grafts is associated with impaired graft function after transplantation (10-13).

Assessment of fatty liver grafts during OLT is still a challenge for the transplant team. Surgical evaluation of fat accumulation by visual inspection and palpation during organ procurement has low predictive values and remains subjective (14). In addition, conventional imaging technologies also have their limitations in steatosis analysis and quantification. Ultrasound (US) is widely used in clinical practice to detect fatty infiltration by assessing the echogenicity in the liver. On the other hand, US has some disadvantages; this technique is not quantitative, prone to inter-observer variance and its sensitivity is reduced in morbidly obese patients (15,16). Other imaging techniques, such as computer tomography (CT), magnetic resonance imaging (MRI), and magnetic resonance spectroscopy (MRS) are able to visualize intrahepatic fat very accurately. The limitations of all three techniques are the inability to differentiate between micro- and macrovesicular steatosis and the relatively time-consuming and logistic efforts involved in these methods during a donation procedure (16,17). New techniques like electrical bioimpedance have recently been used to assess hepatic steatosis with high reliability (3). However, only results have been displayed in an animal set-up. Invasive histological evaluation still remains the gold standard for assessment of steatosis in liver tissue (18). Nevertheless, discrepancy in histological analysis has been described due to variability in interpreting the histological assessment per biopsy and the inter-observer variation among expert pathologists (19).

Over the last decade, diffuse reflectance spectroscopy (DRS) has been suggested to be a potential diagnostic tool for objective and quick assessment of tissue lipid concentration (20-22). During DRS, tissue is illuminated by a selected light spectrum. By subsequent analysis of absorption and scattering characteristics,
an “optical fingerprint” is obtained which represents specific biochemical and morphological information of the tissue examined. DRS is consequently able to determine the amount of fat in the tissue that is illuminated. The goal of the present study is to investigate whether DRS allows to quantify steatosis in human liver tissue in an in vivo as well as in an ex vivo clinical setting.

Materials and methods

Clinical Study Design

The study was conducted at The Netherlands Cancer Institute - Antoni van Leeuwenhoek hospital (NKI-AVL) between October 2009 and December 2012, under approval of the protocol and ethics review board. Optical measurements were performed both in vivo and ex vivo.

For in vivo measurements, 17 patients were included that were scheduled for partial liver resection mainly because of metastatic disease. Written informed consent was obtained from all patients. Before liver resection was performed, a 15 Gauge optical needle (Figure 1a, Invivo, Schwerin, Germany) was inserted into normal liver tissue within the planned resection area (Figure 1B and 1C) just below the liver surface. Ultrasound guidance (Hitachi Aloka, ProSound SSD-4000) was used to confirm the location of the tip of the needle to be in normal liver tissue and at least 2 cm from the liver tumor. A total of 242 optical measurements were performed at 49 different measurement locations. After the optical measurements, a twist coil marker (OTM 3.0SA, BIP GmbH, Türkenfeld, Germany) was inserted to mark the exact measurement location.

Ex vivo optical measurements were performed in normal liver tissue from 41 patients after partial liver resection. These patients included the 17 patients from the previously mentioned in vivo analysis combined with 24 additional patients that underwent only ex vivo measurements of resected liver tissue. Immediately after liver resection, DRS measurements were performed within benign liver tissue at least 2 cm from the metastatic sites. Several measurement locations were determined within each tissue specimen and on average 5 consecutive DRS measurements were performed at each measurement location. Thereafter a biopsy was directly taken from each specific measurement location for further histopathological analysis. A total of 636 DRS measurements at 127 different measurement locations were collected (Figure 1D).

Optical Spectroscopy Instrumentation

Recently, Nachabé et al. described the instrumentation and calibration procedure of our DRS system (20,23-25). The DRS system consists of a console comprising a Tungsten/Halogen broadband light source and two spectrometers. The two spectrometers resolve light in the visible wavelength range between 400 nm and
Figure 1. Overview of the optical spectroscopy system and optical measurements. Panel A: Optical needle with close-up of the tip. Panel B: Schematic display of an in vivo measurement performed before liver resection; L - benign liver tissue; RM - planned resection margin; T - tumor; GB - gallbladder; ON - optical needle. Panel C and D: In vivo and ex vivo measurement in “normal” liver tissue.

