MOUSE LIVER DISPERSION FOR THE DIAGNOSIS OF EARLY-STAGE FATTY LIVER DISEASE: A 70-SAMPLE STUDY

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Abstract—The accumulation of fat droplets within the liver is an important marker of liver disease. This study assesses gradations of steatosis in mouse livers using crawling waves, which are interfering patterns of shear waves introduced into the liver by external sources. The crawling waves are detected by Doppler ultrasound imaging techniques, and these are analyzed to estimate the shear wave speed as a function of frequency between 200 and 360 Hz. In a study of 70 mice with progressive increases in steatosis from 0% to 60%, increases in steatosis are found to increase the dispersion, or frequency dependence, of shear wave speed. This finding confirms an earlier, smaller study and points to the potential of a scoring system for steatosis based on shear wave dispersion.

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INTRODUCTION

As a result of the unabated obesity epidemic, fatty liver disease (FLD) is the most common cause of liver dysfunction in the United States and other economically privileged countries (Angulo 2002; Dowman et al. 2010; Schreuder et al. 2008). Accumulation of fat droplets (steatosis) within the liver is most often associated with the metabolic syndrome of obesity, diabetes and dyslipidemia, but can also be caused by toxins such as alcohol and certain chemotherapeutic agents or, rarely, associated with pregnancy (Charlton 2004; Marchesini et al. 1999; Wanless and Lentz 1990). Simple hepatic steatosis is reversible, but can progress to a chronic inflammatory and fibrotic state termed NASH (non-alcoholic steatohepatitis). Cirrhosis caused by NASH is predicted to become the leading cause of end-stage liver failure and indication for liver transplant in this country within the next 8 y (Charlton 2004; Selzner and Clavien 2001).

Liver biopsy currently remains the gold standard for diagnosing and assessing FLD (Minervini et al. 2009). Because this procedure is uncomfortable and can rarely result in serious complications, the current practice is to reserve biopsy for patients in whom the suspicion for NASH is high (based on blood testing or imaging) (Strassburg and Manns 2001). Unfortunately, biochemical assessment and currently available imaging modalities are insensitive in determining the presence or degree of FLD. Furthermore, histologic assessment of liver biopsies is completely subjective (based on the individual pathologist’s estimation of overall steatosis and fibrosis) and, therefore, subject to wide clinical variability.

Non-invasive techniques to assess hepatic steatosis are emerging to meet this critical need and include magnetic resonance elastography (Chen et al. 2011; Salameh et al. 2009; Schwenzer et al. 2009), magnetic resonance...
spectroscopy (Friedrich-Rust et al. 2010), computed tomography elastography (Castera et al. 2008), radiation force methods (Chen et al. 2013) and controlled attenuation parameter transient elastography (de Ledinghen et al. 2012; Friedrich-Rust et al. 2012; Sasso et al. 2010, 2012). We propose an ultrasound-based system that can serve as a point of care screening modality (i.e., available to primary care physicians and gastroenterologists) to complement and increase efficient use of biopsy and other more costly imaging techniques.

Our previous work (Barry et al. 2012) introduced the hypothesis that increasing the amount of fat in the liver would increase the dispersion of shear wave velocity, resulting in an increase in the slope of shear speed and shear attenuation versus frequency. This is a consequence of adding a viscous element, triglycerides, to the liver medium. This previous study reported results from a preliminary study of 14 mice divided into two groups, lean (<5% steatosis) and obese (~65% steatosis). The difference in dispersion or slope of shear speed versus frequency between the two groups was found to be statistically significant ($p < 0.003$). Dispersion was low in lean livers (0.16 ± 0.03 m/s per 100 Hz) and higher in obese livers (0.23 ± 0.04 m/s per 100 Hz), as measured over a shear wave frequency band centered around 260 Hz.

In the present study, we expand the numbers of mice and attempt to titrate the response by examining subgroups with increasing steatosis. Ultimately the goal is to establish a fine gradation scoring of steatosis using shear wave dispersion, in vivo. The present study takes a first step toward that goal.

