CASE STUDY

Automated ultrasound for non-invasive *in vivo* evaluation of liver disease progression in mice.

Millions of Americans currently suffer from chronic liver disease, which accounts for tens of thousands of deaths per year. There are many potential causes for chronic liver disease, including chronic viral hepatitis infection, alcohol abuse, and non-alcoholic steatohepatitis. If left untreated, these conditions can significantly increase the likelihood of developing liver cancer or end-stage liver failure. In general, there are three pathologic hallmarks of liver disease:

1. **Fibrosis** – formation of excess collagen/connective tissue in response to damage (i.e., scarring)
2. **Steatosis** – abnormal retention of fat or triglycerides in the liver
3. **Inflammation** – activation of the body's innate immune system and release of inflammatory mediators

Characterizing the extent of fibrosis, steatosis, and inflammation in the liver non-invasively enables clinicians and researchers to comprehensively stage disease progression and evaluate therapeutic response.

The value of non-invasive *in vivo* liver research tools

Advances in liver disease treatments will have an enormous impact on human health. For instance, there are no FDA-approved therapies for liver fibrosis. Despite substantial efforts of scientists globally, an effective pharmaceutical treatment to directly slow or reverse the process of liver fibrosis has proven to be elusive. For many patients with fibrotic livers, if diet and lifestyle changes fail a liver transplant becomes the only option. The demand for therapeutics is only expected to grow as obesity rates, and consequently, the prevalence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), escalate.

Animal models are still the gold standard for basic and applied liver disease research, as the complex interaction of numerous cell types during steatohepatitis and fibrogenesis is challenging to mimic using in vitro models. However, to date, the use of non-invasive imaging tools has been limited for assessing hepatic injury within these animal models, as researchers still mostly rely on either serum biomarkers or biopsy/sacrifice of animals. True longitudinal studies involving a single animal being monitored repeatedly over time are rare but will increasingly be utilized by the field as protocols for acquisition and analysis are streamlined for users, and multiple parameters of interest can be gleaned from a single workflow.

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The Vega: a turnkey non-invasive ultrasound platform for staging of liver disease

Fortuitously, several phenotypes associated with liver disease progression give rise to physical changes that are detectable via ultrasound waves. First, the presence of fat within the liver causes an increase in the brightness, or "echogenicity," of the ultrasound images collected of the liver. This change in image brightness is proportional to the amount of fat accumulation in the liver, and thus ultrasound can provide a near-instantaneous and non-invasive readout for steatosis. Additionally, excess collagen and scar tissue throughout the liver is the hallmark of fibrosis onset and progression, which introduces bulk mechanical property change as the liver becomes stiffer. This increase in stiffness has been qualitatively assessed in the clinic via palpation of the abdomen for hundreds of years, but more recently fibrosis-induced changes in liver stiffness can be quantitatively assessed in both clinical and preclinical settings using shear wave elastography (SWE) imaging. This is a non-invasive ultrasound-based technology that takes advantage of differing wave speeds between tissues of varying stiffness; the stiffer the tissue is, the faster shear waves travel through it. Despite widescale clinical adoption, the application of SWE by preclinical researchers and drug developers for studies of liver disease progression in small animal models is limited. This is partly due to the experimental, technical, and reproducibility challenges of current imaging systems.

The Vega® ultrasound system has been designed to address the challenges limiting conventional *in vivo* systems' use by researchers. It offers an automated 3D SWE mode in a hands-free *in vivo* imaging workflow for small animal work. In this system, ultrasound scanning is performed using a bottom-up imaging approach through the use of automated hands-free transducers located under the imaging stage. One key advantage of this approach is it allows the Vega to collect image data without the user accidentally "pre-loading" the tissue through physical pressure on the animal with a handheld transducer (artificially biasing the tissue's fibrosis readings). Additionally, imaging in this fashion is highly reproducible as the animal and transducer positioning is very consistent from timepoint to timepoint and a wide field-of-view is captured every time.

