Early detection of fatty liver disease in mice via quantitative ultrasound

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\textit{Abstract} — Nonalcoholic fatty liver disease (NAFLD) is estimated to have a 25-30\% incidence among obese individuals and can lead to inflammation (nonalcoholic steatohepatitis, NASH) and fibrosis. While conventional ultrasound and other imaging techniques can diagnose advanced stages of fatty liver and hepatic cirrhosis (one NASH outcome), even though pathology is the gold standard, existing techniques are not currently able to detect early stages of NAFLD. The objective is to develop a method for early detection of NAFLD in humans by first examining the model-free quantitative ultrasound (QUS) parameters of livers in diet-controlled mice. Two studies were conducted, each with 25 male C57BL/6J mice; 20 fed an atherogenic (Ath) diet and 5 fed a control diet. In Study 1 (a non-QUS study), 10 mice each were fed the Ath diet for 4 and 8 weeks, respectively; there was significant liver steatosis and inflammation of the Ath-diet-fed mice. Therefore, Study 2 was conducted with reduced feeding times to moderate the Ath diet’s liver effect for which 10 mice each were on the Ath diet for 2 and 4 weeks, respectively. For Study 2, liver lipids compared somewhat favorably to pathologist steatosis grades. QUS model-free parameters were functionally related to liver lipids. These preliminary findings suggest that early detection of NAFLD is feasible with QUS.

I. INTRODUCTION

Prevalence rates for obesity, type II diabetes, coronary heart disease and stroke are unacceptably high and increasing in many populations. Progressive injury to the liver develops early in these metabolic diseases. Nonalcoholic fatty liver disease (NAFLD) is estimated to have a 25-30\% incidence among obese individuals and can lead to inflammation and fibrosis (nonalcoholic steatohepatitis, NASH). While conventional ultrasound and other imaging techniques can diagnose advanced stages of fatty liver and hepatic cirrhosis (one NASH outcome), existing techniques are not currently able to detect early stages of NAFLD. The majority of patients are asymptomatic and screening methods are not available; their liver disease is identified incidentally following blood tests or abdominal imaging [1]; blood and imaging tests are not sensitive for NASH or early fibrosis. Currently, many patients with NASH are not identified until they have progressed to advanced liver disease that is difficult to reverse. Development of more sensitive early detection and monitoring of NAFLD would be a medically significant tool with very high impact on clinical care. Over 30 million Americans are afflicted with NAFLD of which 600,000 likely to have cirrhosis [2].

Early NAFLD consists of 5\% or more intracellular lipid. NASH can result following additional fat deposition and infiltration of inflammatory cells. Advanced liver pathology occurs with ballooning hepatocytes, fibrosis and cirrhosis [3\&4]. Biopsies, which are highly invasive and not practical for routine screening, would be the only way to assess early-stages of NAFLD. There is a critical need to identify susceptible/at-risk persons with a more sensitive noninvasive imaging system capable to quantify NAFLD’s early stages. If accomplished, this will alert physicians to those at risk and in need of diet or drug interventions, and allow for noninvasive monitoring of progression of hepatic degradation or improvement with treatment.

The objective herein is to development a method for early detection of NAFLD in humans by first examining the model-free quantitative ultrasound (QUS) parameters (attenuation, ATN, and backscatter, BSC, coefficients) of livers in diet-controlled mice, and examine their relationships to liver pathology and fat content.

II. DIET, LIVER PATHOLOGY AND QUS

Two studies (denoted Studies 1 & 2) were conducted, each with 25 male C57BL/6J mice; 20 fed an atherogenic (Ath) diet (Teklad TD.120156, Harlan) and 5 fed a control diet (chow diet). The Ath diet is similar to the “Paigen” diet developed by Beverly Paigen that is still utilized today [4, [5] and is essentially the same Ath diet that [6] used. In Study 1 (Fig 1), there was no evidence of steatosis or
inflammation in the mice fed the control diet but those fed the Ath diet had progressive pathologies after 4 and 8 weeks of feeding.

