A Metabolomics Approach to Biomarker Discovery for Non-Alcoholic Steatohepatitis

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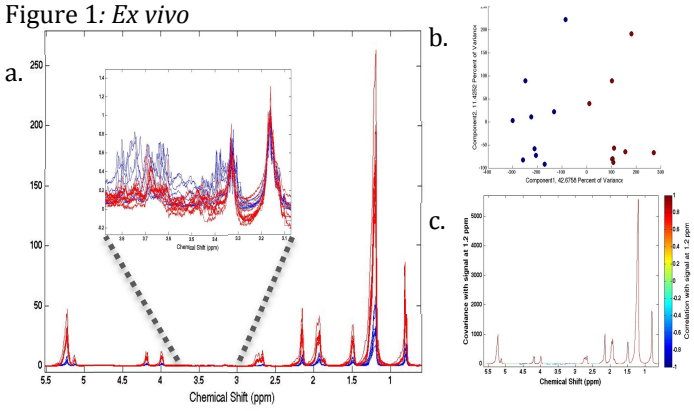
INTRODUCTION: Non-Alcoholic Steatohepatitis (NASH) is a lipid infiltration in the liver causing inflammation and damage [1]. There are few symptoms in the early stages of the disease, but if left undiagnosed it can lead to cirrhosis and loss of liver function [1]. Currently, little is known about the mechanism behind NASH, and the diagnosis can only be made from a biopsy. Metabolomics is the process of identifying and quantifying metabolites produced by an organism [2]. In this study, metabolomics is used to identify possible biomarkers that are indicative of NASH. Magnetic resonance spectroscopy (MRS) allows for a comprehensive and quantitative evaluation of tissues' metabolite content [3]. The present study uses *in vivo* ^{1}H-MRS to identify the metabolic signature in the NASH liver non-invasively, as well as *ex vivo* high-resolution magic angle spinning (HR-MAS) MRS to confirm the *in vivo* observation and to expand upon metabolite resolution. In addition, *in vivo* water and lipid transverse relaxation time constants (T_2) - sensitive reporters of cellular environment - were measured in the liver by ^{1}H-MRS.

METHODS: C57Bl/6 mice were placed on either a methionine choline deficient (MCD, n=9) diet, to mimic the NASH phenotype in the liver, or methionine choline sufficient (MCS, n=9) diet as a control. Metabolic measurements were performed after 4-6 weeks on the diet. ***In vivo*****:** Animals were anesthetized (2% isoflurane in air). The hindlimb was extended and secured in a 13 mm diameter loop gap ^{1}H coil and centered in an 11.1T Agilent system. T_2 and proton MR spectroscopy measurements of ^1H_2O and lipid were acquired from a single voxel (3x4x5mm) using an adiabatic selective refocusing (LASER) pulse sequence and an array of 10 TE's equally spaced from 25 to 70 ms. Lipid fraction was measured by integration of the water (4.2ppm-5.2ppm) and fat (0.7ppm to 1.85ppm) peaks collected with a 40ms TE, and defined as fat/(water+fat). Data was corrected to account for differences in T_2 between water and fat. ***Ex vivo***: Intact liver tissue samples were prepared as previously described in [4] for HR-MAS acquisitions on a 600MHz Bruker magnet with a 4 mm rotor angled at 54.7 degrees and a spinning rate of 5000Hz. 1D nuclear Overhauser effect spectroscopy (NOESY) and 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences were used to quantify liver metabolites. All spectra were processed using the Mestranova software package, including phasing, zero filling and baseline correction. *In vivo* T_2 data was processed with an in-house software written in IDL. Statistical significance was determined using a t-test and a 95% confidence interval. The *ex vivo* HR-MAS processed spectra were then analyzed using an in-house Metabolomics Matlab Toolbox. Analyses, to perform alignment, normalization and statistical tests including principle component analysis and statistical correlation spectroscopy (STOCSY).

Figure 1*: Ex vivo*



RESULTS: The liver lipid fraction measured on week 6 was significantly elevated in the MCD compared to MCS mice. Liver lipid T_2 values (1.3ppm peak) measured *in vivo* were significantly elevated in the MCD compared to MCS mice at all time points (weeks 4 and 6: $p<0.01$, week 5: $p<0.05$). However, the water T_2 was not different between MCD and MCS. *Ex vivo* metabolite profiles were separated using principle component analysis with 43 % variance in the first principle component using HR-MAS MRS spectra from intact liver tissue (Figure 1a and b). The peaks identified to contribute to the separation of the MCD and the MCS mice metabolic profiles, were found to have significant correlations to peaks associated with fatty acids (Figure 1c).

CONCLUSION: This study showed that not only did NASH mice have increased fatty tissue deposition, but also the lipid transverse relaxation rate values from the liver could discriminate the MCD mice from the MCS mice *in vivo*. The *ex vivo* HR-MAS results suggested that this could be due to differences in the lipid profiles between the MCD and MCS groups. Further investigation of this disparity in lipid profiles may allow for a greater understanding of the mechanism of NASH and promote the design of less invasive diagnostic techniques.

Acknowledgments: The Metabolomics Matlab Toolbox was written by Steven Robinette. References: [1] Li et al. J Proteome Res 2011 [2] Dunn et al. Chem Soc Rev 2011 [3] Hamilton et al. NMR Biomed 2011 [4] Beckonart et al. Nature