The Vega contains two integrated transducers offering a suite of imaging modes: SWE, B-Mode, M-Mode, 2D, 3D, and 4D imaging, as well as acoustic angiography, a type of contrast-enhanced ultrasound (CEUS). Here, we demonstrate the preclinical utilization of the Vega *in vivo* imaging system for visualizing and quantitatively analyzing various aspects of liver disease in a range of small animal models.

Evaluation of NAFLD progression in a murine Western diet model

Western diets (WD) containing high fat, fructose, and cholesterol have been widely used to establish murine models of NAFLD/NASH. In a recent study, Dr. Bernd Schnabl and colleagues at the University of California San Diego validated the capability of the Vega system to provide non-invasive measures of NAFLD progression using a WD mouse model.[1] Mice were placed on a standard chow or WD for 48 weeks and imaged with the Vega system every four weeks. A subset of mice was sacrificed at 0, 12, 24, and 48 weeks for histological validation.

In the first 36 weeks, liver echogenicity for mice on the WD increased substantially suggesting a buildup of fat over this time period (Figure 1, left). Echogenicity measures then plateaued and remained elevated thereafter. Livers of mice on WD were also seen to increase in volume during the study period. Notably, SWE liver stiffness readings showed little to no change during the course of the study, suggesting that fibrosis had not developed (Figure 1, right).

These results were supported by histological analyses, which demonstrated substantial increases in triglycerides and liver weight, with little fibrosis development. Correlation analysis indicated that *in vivo* measurements closely reflected the underlying pathological state. Specifically, a good correlation was observed between liver volume/liver weight (R^2=0.88) and echogenicity/triglycerides (R^2=0.80) while moderate agreement was observed between stiffness/picrosirius red (PSR) staining (R^2=0.45).

