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ORIGINAL ARTICLE

Diffuse reflectance spectroscopy: toward real-time quantification of steatosis in liver

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Conflicts of interest
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Summary

Assessment of fatty liver grafts during orthotopic liver transplantation is a challenge due to the lack of real-time analysis options during surgery. Diffuse reflectance spectroscopy (DRS) could be a new diagnostic tool to quickly assess steatosis. Eight hundred and 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. Liver steatosis was quantified from the collected optical spectra and compared with the histology analysis from the measurement location by three independent pathologists. Twenty two patients were diagnosed with <5% steatosis, 15 patients had mild steatosis, and four had moderate steatosis. Severe steatosis was not identified. Intraclass correlation between the pathologists analysis was 0.949. A correlation of 0.854 was found between the histology 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. DRS can quantify steatosis in liver tissue both *in vivo* and *ex vivo* with good agreement compared to histopathology analysis. This analysis can be performed real time and may therefore be useful for fast objective assessment of liver steatosis in liver surgery.

Introduction

Liver steatosis is one of the most important risk factors for primary nonfunction or early graft failure after orthotopic liver transplantation (OLT). Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the world and may ultimately lead to severe nonalcoholic 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* (5–33%), *moderate* (33–66%), or *severe* (>66%) depending 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]. 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. Disadvantages of this technique are that it is not quantitative and prone to interobserver variance and its sensitivity is reduced in morbidly obese patients [14–16]. Computer tomography, magnetic resonance imaging, and magnetic resonance spectroscopy are able to visualize intrahepatic fat very accurately. The limitations of all three techniques are the inability to differentiate between macro- and microvesicular steatosis and the relatively time-consuming and logistic efforts involved in these methods during a donation procedure [16,17]. New techniques such as electrical bioimpedance have recently been used to asses hepatic steatosis with high reliability [2]. Yet, only results in an animal setup have been displayed.

Invasive histological evaluation still remains the gold standard for assessment of steatosis in liver tissue [18]. However, discrepancy in histological analysis has been described due to variability in interpreting the histological assessment per biopsy and the interobserver 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 this 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 (Fig. 1a, In vivo, Schwerin, Germany) was inserted into normal liver tissue *within* the planned resection area (Fig. 1b,c) just below the liver surface. Ultrasound guidance (ProSound SSD-4000; Hitachi Aloka, Tokyo, Japan) 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. Directly 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, five consecutive DRS measurements were performed at each measurement location. A biopsy was then directly taken from all specific measurement locations for further histopathologic analysis. A total of 636 DRS measurements at 127 different measurement locations were collected (Fig. 1d).

Optical spectroscopy instrumentation

Recently, Nachabé *et al.* described the instrumentation and calibration procedure of our DRS system [20,23–25]. The

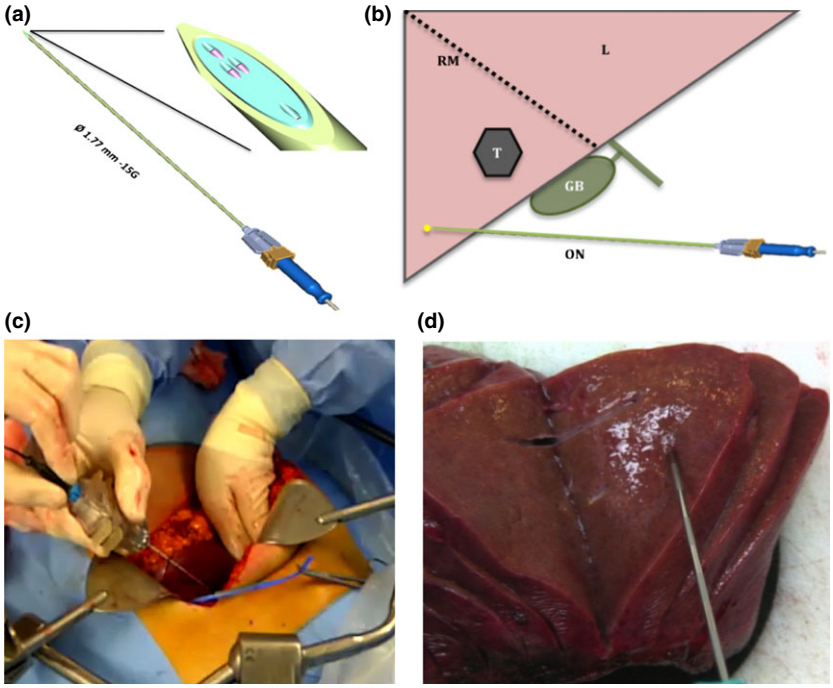

Figure 1 Overview of the optical spectroscopy system and optical measurements performed. (a) Optical needle with closeup of the tip, (b) Schematic display of an In vivo measurement performed before liver resection, L—benign liver tissue, RM—planned resection margin, T—tumor, GB—gallbladder, ON—optical needle, (c) in vivo and (d) ex vivo measurement in "normal" liver tissue.