1100 nm (Andor Technology, DU420A-BRDD) and in the near infrared wavelength range from 800 up to 1700 nm (Andor Technology, DU492A-1.7), respectively. An optical probe that contained four optical fibers was attached to the DRS system for optical measurements (26). One fiber was connected to the light source and two fibers were connected to the spectrometers to capture the diffusely scattered light from the tissue in this study. The remaining fiber was not used. The average tissue volume that is illuminated with the probe is roughly 5 mm³. The acquisition time of each spectrum was on average 0.2 seconds.

Histopathological Analysis
In the resected liver tissue, a pathologist located the twist markers indicating the in vivo measurements and excised the surrounding liver tissue for tissue analysis. These biopsies as well as the biopsies retrieved from the ex vivo measurements were first fixed in formalin, subsequently paraffin embedded and processed for standard hematoxylin and eosin (H&E) staining. Three experienced pathologists, who were blinded for the outcome of the DRS results, individually examined the histological slides and visually determined the amount of steatosis within the benign liver tissue. A semi-quantitative assessment of steatosis was determined by estimating
the percentage of hepatocytes containing lipid droplets (both microvesicular and macrovesicular steatosis droplets) in 10 consecutive fields (magnification 25x). Macrovesicular steatosis was defined as fat vesicles larger than the cell nucleus, often displacing the nucleus. Microvesicular steatosis was defined as fat vesicles with similar size or smaller than the liver cell nucleus. The pathologic degree of steatosis was estimated with increments of 5%. If both steatosis subtypes were evidently present with similar percentages, the steatosis was defined as a ‘mixed type’. The mean of steatosis quantifications for each tissue specimen determined by the three pathologists was used for comparison with the DRS analysis. Each tissue specimen was subsequently divided into one of four preselected steatosis groups; none 0 to 5% steatosis, mild 5 to 33% steatosis, moderate 33 to 66% steatosis and severe > 66% steatosis. Finally, the liver tissue was also categorized as macrovesicular, microvesicular or mixed steatosis type.

**Spectral Data Analysis**
The light delivered to the tissue by the illumination fiber is subject to optical absorption and scattering before being collected by the detection fiber of the optical probe. Optical absorption is determined by the concentration of chromophores in the probed tissue. Each chromophore has its own intrinsic optical absorption characteristic, which is a function of wavelength. Fat and water are the dominant chromophores in the wavelength range between 1100 and 1600 nm (25). Oxygenated and deoxygenated hemoglobin and bile are the dominant chromophores in the wavelength range between 500-900 nm (20). The total absorption of the tissue as a function of wavelength can be written as the summation of the absorption of each chromophore multiplied by their concentrations in the tissue.

Optical scattering in tissue is dependent on the cellular structure of the target tissue and is sensitive to size and density of cellular and subcellular structures. Optical scattering can be described by the reduced scattering coefficient at a certain wavelength. To interpret the acquired DRS spectra, a widely accepted analytic model, introduced by Farrell et al. (27), was used to estimate the various DRS absorption and scattering coefficients. The acquired spectra were fitted and analyzed over the wavelength range from 500 to 1600 nm. Spectral characteristics analysis was performed with a Matlab software package (MathWorks Inc., Natick, MA, USA). Median values for fat, water, oxygenated and deoxygenated hemoglobin, bile, and the scattering parameters were calculated from the obtained spectra of each optical measurement.

**Statistical Analysis**
The lipid fraction scored by the pathologist was considered to be a two dimensional analysis of the same three dimensional volume of liver tissue analyzed with the DRS. To be able to compare the pathological analysis to the DRS analysis, the pathological lipid fractions were recalculated using the principle postulated
by Weibel et al. (28) and the following formula. $L_{\text{area}} = \frac{4}{3\pi}(L_{\text{area}})^{1/3}$. $L_{\text{area}}$ is the lipid fraction from the histological slide of the liver tissue scored by the pathologist and $L_{\text{volume}}$ is the histological volume lipid fraction assuming a homogeneous volume distribution of lipid spheres.