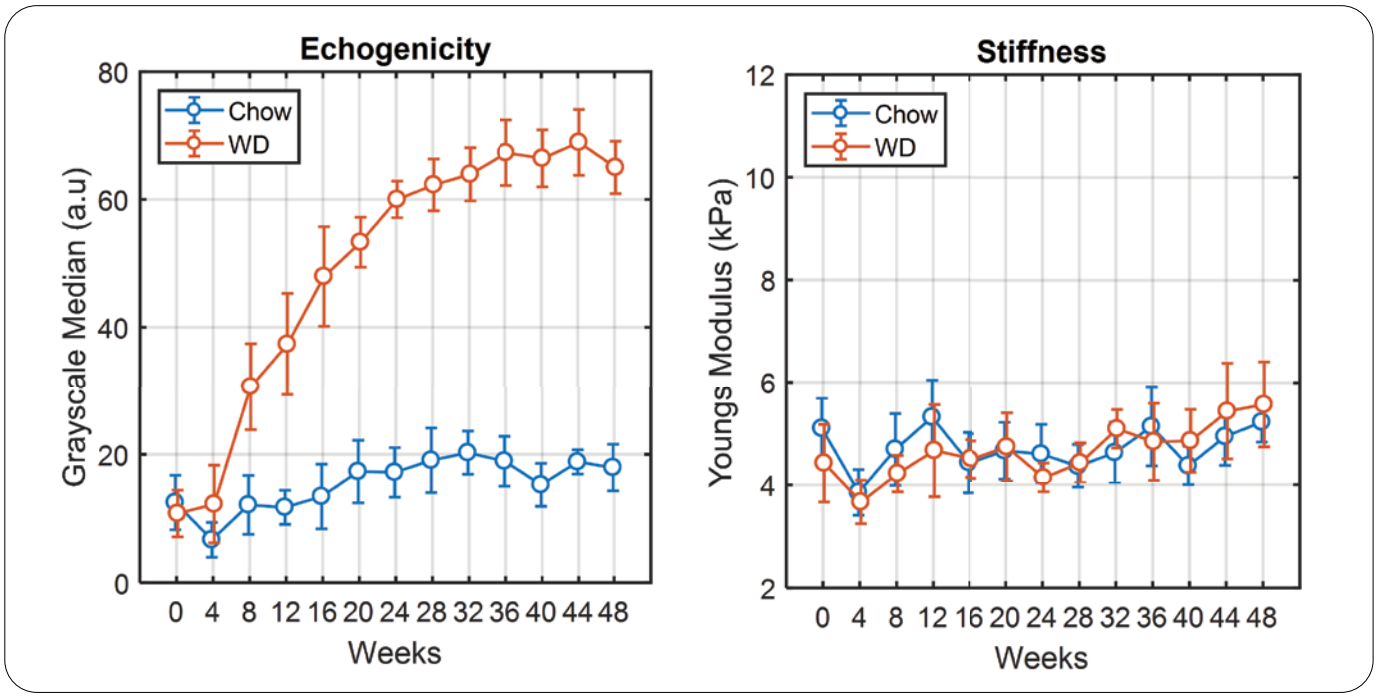

Figure 1: Longitudinal progression of echogenicity (brightness) and stiffness readings over 48 weeks. (Left) Liver echogenicity increased substantially, plateauing at 36 weeks, and remaining elevated suggesting onset and persistence of steatosis. (Right) Liver stiffness showed little to no change suggesting no fibrosis development. Error bar plots represent mean ±1 SD. IMAGE CREDIT: Czernuszewicz T, Wang Y, Jiang L, Aji A, Yu S, Rojas J, et al. 2022.[1]

Evaluation of disease progression in a chemically induced murine fibrosis model

Another group led by Dr. Justin Elstrott at Genentech has used the Vega system to visualize liver fibrosis progression in a carbon tetrachloride (CCl4) mouse model.[2] Twenty mice were included in their investigation, 10 of which were treated with CCl4 three times per week to induce fibrosis in the absence of steatosis. The mice were imaged at baseline, day 17, and day 38.