However, Fig 2 (Study 2: 2 and 4 weeks feeding) shows more varied pathology including steatosis or inflammation with control diet. Liver pathology in the mice fed the Ath diet included steatosis and inflammation. Generally, the steatosis was primarily microvesicular, 2-10 μm in diameter, though a minor component of macrovesicular steatosis was seen. There were several vesicles in the 15-20 μm range, a few in the 20-25 μm range, while single vesicles of ~30 μm were rarely seen.

For Study 1, 95% of mouse livers fed the Ath diet for 4 or 8 weeks were classified as steatohepatitis; for shorter duration feeding, there were 82% in Study 2. Although the histology was recognizable as steatohepatitis by both pathologists, there were some differences between the model and the typical findings in humans with NASH. The fat was predominantly microvesicular as opposed to human NASH, where fat is either predominantly macrovesicular or a mixture of micro- and macrovesicular fat. None of the Ath fed mice had bridging fibrosis found in more advanced cases of human NASH; it is likely that with longer feeding periods, more macrovesicular fat deposition and fibrosis would occur. [6] found significant enhancement of hepatic hydroxyproline after 24 weeks of feeding essentially this same diet to the same strain of male mice; therein it was shown that more enhanced fibrosis, inflammation and hepatocellular ballooning occurred when additional fat was added to the diet.

Study 2 included QUS liver assessments. Liver lipids [7] were compared to steatosis grades (Fig 3). Prior to euthanasia, the mouse liver was scanned in vivo (Vevo 2100) to estimate ATN and BSC. Following euthanasia, QUS estimates of the ex vivo liver were made with a single-element technique [8], [9]. Three in vivo (15-17, 19-21 & 23-25 MHz) and four ex vivo frequency ranges (24-26, 31-33, 38-40 & 45-47 MHz) were evaluated. Fig 4 shows 23-25 MHz (in vivo) and 24-26 MHz (ex vivo) outcomes. The full six-variable (3 freq ranges) in vivo regression model for predicting number of weeks on cholesterol was highly statistically significant ($R^2 = 0.57$, $F = 4.03$ on 6 & 18 degrees of freedom, $p = 0.0098$). The full eight-variable (4 freq ranges) ex vivo regression model for predicting number of weeks on cholesterol was highly statistically significant ($R^2 = 0.83$, $F = 15.12$ on 8 & 16 degrees of freedom, $p < 0.0001$). Overall the predictions were well separated between 0 weeks and 2-4 weeks, while 2 and 4 weeks were more difficult to separate.

In vivo, the full linear discriminant analysis [10] used ATN and BSC for all 3 freq ranges. The analysis produced two linear discriminant functions with 98.16% of the total variation explained by the first linear discriminant. This discriminant function loaded most heavily on ATN and BSC at 23-25 MHz. While there was some difficulty in distinguishing 0 vs. 2 weeks on Ath diet, and in distinguishing 2 vs. 4 weeks on Ath diet, there were no misclassifications of control as 4 wk or vice versa. Ex vivo,
the full linear discriminant analysis used ATN and BSC for all 4 freq ranges. The analysis produced two linear discriminant functions with 72.8% of the total variation explained by the first linear discriminant and 27.2% explained by the second. Only 1 of the 25 livers was misclassified (as 2 weeks instead of 4 weeks) in this ex vivo analysis.

III. LIVER QUS AND FAT CONTENT

Fig 5 shows the attenuation coefficient and backscattered coefficient as a function of fat content for 25 C57BL/6J mouse livers. The same livers were scanned in vivo, then scanned ex vivo within one hour of the mouse being euthanized. These mice were those of Study 2. Both ATN and BSC increase as the fat content increases. The best-fit linear equations indicate there is a greater change with fat content for ATN compared to BSC. While the idea of fatty liver disease does not pertain to mice, these ATN and BSC observations strongly suggest that model-free QUS parameters are sensitive to liver fat. Further, animal models offer the possibility to model fatty liver disease, specifically because even near normal fat content (~4-5%) in these mice for which these five mice were fed the chow diet), it is possible to study the livers via biopsy and biochemical procedures, procedures not likely with human participants who appear to have normal livers.

These observations point out why it is critical to study the acoustical properties of tissue under in vivo conditions.