Inter-observer variability between pathologists was determined using a one-way single score intraclass correlation (ICC). We used a Spearman’s rank correlation test for the correlation between both the DRS ex vivo measurements and the pathologists’ quantification of steatosis as well as for the correlation between in vivo and ex vivo measurements within the same 17 patients. Analyses were performed using SPSS software (version 16.0, Chicago, IL, USA).

**Results**

A total of 41 patients (24 male and 17 female) were included in this study. The average age of all patients was 64 years (range 38–83 years). Patient characteristics as well as the histological characterization of the liver tissues are displayed in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Patient and Histological Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>Included patients</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Indications for resection</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Neoadjuvant chemotherapy</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Histological steatosis quantification</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Histological steatosis characterization</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*Chemotherapy regime for colorectal metastases consisted of a combination of Capecitabine and Oxalipatin. Seven patients also were treated with Bevacizumab. Mesothelioma was pretreated with Cisplatin and Pemetrexel and mamma carcinoma metastases were pretreated with Capecitabine and Lapatinib.*
Histological Characteristics

To be able to assign specific DRS patterns to differences in liver tissue composition, detailed histopathological examinations were performed for the tissue areas measured. Examples of the steatosis patterns encountered are displayed in Figure 2. The generally observed pattern of steatosis was a diffuse and relatively homogenously spread of clusters of lipid droplets as depicted in Figure 2A and 2B. Within a liver lobule the lipid droplets particularly accumulate near the central vein (Figure 2C). Histological analysis determined 22 patients in the group with less than 5% steatosis (represented as the group ‘none’). Twelve of these 22 patients...
had between 1 and 5% steatosis, the other 10 patients had 0% steatosis. Fifteen patients had ‘mild’ steatosis (5 to 33%), four had ‘moderate’ steatosis (33 to 66%), and no patients were diagnosed with ‘severe’ steatosis (> 66%). Differentiation of the steatosis type was performed on the 19 patients diagnosed with ≥ 5% steatosis. Most of these patients (n = 12) displayed a mixed pattern of both microvesicular and macrovesicular steatosis (Figure 2D). Macrovesicular steatosis, with lipid droplets up to 80 µm, was observed in 6 of these 19 cases. The steatosis percentages of these patients ranged from 6 to 47%. Microvesicular steatosis was only observed in 1 patient. High magnification illustrations of both steatosis subtypes are respectively displayed in Figures 2E and 2F. Three independent pathologists determined the quantification of steatosis for each individual patient. The calculated intraclass correlation (ICC) between the pathologists was 0.949, indicating a good agreement with each other.

**DRS Steatosis Analysis**

On average, 15 DRS measurements were performed within each liver specimen ex vivo. Examples of the optical spectra from one patient of each defined group and their corresponding histopathological slides are displayed in Figure 3 (next page). The spectrum in the vicinity of 1200 nm is dominated by the absorption of light by lipid cells. A more prominent inverse sharp peak in the light spectrum at this wavelength corresponded to a higher fat concentration in the tissue and consequently a higher steatosis score was observed. Figure 4 shows box plots of the calculated concentrations of fat, water and bile as well as the scattering coefficient from all tissue measurements for each defined steatosis group. The amount of fat, as determined by DRS, clearly increased with a higher grade of steatosis on histopathology. In addition, with an increasing steatosis score a significant decrease in water and bile concentration was observed together with an increase in scattering at 800 nm. The results of the average concentration of steatosis determined by both DRS and histology for each measured liver tissue specimen are displayed in Figure 5. A high level of agreement is presented with a correlation of 0.854 when comparing the results of both quantification methods for each measured tissue specimen. The comparison of the DRS analysis for each of the 17 patients for which steatosis was determined both in vivo and ex vivo is depicted in Figure 6. The correlation of 0.925 indicates a little difference in the quantification of liver steatosis by the optical needle before and after resection.