THEORY

Shear wave dispersion

To model shear wave propagation in sinusoidal steady state in an elastic material with losses, the general stress-strain relationship is

$$ T(\omega) = \mu S(\omega) \tag{1} $$

where $T$ and $S$ are the shear stress and strain, respectively, $\omega$ is the frequency and $\mu$ is the shear modulus; the shear wave speed $c_s = \sqrt{\mu/\rho}$, where $\rho$ is density. Assuming that $\mu$ can be described as $\mu(\omega) = K(\omega) + jH(\omega)$, where $K$ is the real part and $H$ is the imaginary part of the shear modulus, then the complex wavenumber is

$$ k = \frac{\omega}{c_s} = \beta - j\alpha = \frac{\omega}{\sqrt{K(\omega) + jH(\omega)}} \tag{2} $$

Here, $k$ is the wavenumber with real ($\beta$) and imaginary ($\alpha$) parts (Blackstock 2000). The attenuation coefficient, $\alpha$, of a propagating wave will therefore be a function of frequency depending on $K(\omega)$ and $H(\omega)$. Expanding on the real and imaginary parts of eqn (2), we have

$$ \beta = \omega \sqrt{\frac{\rho}{K^2 + H^2}} \left[ \frac{1}{2} \left( 1 + \frac{1}{\sqrt{1 + \frac{H^2}{K^2}}} \right) \right]^{1/2} \tag{3} $$

and the wave speed

$$ c = \sqrt{\frac{K^2 + H^2}{\rho}} \left[ \frac{1}{2} \left( 1 + \frac{1}{\sqrt{1 + \frac{H^2}{K^2}}} \right) \right]^{1/2} \tag{4} $$

and the absorption coefficient

$$ \alpha = \omega \sqrt{\frac{\rho}{\sqrt{K^2 + H^2}}} \left[ \frac{1}{2} \left( 1 - \frac{1}{\sqrt{1 + \frac{H^2}{K^2}}} \right) \right]^{1/2} \tag{5} $$

Note that if $H(\omega)$ is zero, then $c$ and $\beta$ are constant (over frequency), and $\alpha$ is zero. However, if $H(\omega)$ is non-zero, then $c$ and $\alpha$ have a slope versus frequency and are termed “dispersive.” From these relations, dispersion measurements are found to indicate the presence of a loss term in the material properties.

Crawling waves

The crawling wave technique, introduced by Wu et al. (2004), is an elasticity imaging method used to map elastic properties within biomaterials. It is a slowly propagating pattern of interfering shear waves, generated in the medium via non-invasive sources. Crawling wave (CrW) propagation can be implemented using external mechanical vibrations (Hoyt et al. 2008b; Partin et al. in press; Wu et al. 2006) as well as acoustic radiation force (Hah et al. 2012; Hazard et al. 2012). CrW data acquired by an ultrasound system can be further analyzed to estimate shear parameters (shear speed or shear modulus) within the region of interest (ROI) and, thereby, used to quantify the elasticity of the scanned medium.

In this study, mechanical vibration sources are placed at opposite sides of a phantom to induce plane shear waves into a ROI using continuous harmonic vibrations, as illustrated in Figure 1. The sources are driven by sinusoidal signals with a slight difference between the frequencies $f_1$ and $f_2$, such that $(f_2 - f_1) \ll f$. Subscripts 1 and 2 correspond to the left and right sources,
respectively. The plane shear waves, propagating along the lateral direction \((x\text{-axis})\), are represented by

\[
    u_1(x, t) = A_1 e^{-\alpha_s(x-d_1)} e^{i [u_1 - k_1(x-d_1) + \phi_1]} \quad (6)
\]

and

\[
    u_2(x, t) = A_2 e^{-\alpha_s(x-d_2)} e^{i [u_2 + k_2(x-d_2) + \phi_2]}, \quad (7)
\]

where \(A\) is the vibration amplitude, \(\alpha_s\) is the attenuation coefficient for shear waves, \(d\) is the lateral location of the vibration source, \(\omega\) is the angular frequency of the shear wave \((\omega = 2\pi f\), where \(f\) is the vibration frequency), \(k_1\) and \(k_2\) are the shear wavenumbers, and \(\phi\) is an arbitrary phase term. The shear waves are superimposed, and a moving interference pattern is created that is displayed in Doppler mode as “crawling” parallel stripes.