IV. QUS METHODOLOGY

ATN in vivo method: ATN estimates were obtained using the spectral difference reference phantom method [11]. An area (denoted field of interest, FOI) of each image was manually segmented to carefully avoid local areas of high brightness in the B-mode images. The FOI was divided into 50%-overlapped sub-regions of interest (denoted sub-ROIs) with dimensions 1.5 x 1.5 mm. Each sub-ROI was divided axially into three overlapping window regions that were 0.75 mm in length, as required by the spectral difference method, in order to obtain attenuation estimates. All of the estimates from each of the sub-ROIs of one liver were averaged to obtain the mean ATN versus frequency over the bandwidth 15-25 MHz. The mean ATN (dB/cm-MHz) was then averaged over the indicated frequency range, for example, ATN.23.24 was the ATN average over 23 and 24 MHz.

BSC in vivo method: BSC estimates were obtained using the reference phantom method [11]. The FOI that was used for ATN estimation in each image was the same FOI that was segmented for BSC estimation. The FOI was divided into 75%-overlapped sub-ROIs with dimensions 1.16 x 1.16 mm (equivalent to 15 x 15 wavelengths at 20 MHz). Attenuation was compensated for by using the power-law fit values of the mean attenuation versus frequency curve for each liver. All the BSC estimates from data of one liver were averaged together to obtain the mean BSC versus frequency curve over the bandwidth 15-25 MHz. The mean BSC (1/cm-sr) was then averaged over the indicated frequency range, for example, BSC.23.24 was the BSC average over 23 and 24 MHz.

ATN ex vivo method: The ex vivo ATN was estimated using a broadband insertion-loss technique [12] with a 40-MHz f/3 focused transducer. A piece of liver sample 2- to 3-mm thick was placed on a planar Plexiglas® reflector. The liver sample, the Plexiglas® reflector and the transducer were all submerged in 0.9% degassed saline. The transducer focus was positioned at the liver-Plexiglas® interface. The insertion loss was determined by comparing the power spectra of the echoes reflected off the Plexiglas® surface with and without the sample being present in the acoustic path. The effect of saline attenuation was compensated for, where the saline attenuation was assumed to be the same as water at 20°C [13]. Thirty-six independent lateral locations across the sample were scanned and the attenuation estimates from all locations were averaged together to obtain the mean attenuation (dB/cm) versus frequency curve over the bandwidth of 22 – 49 MHz. The mean attenuation (dB/cm) versus frequency (MHz) slope was calculated, and then averaged over the indicated frequency range, for example, ATN.23.24 was the ATN (dB/cm-MHz) average over 23 and 24 MHz.

BSC ex vivo method: The ex vivo BSC was estimated using the planar reference technique [14]. First, reference signals were obtained from the planar Plexiglas® plate whose pressure reflection coefficient relative to 0.9% saline at room temperature is known (= 0.37). The reference scan...
was taken by recording the reflection off the saline-Plexiglas® interface at the set of positions that covered the -6 dB depth of focus with a step size of a half wavelength. Second, a raster scan on the sample was performed to acquire 11 independent images at different locations from each sample, and the lateral step size within each image was one beam width. Third, the BSC was estimated from the recorded echo data. The analyzed liver sample area was divided into 75%-overlapped sub-ROIs with dimensions 0.578 x 0.578 mm (equivalent to 15 x 15 wavelengths at 40 MHz). Attenuation was compensated for by using the power-law fit values of the insertion-loss attenuation for each sample. The BSC was estimated for each sub-ROI, and all the BSC estimates from the data of one sample were averaged together to yield the mean BSC versus frequency curve over the bandwidth 22 - 49 MHz. The mean BSC (1/cm-sr) was then averaged over the indicated frequency range, for example, BSC.23.24 was the BSC (1/cm-sr) average over 23 and 24 MHz.

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Illinois and satisfied all campus and National Institutes of Health rules for the humane use of laboratory animals.

The authors would like to thank Rita J. Miller, DVM and Sandhya Sarwate, MD from the University of Illinois at Urbana-Champaign and Michael R. Peterson, MD, PhD from the University of California at San Diego for their valued assistance.

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