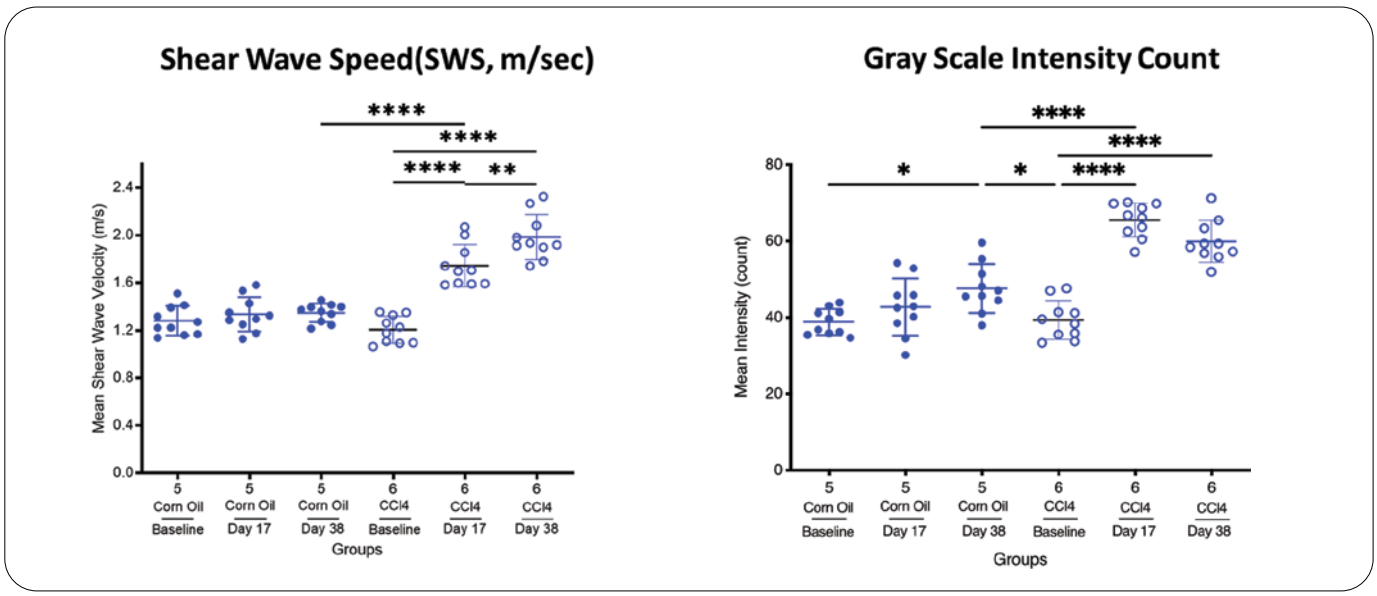

Figure 2: Increased shear wave speeds (SWS) and echogenicity (grayscale intensity) in CCl4-treated mice. IMAGE CREDIT: Gandham V, Wong A, Brightbill H, Elstrott J. 2022.[2]

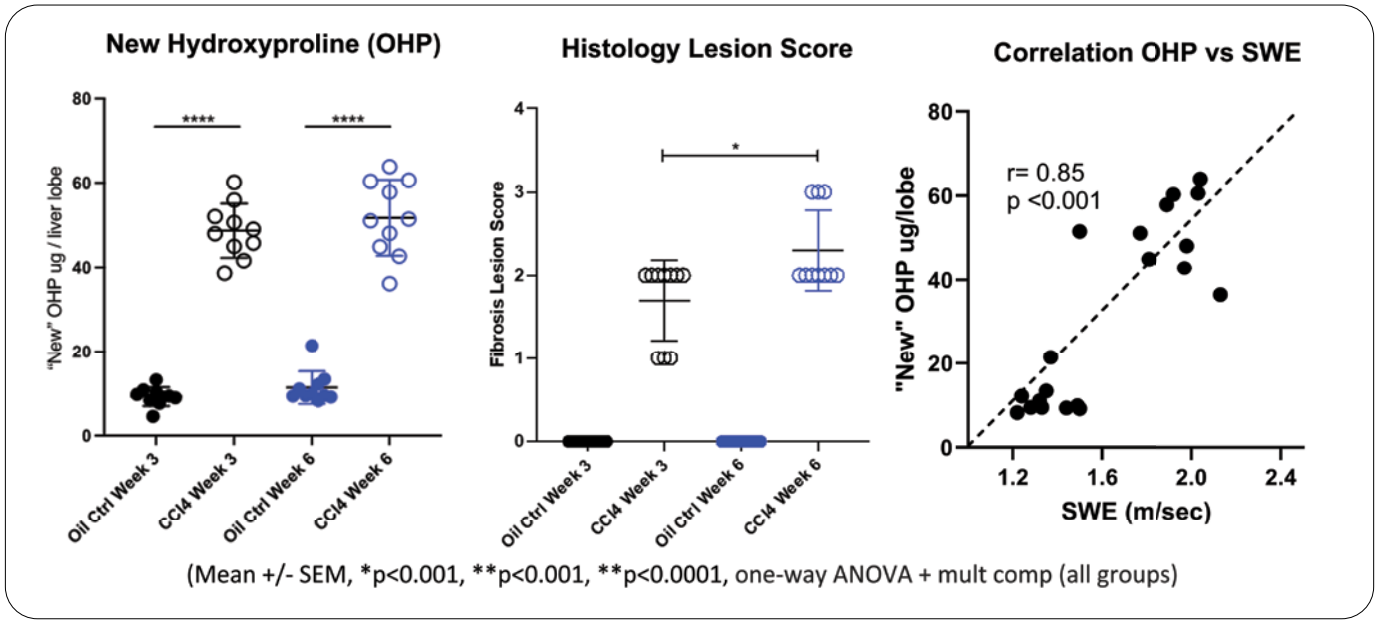

Figure 3: Terminal endpoints showing significant fibrosis in CCL4-treated mice. IMAGE CREDIT: Gandham V, Wong A, Brightbill H, Elstrott J. 2022.[2]

Ultrasound imaging showed significantly increased liver stiffness and echogenicity in the CCl4-treated over the 38-day study period (Figure 2). In order to validate their results, the team sacrificed a subset of mice at days 17 and 42. Histological and hydroxyproline analysis confirmed increased collagen/fibrotic tissue in the CCl4 mice, which correlated with the findings from their ultrasound analysis (Figure 3).