\[
    \left| u_T(x, t) \right|^2 = (u_1 + u_2) \cdot (u_1 + u_2)^* = A_1^2 e^{-2\alpha_s(x-d_1)} + A_2^2 e^{-2\alpha_s(x-d_2)} + 2A_1A_2 \cos[(\omega_1 - \omega_2) t - (k_1 + k_2)x + k_1d_1 + k_2d_2 + \phi_1 - \phi_2]
\]

\[
    = B(x) + 2A_1A_2 \cos[\Delta \omega t - 2kx + \phi] \quad (9)
\]

\(k\) is the shear wavenumber and \(\phi\) is an arbitrary phase term. The shear waves are superimposed, and a moving interference pattern is created that is displayed in Doppler mode as “crawling” parallel stripes.

A 3-D matrix of power spectrum variance (axial \(\times\) lateral \(\times\) temporal), illustrated in Figure 2, is computed from the acquired Doppler data using an equation derived by Kasai et al. (1985):

\[
    \sigma^2 = \frac{2}{T_{PRF}} \left(1 - \frac{|R(T_{PRF})|}{R(0)}\right), \quad (8)
\]

Here \(T_{PRF}\) is the time interval between the successive ultrasound Doppler pulses, and \(R\) is the autocorrelation of the backscattered signals of a given Doppler packet. The Doppler imaging is employed with the following parameters: \(f_{Doppler} = 10\) MHz, \(PRF = 1/T_{PRF} = 1.3\) kHz and a Doppler packet of 16 echoes. The size of the 3-D spectrum variance matrix is on the order of \(7\) mm \(\times\) \(4\) mm \(\times\) \(10\) s. The variance of the Doppler power spectrum is proportional to the square amplitude of the particle displacements. By use of eqns (6) and (7), the displacement square of the interfering shear waves is

\[
    B(x) \quad \text{is a baseline term, } \omega_1 - \omega_2 = \Delta \omega, k_1 \approx k_2 \approx k \quad \text{(as } \Delta k = \Delta \omega/c_i \ll k) \quad \text{and } k_1d_1 + k_2d_2 + \phi_1 - \phi_2 = \phi.
\]

Several approaches that estimate local shear speed have been developed and applied in previous studies.
(Hoyt et al. 2007, 2008a; Partin et al. in press; Wu et al. 2004). Here, however, we assume that the medium is macroscopically homogeneous because liver steatosis and liver fibrosis are characterized by gross overall changes in liver viscoelastic properties.

Each vertical stripe of the CrW pattern corresponds to a set of data points of constant phase. A motion slice image, outlined in Figure 2, is extracted from the 3-D CrW data. The CrW stripes are displayed as diagonal lines in the motion slice, as illustrated in Figure 3. The diagonal lines represent data points of constant phase over the temporal-lateral plane. The average shear speed \( c_s \) within the scanned ROI is calculated as

\[
c_s = 2 \frac{\omega}{\Delta \omega} \frac{dx}{dt},
\]

where \( dx \) and \( dt \) are extracted from the motion slice. Equation (10) was derived by differentiating the phase term of eqn (9), \( \Delta \omega = 2k + f \), with respect to \( t \) as follows:

\[
\Delta \omega \frac{dx}{dt} = -\frac{4 \pi f}{c_s} \Delta \omega = 0.
\]