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 1100 nm (DU420A-BRDD; Andor Technology, Belfast, UK) and in the near infrared wavelength range from 800 up to 1700 nm (DU492A-1.7; Andor Technology, Belfast, UK), respectively. An optical probe containing four optical fibers is 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 mm^3. The acquisition time of each spectrum was on average 0.2 s.

Histopathologic analysis

A pathologist located the twist markers inserted into the resected liver tissue after 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, then 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% steps. If both steatosis types 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 then divided into one of four preselected steatosis groups: "none" 0–5% steatosis, "mild" 5–33% steatosis, "moderate" 33–66% steatosis, and severe >66% steatosis. Finally, the liver tissue was also categorized by 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 and 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 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 and Gomez [28] and the following formula $L_{\text{volume}} = \frac{4}{3\sqrt{\pi}}(L_{\text{area}})^{3/2}$. L_{area} is the lipid fraction from the histological slide of the liver tissue scored by the pathologist, and L_{volume} is the histological volume lipid fraction assuming a homogeneous volume distribution of lipid spheres.

Interobserver 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 (Statistical Package for the Social Sciences, 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 tissue are displayed in Table 1.

Histological characteristics

To be able to assign specific DRS patterns to differences in liver tissue composition, detailed histopathologic examinations were performed for the tissue areas measured. Examples of the steatosis patterns encountered are displayed in Fig. 2. The generally observed pattern of steatosis was a diffuse and relatively homogenously spread of clusters of lipid droplets as depicted in Fig. 2a,b. Within a liver lobule, the lipid droplets particularly accumulate near the central vein (Fig. 2c). Histological analysis determined 22 patients in the group with <5% steatosis (represented as the group "none"). Twelve of these 22 patients had between 1% and 5% steatosis, and the other 10 patients had 0% steatosis. Fifteen patients had "mild" steatosis (5–33%), four had "moderate" steatosis (33–66%), 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 (Fig. 2d). Macrovesicular steatosis, with lipid droplets up to 80 μm, was observed in six of these 19 cases. The steatosis percentages of these patients

Table 1. Patient and histological characteristics.

		N (%)
Included patients	Total	41
	Male	24 (58.5%)
	Female	17 (41.5%)
Indications for resection	Colorectal metastases	38 (92.8%)
	Mesothelioma	1 (2.4%)
	Mamma carcinoma metastases	1 (2.4%)
	Hepatocellular carcinoma	1 (2.4%)
Neoadjuvant chemotherapy	Yes	22 (53.7%)*
	No	19 (46.3%)
Histological steatosis quantification	None (0–5%)	22 (53.7%)
	Mild (6–33%)	15 (36.6%)
	Moderate (34–66%)	4 (9.7%)
	Severe (>67%)	0
Histological steatosis characterization	No steatosis	10 (24.4%)
	Microsteatosis	2 (4.9%)
	Macrosteatosis	14 (34.1%)
	Mixed steatosis	15 (36.6%)

*Chemotherapy regime for colorectal metastases consisted of a combination of capecitabine and oxaliplatin. Seven patients also were treated with Bevacizumab. Mesothelioma was pretreated with cisplatin and pemetrexel and mamma carcinoma metastases were pretreated with capecitabine and lapatinib.