Monitoring treatment response in Mdr2 knockout mice

Genetic knockout of the Mdr2 gene in mice causes an absence of phosphatidylcholine from bile, leading to liver injury. Using an Mdr2 knockout model, Dr. Bernd Schnabl and colleagues at the University of California San Diego monitored the effect of nor-ursodeoxycholic acid (nor-UDCA) treatment on liver stiffness with the Vega platform.[3] The team imaged wildtype and Mdr2 knockout mice longitudinally from 6 to 28 weeks old. At 20 weeks, a subset of the knockout mice (all female) was dosed with nor-UDCA forming the treatment arm.

Imaging confirmed that Mdr2 knockout mice had slightly larger and brighter livers at 28 weeks compared to wildtype mice, with liver stiffness being highest in the female knockouts. Interestingly, longitudinal analysis also revealed distinct differences in liver stiffness between sexes, with female knockout mice exhibiting higher levels of liver stiffening over time compared to their male counterparts (Figure 4, left).

When the researchers compared the effect of treatment on liver stiffness, they found that the treated knockout mice experienced an inflection point followed by a plateau effect on liver stiffening suggestive of treatment response, while the liver stiffness of their untreated littermates continued to increase over the 8-week treatment period (Figure 4, right).

At the end of the study, the mice were sacrificed for histological validation of non-invasive ultrasound measures. Histology confirmed that liver stiffness was statistically lower in the treated mice compared to the untreated mice and that untreated female mice had statistically significantly higher levels of fibrosis compared to treated female mice and their untreated male littermates.