**METHODS**

Seventy C57BL/6J mice purchased from Jackson Laboratories were housed in a micro-isolator room on a 12-h light/dark cycle at the University of Rochester. The University Committee on Animal Resources approved all protocols. At the age of 5 wk, the normal chow diet was switched to a high-fat diet (16.6% kcal/g protein, 59.3% kcal/g fat, 24.48% kcal/g carbohydrate) (No. S3282, Bioserve, Frenchtown, NJ, USA). The latter resulted in diet-induced obesity. Seventy mice were sacrificed for hepatectomy (surgical resection of the liver) 0, 4, 8, 11, 12, 13, 14, 15, 20 and 25 wk after being fed the high-fat diet. Whole-liver weights were in the range of approximately 10–20 g. This corresponds roughly to volumes of 10–20 mL.

After hepatectomy, two small portions of the liver were evaluated histologically and biochemically. Histologic examination of percentage hepatic steatosis was performed by a single, experienced pathologist (W.Q.C.) using hematoxylin and eosin-stained tissue sections (Klain et al. 1989). Triglyceride (TG, a representative of fat concentration [Levene et al. 2012]) assay was performed, and the results were reported as milligrams of TG per milligram of liver. The rest of the liver was suspended in a gelatin phantom for CrW scanning. The overall procedure executed for each liver sample is summarized in Figure 4.

The TG extraction protocol was modified from Burant et al. (1997). We weighed the frozen liver pieces and homogenized them in chloroform:methanol (2:1 v/v). Then we filtered extracts through fluted filter paper. Sulfuric acid (0.05% in saline) was added to filtered extract at a ratio of 1:5 (v/v). After centrifugation, the chloroform layer was removed, dried down and resuspended in fresh chloroform. Samples were then diluted in 5% Triton X-100 (Sigma) (in chloroform) and evaporated. Finally, we measured TG in duplicate using the L-Type Triglyceride Kit (Wako Chemicals, Richmond, VA, USA).
Separately, for crawling wave measurements, liver specimens were suspended in cubical molds using a 9.3% gelatin phantom. The phantom consisted of 2 L of degassed water, 409 g porcine gelatin (300 bloom pork gelatin, Gelatin Innovations, Schiller Park, IL, USA), 19.66 g salt (sodium chloride, BDH, West Chester, PA, USA) and 3.27 g agar (Difco Agar Technical Solidifying Agent, BD, Sparks, MD, USA). The components were mixed together and heated to 55°C. When it had been cooled to 32°C, the mixture was poured into the mold and placed in a refrigerator (approximately 5°C) for hardening. When solidified, the phantom was removed from the mold and allowed to rest at room temperature for about 7 h. CrW scanning was performed when the phantom reached room temperature, usually in the range 17°C to 19°C.

The CrW experimental setup is illustrated in Figure 1. The axes $z$ and $x$ correspond to the axial and lateral directions, respectively. A dual-channel function generator (Model AFG3022 B, Tektronix, Beaverton, OR, USA) was used to produce two sinusoidal signals with a slight difference of 0.6 Hz between the frequencies. The signals were passed through a power amplifier (Model 5530, AE Techron, Elkhart, IN, USA) and subsequently were supplied to piston vibration sources (Model 2706, Brüel and Kjær, Naerum, Denmark). Two elongated bars with rough surfaces of $8 \times 1 \text{ cm}^2$ were mounted on the pistons and placed in close contact at opposite sides of the phantom. The bars oscillated along a direction parallel to the sides of the phantom ($z$-axis), thereby generating shear wave propagation in the lateral direction ($x$-axis), from each side of the phantom. A linear array ultrasound transducer (L40-8/12 linear, Ultrasonix, Richmond, BC, Canada) was positioned between the vibration sources and scanned the medium.