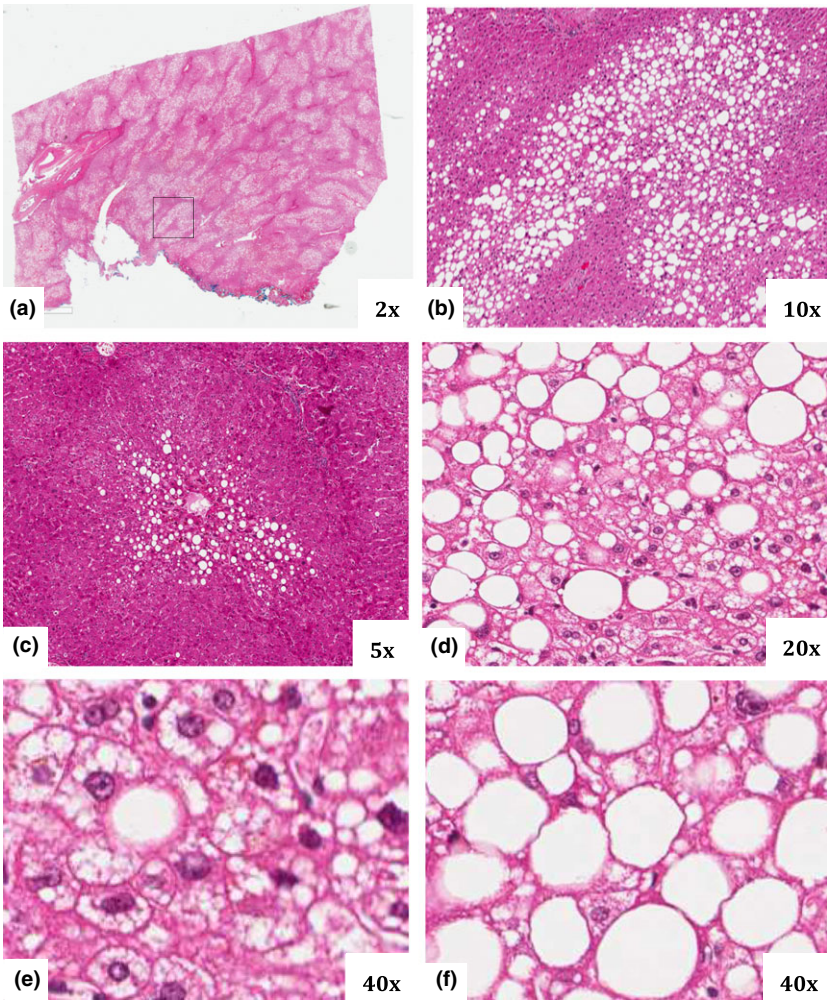

Figure 2 Examples of steatosis after standard hematoxylin and eosin (H&E) staining. (a, b) Typical example of the diffuse pattern of steatosis in liver tissue in one patient specimen, (c) lipid droplets generally accumulate in the zone around the central veins in each liver lobule (d) normal liver with mixed pattern of both macro- and microsteatosis, high magnification of (e) microsteatosis, and (f) macrosteatosis. Magnifications are added in the bottom right corner of each photograph.

ranged from 6% to 47%. Microvesicular steatosis was only observed in one patient. High magnification illustrations of both steatosis subtypes are respectively displayed in Fig. 2e,f.

Three independent pathologists determined the quantification of steatosis for each individual patient. The calculated intraclass correlation (ICC) between the pathologists was 0.949, indicating 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 histopathologic slides are displayed in Fig. 3. 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 corresponds to a higher fat concentration in the tissue, and consequently, a higher steatosis score was observed.

Figure 4 shows boxplots 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 increases with a higher grade of steatosis on histopathology. In addition, with an increasing steatosis score, a significant decrease in water and bile concentration is 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