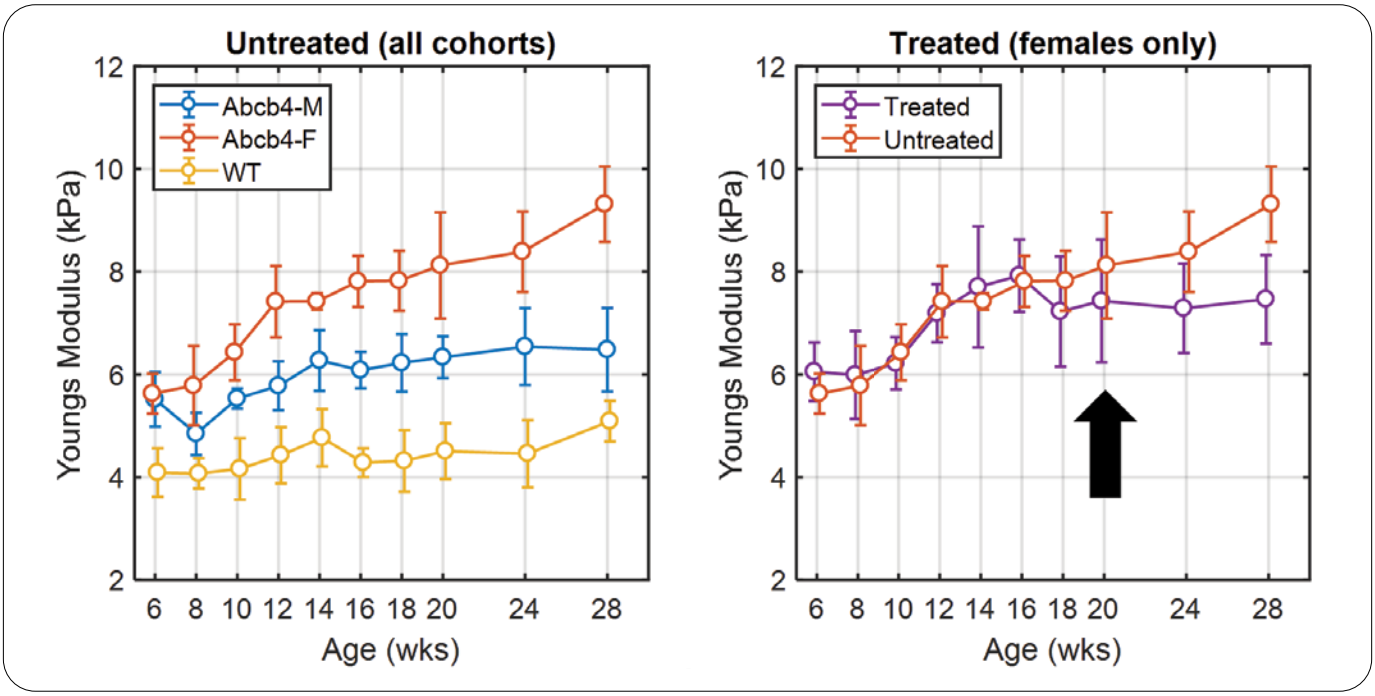

Figure 4: Liver stiffness over time for both treated and untreated cohorts. (Left) Female (F) Abcb4 mice were observed to have higher levels of liver stiffening compared to their male (M) littermates, and both were higher than wildtype (WT) controls. (Right) Treated mice experienced a plateau effect on liver stiffening, while untreated mice continued to increase. The black arrow indicates the week that treatment was started. IMAGE CREDIT: Czernuszewicz T, Wang Y, Jiang L, Fuchs C, Trauner M, Aji A, et al. 2022.[3]

Multimodal non-invasive imaging of NAFLD/ NASH disease progression in a mouse model

Studies also support the use of automated ultrasound imaging alongside other imaging modalities to analyze liver disease progression in small animal models. For example, Dr. Jeffrey Peterson and co-workers at Revvity Inc. have used a multimodality approach of fluorescence (IVIS Spectrum), ultrasound (Vega), and X-ray uCT (Quantum GX2) imaging to study the progression of inflammation, steatosis, and fibrosis in murine disease models.[4] Mice were either fed regular chow (control), a high-fat diet to induce NAFLD (HFD), or a high-fat diet with CCl4 injections to induce NASH (HFD-CCl4) for 14 weeks and imaging was performed throughout the study.

After seven weeks on the diet, histological analyses revealed only the HFD-CCl4 mice developed all three major pathological characteristics of NASH: inflammation, steatosis, and fibrosis. Notably, *in vivo* fluorescence imaging detected inflammation in the livers of HFD-CCl4 mice as early as week two of NASH development.

Over the first three weeks of the study, ultrasound imaging detected a reduction in the stiffness of HFD-CCl4 livers. After eight weeks, the stiffness of the HFD-CCl4 livers started to increase and became stiffer than that of the control mice, suggesting extensive tissue fibrosis. B-mode imaging showed a gradual increase of grayscale intensity over the 14-week period as fat accumulated during NASH onset. The SWE findings were supported by uCT HU assessment of liver density. Specifically, the HFD-CCl4 livers showed lower HU readings at week three, but by week 12 they showed recovery in HU suggesting the development of denser fibrosis tissues. The study demonstrates how the three imaging modalities can be used together to acquire non-invasive readouts relating to inflammation, steatosis, and fibrosis in obesity and NAFLD/NASH models.

Conclusion

The Vega imaging system is an automated, hands-free, high-throughput ultrasound system that can be used for longitudinal and quantitative measurements of changes in liver size, brightness, and stiffness, all of which are biologically important phenotypes in liver disease progression. As demonstrated by the work of several groups across North America, the Vega has proven to be sensitive to small changes in liver disease state, can be used in different types of animal models, and produces measurements that closely reflect the underlying pathological disease state.

References

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Revvity
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com