A ROI within the liver specimen was chosen using the 15-MHz B-scan image of the liver for guidance. Then the color Doppler mode was turned on to scan the sample to produce a CrW movie. A single frame of the CrW movie is provided in Figure 5. Multiple frames (at least 100 frames; the maximum number of frames that can be saved depends on the color-flow ROI size) were acquired for each CrW movie by an ultrasound system (SonixTablet, Ultrasonix). The data were post-processed to generate a 3-D variance matrix, and subsequently the average shear speed was calculated using eqn (11). CrW experiments and subsequent shear speed estimations were performed for each liver sample using multiple discrete frequencies in the range [200, 360] Hz with frequency shifts of 0.6 Hz ($f_1 = 200$ Hz and $f_2 = 200.6$ Hz, and $f_1 = 240$ Hz and $f_2 = 240.6$ Hz, and so on). The CrW frequency range was appropriately chosen to increase the signal-to-noise ratio and reduce the reflections of the propagating shear waves at boundaries.

The raw data for each group consist of shear wave speed estimates at six discrete frequencies between 200 and 360 Hz. These data are fit to a linear regression to obtain the slope (dispersion in m/s per 100 Hz) and reference value at 250 Hz. The small size of each liver and limited frequency range studied make the dispersion (slope) calculation of each group more robust than use of the slopes of individual liver measurements.

On completion of the shear speed estimations and TG measurements for all liver samples, the data were organized with respect to the fat concentration for analysis as a group.

**RESULTS**

The histogram distribution of the specimens with respect to TG concentration (mg/mg liver) is given in Figure 6. According to the histogram, it is natural to divide the livers into three groups. The average fat content in these three groups, as assessed by hematoxylin and eosin analysis of each specimen by our pathologist, was 19%, 53% and 78%. Dispersion analyses were performed within each group. All shear speed data are placed on the plot of shear speed versus frequency.

Figure 7(a–c) illustrates the shear speed estimates versus frequency and linear fits for the three groups (low, medium and high TG assays as denoted in Fig. 6). It can be seen that the slope, or dispersion, increases among the groups with higher triglyceride levels. However, the raw shear speeds in the range 200–250 Hz remain around 2.5 m/s in all three cases.
for all liver samples. The livers were divided into three groups
togram was generated on completion of the TG measurements
milligrams of triglyceride (TG) per milligrams of liver. This his-
Fig. 6. Histogram of number of liver samples with respect to
speed (\(c_s\)) for the three groups in Figures 6 and 7.

\[ \text{Number of Samples} \]

\[ \text{mg TG per mg liver} \]

These boxes illustrate that dispersion slopes tend to in-
crease with increasing triglyceride levels; however, the
these high outliers at high frequency in Figure 7c are removed
from the group analysis.

Figure 8 provides a 2-D parameter space of disper-
sion (vertical axis) versus reference value of shear speed
at 250 Hz (horizontal axis). The three boxes define the
90% confidence intervals for the linear fits of (slope,
reference value) for the three groups in Figures 6 and 7.
These boxes illustrate that dispersion slopes tend to in-
crease with increasing triglyceride levels; however, the
reference shear speed at 250 Hz is relatively unchanged.

**Statistical analysis**

Paired series of scanning frequency (Freq) and shear
speed (\(c_s\)) were available for \(n = 68\) mice (2 of 70 were
excluded for insufficient signal-to-noise), for each of
which the TG/liver measurement was available. A group
variable, Gr, was constructed by defining levels 1, 2 and 3
using respective rules TG/liver \(\epsilon (0, 0.1)\), TG/liver \(\epsilon (0.1,
0.25)\) and TG/liver \(\epsilon (0.25, 1)\), according to the histogram
in Figure 6. Plotting aggregate data for each individual
mouse revealed a tendency toward more positive slopes
for groups 2 and 3. In addition, the variance in \(c_s\) attribut-
able to within-subject variation of Freq appears smaller
than the variance in \(c_s\) across patients. For the subsequent
analyses, we discarded a distinct subgroup of anomalous
series within group 1, which is characterized by sharply
discontinuous values of \(c_s\) approaching \(\geq 3.5 \text{ m/s at}
\text{Freq values } \geq 320\). This rule was used to define anom-
alous patients, of which there were 7. No similar definition
of anomaly was considered for the remaining groups. Af-
ter removing anomalies, we had respective group sizes
25, 20 and 16 (\(= 61\)). Although it is preferable that sample
sizes are balanced by group, the correctness of the re-
ported \(p\)-values and confidence intervals does not depend
on this. All of the following statistical methods correctly
account for the respective sample sizes, using standard
methods. The linear regression fit \(c_s = \beta_0 + \beta_1(F \text{req} –
360)\) was calculated, demonstrating a separation between
group 1 and the remaining groups, but not between
groups 2 and 3.