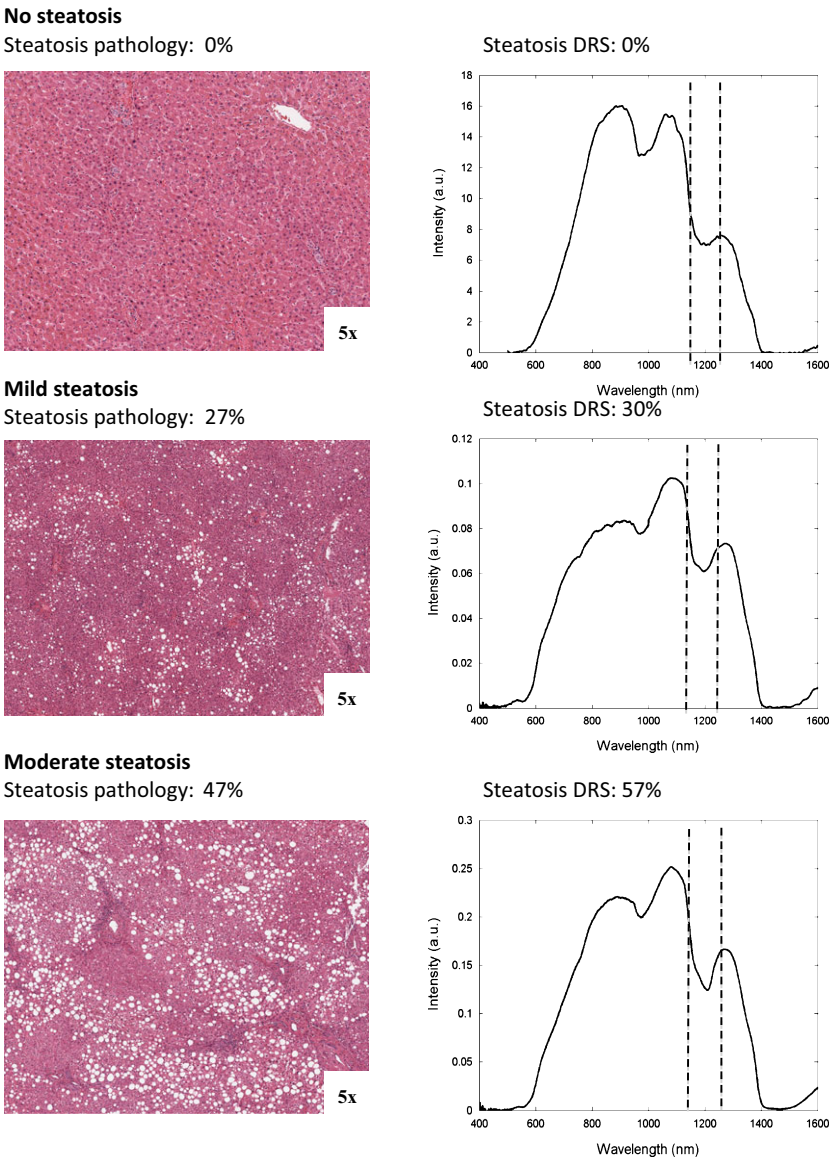

Figure 3 Examples of steatosis of the liver of increasing severity and the corresponding light spectra of the tissue generated with DRS. The estimated steatosis percentages for each tissue sample by three specialized pathologists and the corresponding DRS spectra are displayed. Specific wavelengths from which the fat volume concentration was calculated are indicated between the dashed lines. Magnifications are added in the bottom right corner of each photograph.

liver tissue specimen are displayed in Fig. 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 Fig. 6. The correlation of 0.925 indicates 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 steatosis of significant clinical relevance. 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 tools capable of accurate quantification of fat in liver tissue [19]. DRS has

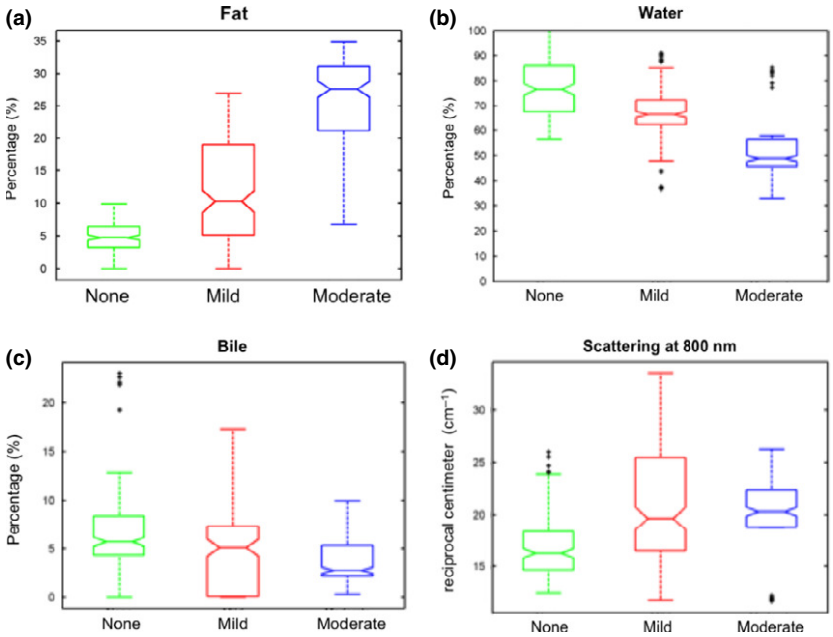

Figure 4 Boxplots of the concentrations of (a) fat, (b) water, (c) Bile, and (d) the Scattering at 800 nm of all included patients by steatosis group as defined by histology.