**Discussion**

Liver steatosis may significantly affect the function and survival rate of donor livers after transplantation. This renders identification of moderate and severe macrovesicular steatotic donor livers. Invasive histological evaluation remains the gold standard for the assessment of steatosis in liver tissue (18). However, limitations in the histological assessment have necessitated the search for novel
Figure 3. Examples of steatosis of the liver of increasing severity and the corresponding light spectra of the tissue generated with DRS. The estimated steatosis percentages for each tissue sample by three specialized pathologists and the corresponding DRS spectra are displayed. Specific wavelengths from which the fat volume concentration was calculated are indicated between the dashed lines. Magnifications are added in the bottom right corner of each photograph.
Figure 4. Box plots of different tissue components compared in different degrees of steatosis: none, mild, and moderate steatosis. Panel A: Fat. Panel B: Water. Panel C: Bile. Panel D: With an increasing degree of steatosis, an increase in scattering was observed at 800 nm.

Figure 5: Comparison of steatosis analysis by DRS versus pathological evaluation in 41 patients. Between pathological and DRS analysis a Spearman’s rank correlation of 0.854 was calculated.

Figure 6. Measurements with the DRS were performed in liver tissue of 17 patients in vivo as well as ex vivo. Between in vivo and ex vivo measurements a Spearman’s rank correlation of 0.925 was calculated.
tools capable of accurately quantification of fat in liver tissue (19). DRS has the ability to determine the lipid fraction within a tissue specimen with high accuracy (21,23,24,29). Our group recently compared the accuracy of DRS quantification of liver steatosis in murine livers with analysis by magnetic resonance spectroscopy, magic angle spinning - nuclear magnetic resonance, high-performance thin-layer chromatography, and histopathology. A good agreement of the estimated lipid fractions was demonstrated between DRS, the various imaging techniques and histopathological analysis (22). The next step towards the introduction of DRS into daily clinical practice of liver surgery, such as liver transplantation, is to first explore the accuracy quantifying liver steatosis in human liver tissue in vivo.

In this present study we have analyzed liver tissue in comparable conditions to those during liver transplantation: in a controlled situation in the operating theater during abdominal surgery immediately before and after liver tissue resection. Our results confirmed that DRS shows good agreement (correlation of 0.854) in fat quantification of liver tissue in comparison with the mean histological quantification of three independent expert pathologists. We subsequently demonstrated that DRS could quantify liver steatosis in vivo and ex vivo with comparable accuracy. The results of this preliminary study demonstrate that DRS can have the potential to improve real-time quantification of steatosis during organ donation procedures.

The main advantages of DRS compared to other available imaging techniques as well as to histopathology, are that the quantification of steatosis can be performed in real-time. In contrast, histological analysis requires specific staining at the pathology department and will generally take at least 30 minutes before reliable conclusions can be drawn. Similarly, CT, MRI, and MRS, are also relative time-consuming procedures during the donation procedure. In contrast, analysis of the degree of steatosis with the DRS can be performed much faster, especially during donation procedures, where every second counts.

It is known that liver steatosis is heterogeneous distributed throughout the liver parenchyma. However, during organ procurement, a needle biopsy is performed one or two times (30). Compared to these biopsies, with the DRS it is easier to perform multiple measurements. Therefore, we postulate that with the DRS a better estimation of the steatosis content of the whole liver can be made compared to histological analysis. In the current study, in liver specimens of the same patients, there was little variation of the steatosis concentration determined with DRS between different locations. The individual standard variation of the fat fraction varied from 1 and 13.0% steatosis. However, it should be noticed that for the most optimal result with the DRS, we have to validate the number of necessary measurements.
The main disadvantage regarding DRS in this study is that it concerns an invasive technique. During the invasive measurements in vivo, we did not observe any bleeding complications of the examined liver tissue. Microscopic analysis of the examined tissue specimen did not reveal obvious tissue damage of local bleeding. Moreover, an improved version of our optical needle has recently been developed for clinical applications, 20 G (= 0.8 mm) instead of 15 G (31). Biopsy needles generally used during invasive hepatic procedures nowadays range up to 14 G (or 1.6 mm). These arguments render our current DRS system as a similar invasive technique when comparing it to the generally performed core biopsy for histopathology analysis.