**Linear effects model**

To more precisely quantify the \(c_s\) gradients, a linear
mixed effects model was used. Within a single group, this
is defineable as

\[ c_{ij} = \beta_{0i} + B_{0i} + (\beta_{1i} + B_{1i})(\text{Freq}_{ij} – 360) + \epsilon_{ij} \]  (12)

where \(i\) denotes the \(i\)th subject, and \(j\) identifies the \(j\)th data
pair (Freq\(_{ij}\), \(c_{ij}\)) of subject \(i\). For consistency, the model is
similarly offset so that the intercept represents the fit at
Freq = 360. The model is interpreted as follows: each
subject \(i\) is assumed to have a distinct intercept \(\beta_{0i} + B_{0i}\) and slope \(\beta_{1i} + B_{1i}\), where \(\beta_{0i}\) and \(\beta_{1i}\) are constant
fixed effects, and the values of \(B_{0i}\) and slope \(B_{1i}\) are
random effects assumed to be sampled from a normal dis-
tribution of mean 0. The model is therefore hierarchical
in the sense that the slopes and intercepts for each subject
are taken to be random samples from normal distributions
of mean \(\beta_{0i}\) and \(\beta_{1i}\). The fixed effects may be interpreted in
the same way as for standard linear models, the role of the
random effects being to model variation attributable to
patients. The remaining term, \(\epsilon_{ij}\), then models variation
not explained by the fixed and random effects combined,
and the values of \(\epsilon_{ij}\) are pooling a random sample from a zero mean
normal distribution and is independent of all other model
elements. Group interactions may then be introduced in
the standard manner as interaction terms, permitting the
estimation of group effects for the slope and intercept.

Two models are considered, one using the three
groups already defined and one in which groups 2 and 3
are pooled. The models (eqn [12]) were fit using the
lme (linear mixed effects) function from the \(R\) statistical
library (Pinheiro and Bates 2000). The fixed effects esti-
mates are listed in Table 1. The \(p\)-value refers to the dif-
fences in \(\beta_{0i}\) and \(\beta_{1i}\) between group 1 and groups 2 and 3
in the first model and between group 1 and the pooled
group for the second model (these differences are of
more relevance than the absolute values). Examining
both models, we can see that both \(\beta_{0i}\) and \(\beta_{1i}\) differ
significantly between group 1 and group 2 and between group 3 and groups 2 and 3 pooled. However, the parameters for groups 2 and 3 are approximately equal, the differences being within the respective standard errors. We therefore conclude that statistically significant differences in the $c_s$/Freq gradients and magnitudes exist between group 1 and the remaining groups, but also that groups 2 and 3 are not significantly different in this regard.

**Discriminant analysis**

We next consider the design of a classification rule. Following the previous analysis, groups 2 and 3 were pooled, and the classifier was based on the estimated values of $\beta_0$ and $\beta_1$. Linear discriminant analysis (LDA) was used to estimate a linear classifier of the form $\alpha_0\beta_0 + \alpha_1\beta_1$. The coefficients $\alpha_0 = 2.382$ and $\alpha_1 = -14.79$ were obtained using the lda function from the R statistical library (Venables and Ripley 2002), leading to classification score:

$$\text{score} = 2.382\beta_0 - 14.79\beta_1$$

(Figure 9) is a plot of the individually estimated values of the pairs ($\beta_0, \beta_1$), with anomalies removed, and the group symbols indicate the two-group model. The dashed line illustrates the classification induced by setting score $= 6.5$, using eqn (13).