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 histopathologic analysis [22]. The next step toward 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 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 directly before and after liver tissue resection. Our results confirmed that diffuse reflectance spectroscopy 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 could have the potential to improve real-time quantification of steatosis during liver surgery.

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 the operating theatre during surgery and it can be performed real time with feedback of the estimated lipid fraction within seconds. In contrast, histological analysis requires specific staining at the pathology department and will generally take at least 30 min before reliable conclusions can be drawn. This is an important feature considering that the time factor is critical in all transplantation surgery.

In addition, incorporation of DRS into a needle as shown in this study allows a single-point measurement of several mm^3 at the tip of the optical needle. This could arguably be assumed a disadvantage considering the aim to determine the steatosis level in the whole liver. Yet, performing continuous measurements with direct feedback of the tissue parameters during the insertion of the optical needle into the target tissue allows direct characterization of tissue all along the whole needle path. Liver steatosis is known have a heterogeneous distribution throughout liver parenchyma. When we take this most common diffusely clustered pattern of liver steatosis (Fig. 2a) into account, multiple DRS measurements along the needle tract could arguably provide a good representation of the average steatosis fraction throughout a larger area of the liver specimen compared with the advised standard histological analysis of a double core biopsy [30]. A notable observation in the liver specimens of the patients included in this study was that there was little variation of the steatosis concentration determined with DRS between different locations within each individual patient. The individual standard variation of the fat fraction varied from 1.0% to 13.0% steatosis. We

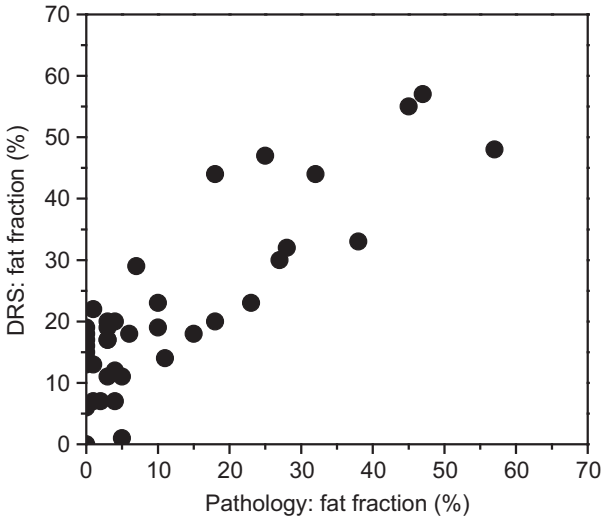

Figure 5 Comparison of the steatosis analysis by DRS versus pathology in 41 patients. Spearman's rank correlation = 0.854.

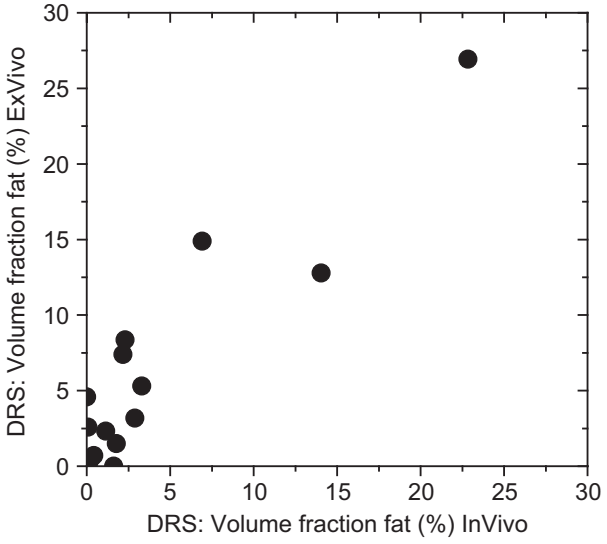

Figure 6 Comparison of steatosis analysis by DRS per patient for 17 patients *in vivo* versus *ex vivo.* Spearman rank correlation = 0.925.

will focus on the number and depth of measurement locations for optimal result in future analysis.