Interestingly, when comparing pathological assessment with DRS, higher percentages of steatosis were detected with the DRS system (Figure 5). Percent differences up to 20% were observed between the analysis methods. An explanation for this discrepancy could be the staining method that we used to visualize steatosis. The staining method was a standard H&E staining. Levene et al. (32) demonstrated that this staining method could cause an underestimation of the quantification of steatosis by expert pathologists when the lipid droplets are mainly microvesicular, as shown by an alternative staining method with Oil Red-O on frozen liver samples. However, Oil Red-O staining is not routinely used in clinical practice. In addition, the good ICC between the three pathologists is notable when compared to a previous study (19). We believe that this high correlation is due to the fact that more than half (n = 22) of the included patients were assigned to the ‘none’ steatosis group. This results in a relatively low variation in steatosis concentration over the entire cohort.

Within the steatosis groups as defined by histology, important differences were displayed in concentrations of water and bile (Figure 4). Both tissue parameters significantly decreased with an increase of lipid deposit in the hepatic tissue. The decrease in water concentration together with an increase in the degree of steatosis is in line with observations made by Marsman et al. (33). In a subsequent study, the same group confirmed these results, hypothesizing that the decrease in water is a result of exudation (34). In addition, the low concentrations of bile in steatotic liver tissue could be caused by a decreased hepatic uptake of serum bilirubin, as shown by McCormack et al. (35).

It has been shown that inferior graft function after transplantation is associated with macrovesicular steatosis. On the other hand, solely microvesicular steatosis has not this association (10-13). Therefore, it is necessary to determine the steatosis subtype during organ procurement. In our study, the incidence of livers with microvesicular steatosis was low. Although the predominance of macrovesicular and mixed steatosis in our patient cohort is comparable to previously published papers (3,35,36), it was in our experiment not possible to distinguish between
micro- and macrovesicular steatosis. Due to this limitation, we could not draw any conclusions towards a possible applicability of DRS distinguishing micro- and macrovesicular steatosis.

The results of this study provide a proof-of-principal that the DRS has the potential to enhance liver steatosis quantification. However, several questions remain to be answered before DRS can be translated into clinical practice. First, in future studies we must first reconfirm our results with specific evaluation of safety. Moreover, another future direction is to determine whether DRS can qualify both steatosis subtypes. We hypothesize that discrimination of microvesicular from macrovesicular steatosis could be made based on differences in the scattering of light. Optical scattering depends on the size and distribution of cellular particles compared to the wavelength of light \((23)\). Notable differences in the scattering of light at 800 nm between the defined steatosis groups were apparent as displayed in Figure 4. Graaff and coworkers \((37)\) demonstrated that the wavelength dependence of the scattering parameters depends on the size of the scattering particles. Since microvesicular and macrovesicular steatosis droplets significantly differ in size, careful analysis of the wavelength dependence of the scattering parameters might allow discrimination between the two subtypes of steatosis. In addition, we further aim to assess relevant transplantation related questions e.g. if the DRS is able to quantify steatosis after perfusion and cold storage. Finally, before DRS can be implemented into clinical practice, important focus must be put towards development of practical DRS hardware with minimization of user costs.

In conclusion, we have demonstrated that DRS can quantify steatosis in liver tissue both \textit{in vivo} and \textit{ex vivo} with good agreement in comparison to histopathology analysis. DRS analyses of liver steatosis can be performed within seconds and could therefore be used for a rapid clinical assessment of liver tissue during donation and prior to transplantation. Future studies need to be carried out to in order to establish whether DRS can distinguish between different subtypes of steatosis and to examine how the DRS can be implemented in the field of organ procurement and transplantation.

\textbf{Acknowledgements}

We would like to thank all the collaborators of the NKI surgery, pathology and radiology departments as well as the Philips Research project members for their contribution in the optical data collection and assessment. In particular, we would like to thank Gerard Lucassen and Rami Nachabé for their involvement in the study design and optical measurement process; Bart van de Wiel, MD and Joyce Sanders, MD for the histology assessment of the liver steatosis; Vincent van der Noort and Erik van Werkhoven for their help in the statistical analysis of the clinical data; Torre Bydlon for the assessments of English language and finally Walter Bierhoff and Jeroen Horikx for the respective probe and console development.
References