The LDA method may be used to estimate values of Freq at which observations of $c_s$ have the greatest discriminating ability. We may express the linear discrimination function

$$\text{score} = \alpha_0\beta_0 + \alpha_1\beta_1 = c[\beta_0 + \beta_1(x - 360)]$$

so that in our model the score (eqn [13]) may be seen to be equivalent to selecting a value Freq $= x$ at which a fitted...
regression line has greatest discriminating ability. Given our values of $a_0$ and $a_1$, eqn (14) may be solved to yield $x = 353.8$, from which we conclude that values of $c_s$ observed near the upper limit of $\text{Freq} = 360$ are to be preferred for classification.

In Figure 10(a) are receiver operating characteristic (ROC) curves, obtained by estimating true- and false-positive rates obtained by varying the score classification threshold. For the LDA regression method, rates were estimated using leave-one-out cross validation. The area under curve (AUC) statistic obtained was 83%, and estimates the probability that a pair of classification scores drawn randomly from each group are in the correct order. For comparison, the observed values of $c_s$ at $\text{Freq} = 280$ (the midpoint of the observed range) and $\text{Freq} = 360$ were also used as classifiers, and the resulting ROC curves are also provided in Figure 10(b, c), with respective values of AUC = 72% and AUC = 88%. This supports the conclusion of the LDA analysis that values of $c_s$ near $\text{Freq} = 360$ have greater discriminating ability.

**DISCUSSION AND CONCLUSIONS**

The dispersion within groups of livers is found to be near zero for normal livers (steatosis < 5%), increasing to 0.2–0.5 m/s per 100 Hz in the group with triglyceride levels >0.25 mg/mg liver. The possibility exists for staging progressive grades of steatosis by careful measurement of shear wave dispersion.

One limitation of our study was the difficulty in predicting steatosis accumulation simply based on the length of time mice were fed high-fat diets. Although we attempted to capture a range of steatotic livers by sampling at frequent time points, we encountered a rapid rise in steatosis over time and individual mouse variations in steatosis accumulation over time (data not shown). Future experiments should overcome these phenomena by increasing the number of animals studied and increasing the frequency of sampling, especially between weeks 6 and 16.

The lower range of clinically significant steatosis is generally regarded to be 30% of hepatocytes containing macrovesicular fat inclusions on hematoxylin and eosin staining of liver biopsy specimens. Above this range, patients are at higher risk of developing liver biochemical abnormalities and progression from simple steatosis to the fibro-inflammatory changes of non-alcoholic steatohepatitis (NASH). Also, the rates of primary liver
allograft dysfunction increase when organs with >30% macrosteatosis are transplanted. Our study found that low (<20%) and high (>50%) steatosis can be distinguished with crawling wave shear speed dispersion and that further study, including refinements in our technique and experimental design, is merited.

Other limitations of the study include the small sample volumes of mouse liver, the limited frequency range of the shear wave dispersion measurements and the use of ex vivo livers at room temperatures. In vivo liver values at body temperature will undoubtedly have different absolute values, and this may also alter the slope or dispersion dependence on the accumulation of fat. In addition, in some cases, shear wave reflections from adjacent boundaries (gel-liver) perturb the crawling wave patterns. These tend to be dependent on frequency, leading to higher variance in the resulting estimates of dispersion. This problem of proximal side boundaries is reduced in larger livers, and a modified configuration has been proposed to apply crawling waves through the abdominal wall in larger patients (Partin et al. in press). In this modified configuration, two shear wave sources are applied to the surface of the skin. The crawling wave pattern and estimates have been re-derived for this configuration, which is more suitable for in vivo human studies. Nonetheless, the results support the hypothesis that fat adds a viscous, lossy and therefore dispersive element to the liver, which can be estimated by measurement of dispersion.

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