The main disadvantage regarding DRS in this particular 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 caused by the optical needle. Moreover, an improved version of our optical needle has recently been developed for clinical study, now 20G (=0.8 mm) instead of 15G [31]. Biopsy needles generally used during invasive hepatic procedures nowadays range up to 14G (or 1.6 mm). These arguments render our current DRS system a similar invasive technique when comparing it to the generally performed core biopsy for histopathology analysis, the current gold standard.

Our results displayed in Fig. 5 show that liver specimens with histologically small percentages of steatosis appear to have higher percentages of steatosis detected with the DRS system. Percent differences up to 20% were observed between the analysis methods. The staining method we used 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, this method is not routinely used in clinical practice. The discrepancies in steatosis concentrations in our study could be caused by an underestimation of the hepatic steatosis by the pathologists due to the standard staining method used. The clinical consequences of this discrepancy, however, are limited as it is well known that minimal degree of steatosis has no impact on outcome after liver transplantation [10–13]. The good ICC between the three pathologists is notable when compared to previous studies. 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 "no" steatosis group, thus resulting 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 (Fig. 4). Both tissue parameters significantly decreased with an increase of lipid deposit in the hepatic tissue. The decrease in water concentration with increased steatosis is in line with observations made by Marsman *et al.* [33] in murine studies. In a subsequent study, the same group confirmed these results, hypothesizing the decrease of water concentration to be a result of exudation from the liver tissue [34]. The decrease of bile within the liver tissue could be caused by decreased hepatic uptake of serum bilirubin due the lipid deposits within the hepatocytes. This hypothesis is supported by several groups who have reported increased serum bilirubin levels in patients with moderate and severe hepatic steatosis [35,36].

The predominance of macrovesicular and mixed steatosis in our patient cohort is comparable to previously published articles [3,37,38]. Poor graft outcome which is commonly associated with macrovesicular steatosis has proven the clinical relevance to determine steatosis subtype [10–13]. Because of the minimal number of patients with microvesicular steatosis, an analysis to distinguish both steatosis subtypes by DRS within our patient cohort was not possible. Due to this limitation in our study, we could not draw any conclusions toward a possible applicability of DRS distinguishing micro- and macrovesicular steatosis.

The results of this experimental technique based on a mature optical fiber technology arguably provide proof of principal that DRS has the potential to enhance liver steatosis quantification. Yet, several questions remain to be answered before DRS can be translated into clinical practice. In future studies with more tissue specimens, we must first reconfirm results displayed in this study with specific evaluation of the safety of the methods. We further aim to determine whether DRS can qualify both steatosis subtypes based on the described scattering properties of DRS. We hypothesize that discrimination of microvesicular from macrovesicular steatosis could be made using DRS based on differences in the scattering of light. Optical scattering depends on the size and distribution of cellular particles compared with the wavelength of light [36]. Notable differences in the scattering of light at 800 nm between the defined steatosis groups were apparent as displayed in Fig. 4. Graaff *et al.* [39] demonstrated that the wavelength dependence of the scattering parameters depends on the size of the scattering particles. As 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 types of steatosis. We further aim to assess relevant transplantation-related questions, for example whether DRS is a reliable technology for steatosis quantification in liver parenchyma after perfusion and cold storage. Finally, before DRS can be implemented into clinical practice, important focus must be put toward development of practical DRS hardware with minimization of user costs.

Conclusion

In a preliminary study, we have demonstrated that DRS can quantify steatosis in liver tissue both *in vivo* and *ex vivo* with good agreement when compared to histopathology analysis, the current gold standard. DRS analyses of liver steatosis can be performed within seconds and could therefore be used for a rapid clinical assessment of the liver tissue during liver donation and prior to transplantation. Future studies will focus on the question of whether DRS can also distinguish between microvesicular and macrovesicular steatosis in real time and to explore effects on transplantation of a liver organ.

Authorship

DJE: designed research/study, performed research/study, collected data, analyzed data and wrote the article. ACW: wrote and reviewed the article. JWS and M-LFvV: performed research/study, collected data, analyzed data and reviewed the article. VVP: performed research/study, collected data, analyzed data and wrote and reviewed the article. DH and WP: performed research/study, collected data and reviewed the article. BHWH: designed research/study, performed research/study, collected data, analyzed data and reviewed the article. RJP: reviewed the article. TJMR: designed research/study, performed research/study, collected data and reviewed the article.

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